Indicator Amino Acid Derived Estimates of Dietary Protein Requirement in Exercise-Trained Individuals

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Graduate Program in Kinesiology
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy
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Abstract

Despite a number of nitrogen balance (NB) studies indicating increased dietary protein needs in Endurance (ET) and strength (ST) trained athletes, the Institute of Medicine (2005) has concluded, based largely on methodological concerns, that “no additional dietary protein is suggested for healthy adults undertaking resistance or endurance exercise”. Indicator amino acid oxidation method (IAAO) has been recently used for determination of protein requirement in humans. This method is based on the concept that when one indispensable amino acid (AA) is deficient for protein synthesis, then all other AA, including the indicator, will be considered excess and oxidized. With increasing intakes of protein, oxidation of the indicator will decrease, reflecting its increasing incorporation into protein. Once the requirement for the protein is met there will be no further change in the indicator oxidation. The objectives of this dissertation were to determine the dietary protein requirement of young ET and ST men and to compare their results with that of previously determined sedentary young men using IAAO. Eight healthy young ST men (≥3 y training experience with ≥90% muscularity based on past published Mr. USA winner) and 8 ET men (≥1 y training experience with a $V_{O2max} = 64.1 \pm 3.7$) participated in 85 experiments composed of two adaptation days followed by a study day. The adaptation day diets provided energy at 1.7 times each individual’s measured resting energy expenditure (REE) with dietary protein intake of 1.5 g•kg\(^{-1}\)•d\(^{-1}\). On the study day energy was provided at 1.5 times each individual’s REE and dietary protein requirement was determined by measuring the oxidation of ingested L-[1\(^{-13}\)C] phenylalanine to \(^{13}\)CO\(_2\) in response to variable intakes of protein (0.1 to 3.5 g•kg\(^{-1}\)•d\(^{-1}\)). The requirement (breakpoint) was defined by applying a mixed-effects change-point regression analysis to label tracer oxidation in \(^{13}\)CO\(_2\) breath. The estimated average protein requirement (EAR) for these young ET and ST men were 2.0 and 1.7 with an RDA of 2.6 and 2.2 g•kg\(^{-1}\)•d\(^{-1}\), respectively. These results are about twice the EAR (0.93 g•kg\(^{-1}\)•d\(^{-1}\)) determined by IAAO in sedentary young men and about 2.6 - 3 fold greater than EAR recommendation by the institute of medicine.

Keywords: Protein requirement, Indicator amino acid oxidation, Endurance athletes, strength athletes, stable isotope
Co-Authorship Statement

Arash Bandegan was the first author and Dr. Peter W. R. Lemon was the senior author on all papers included in this thesis with Glenda Courtney-Martin, Mahroukh Rafii and Paul B Pencharz as co-authors.
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<tr>
<td>AA</td>
<td>amino acids</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BCAA</td>
<td>branched chain amino acid</td>
</tr>
<tr>
<td>BCOAD</td>
<td>branched chain oxo-acid dehydrogenase enzyme</td>
</tr>
<tr>
<td>BM</td>
<td>body mass</td>
</tr>
<tr>
<td>DAAO</td>
<td>direct AA oxidation</td>
</tr>
<tr>
<td>DRI</td>
<td>dietary reference intake</td>
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<tr>
<td>EAR</td>
<td>estimated average requirement</td>
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<tr>
<td>ET</td>
<td>endurance-trained athlete</td>
</tr>
<tr>
<td>FFM</td>
<td>fat-free mass</td>
</tr>
<tr>
<td>IAAO</td>
<td>Indicator amino acid oxidation method</td>
</tr>
<tr>
<td>IOM</td>
<td>Index of Muscularity</td>
</tr>
<tr>
<td>NB</td>
<td>Nitrogen balance</td>
</tr>
<tr>
<td>PAR-Q</td>
<td>physical activity readiness questionnaire</td>
</tr>
<tr>
<td>RDA</td>
<td>recommended daily allowance</td>
</tr>
<tr>
<td>REE</td>
<td>resting energy expenditure</td>
</tr>
<tr>
<td>SED</td>
<td>sedentary young men</td>
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<tr>
<td>ST</td>
<td>strength-trained athlete</td>
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Chapter 1.

General Introduction
1.1 Introduction

Proteins are an important component of all living organisms. Specifically, they function as catalysts, are part of the structural framework of cells, act as antibodies, control metabolism and act as mediators in their own metabolic control. Skeletal muscles are the major depot of proteins in the body, comprising about 40% of body mass (BM). In addition, other organs or tissues also contain protein including liver (which synthesizes plasma proteins), immune cells, digestive enzymes, bone and dermal collagen (Guillet, et al., 2004). The proportion of the twenty common amino acids (AA) found in proteins vary as a characteristic of a specific protein, but most proteins contain some of each. All 20 AA are required for the synthesis of protein as well as important nitrogen-containing compounds, such as creatine, peptide hormones, and neurotransmitters (Poortmans, et al., 2012). Some of these AA can be produced in the body (dispensable AA) but others (indispensable AA) must be consumed as part of one’s diet or protein synthesis becomes insufficient to replace regularly degraded body protein. This situation would affect exercise performance and eventually health adversely.

Continuously, protein and other nitrogenous compounds are degraded and resynthesized at varying rates by body tissues (Schoenheimer, 1942). More protein is turned over daily within the body than is consumed routinely, indicating that the reutilization of circulating AA is a major feature of protein metabolism. Further, some AA are lost by oxidative catabolism because the mechanism of reutilization is not absolute. The metabolic end-products of AA degradation, such as urea, ammonia, creatinine and uric acid, are excreted in the urine primarily but nitrogen can also be found in faeces, sweat, skin, hair, nails as well as in other body secretions. Further, the relative importance of these routes is altered with exercise. For example, urinary losses are reduced acutely and sweat losses increased substantially (Lemon & Mullin, 1980). Also as mentioned, body proteins must be synthesized continuously, to allow growth and/or to replace degraded molecules, so a continuous supply of dietary AA is required even after adult height is attained (Felig, 1975; McMurray, 1977).

Amino nitrogen accounts for approximately 16% of the mass of proteins and its whole body balance is used classically as an indicator to study the human protein metabolism. Nitrogen balance (NB) methodology is based on the measurement of the difference between nitrogen
intake and the amount excreted in urine, faeces, and sweat, together with minor losses occurring by other routes. Classically, one’s dietary protein requirement is determined by measuring NB at varying dietary quantities of protein, ranging from deficient to excess, while consuming an otherwise nutritionally complete/adequate diet. The requirement estimate is taken to be the amount of dietary protein which results in nitrogen equilibrium (or the zero balance point) and is determined using a linear regression line typically. Although a useful tool, the NB method has many inherent flaws (Fuller & Garlick, 1994; Hegsted, 1976; Millward, 2001). Moreover, it is quite laborious not only because of the need to collect all routes of excretion while controlling energy expenditure but also because each protein intake manipulation must be quantified meticulously and provided for 10 or more days due to adaptations that result from changing protein intakes. As such, this methodology is often criticized as an unsuitable technique to determine protein requirements. Regardless, the current protein requirement for adult humans (0.6 g•kg⁻¹•d⁻¹) established by the Institute of Medicine (Institute of Medicine, et al., 2005) is based on NB derived data. Further, the recommended dietary allowance (RDA) which includes a buffer equal to two standard deviations (SD) has been calculated (0.8 g•kg⁻¹•d⁻¹) in order to ensure that even those with greater than average needs will not be deficient. To date, there is no additional protein intake recommendation for physically active individuals despite consistent NB results on both endurance (ET) and strength (ST) athletes which exceed these values (Thomas, et al., 2016). The rationale for this inconsistency centres on the methodological problems associated with the NB technique. Consequently, there is a conflict between those who establish dietary protein requirements (Institute of Medicine, et al., 2005) and those who issue guidelines for exercise-trained individuals (Rodriguez, et al., 2009; Thomas, et al., 2016). Clearly to resolve this debate which has existed for years (Lemon & Nagle, 1981), other methods to assess protein requirements in all populations are needed.

Recently, the indicator amino acid oxidation method (IAAO) has been used to determine protein requirements of humans at different ages (Elango, et al., 2011; Humayun, et al., 2007; Rafii, et al., 2016; Rafii, et al., 2015; Stephens, et al., 2015; Tang, et al., 2014). This ¹³C stable isotope method measures protein requirement based on the oxidation of ¹³C labeled phenylalanine (indicator), a reverse proxy for whole body protein synthesis, in response to variable protein intakes. The results generally indicate 30-40% greater requirement estimates compared with
those of the NB method (Humayun, et al., 2007; Rafii, et al., 2016; Rafii, et al., 2015; Tang, et al., 2014). The IAAO method is accepted as valid by the Institute of Medicine (Food And Agricultural Organization, 2007; Institute of Medicine, et al., 2005) but to date it has been utilized primarily in sedentary participants (both animals and humans) while at rest. Use of this technique in exercise-trained individuals on both exercise and non-exercise days might be able to shed some light on whether and how much exercise affects protein requirements/recommendations.

Interestingly, gender may also be an important consideration, especially in those who exercise regularly because several studies show that women oxidize proportionately more fat and less carbohydrate during endurance exercise compared with men (Friedlander, et al., 1998; Horton, et al., 1998; Lamont, et al., 2001; Phillips, et al., 1993). Further, post-exercise urinary urea excretion is often used as a marker of total AA oxidation and is increased in men typically but not in women (Tarnopolsky, et al., 1990). In addition, oxidation of the indispensable AA leucine in women during exercise has been reported to be lower than men (Lamont, et al., 2001, 2003). Collectively, these reports suggest that physically active women might have a lower protein requirement than their male counterparts. However, there are no IAAO studies comparing male and females. Consequently, although there are NB data indicating protein requirements are increased in physically active individuals, data need to be collected using the IAAO methodology before current protein dietary recommendation estimates for the physically active from NB studies (1.2-1.8 g•kg\(^{-1}•d^{-1}\)) can be confirmed as well as to determine whether gender differences exist.
1.2 References


Chapter 2.

Literature Review
2.1 Historical review

According to legend, the earliest historical report on the importance of protein intake for the physically active goes back as far as the 6th century B.C. in Greece when Milo, multiple Olympian wrestling champion, supposedly strengthened his musculature by regularly carrying a calf as it grew into a bull together with consuming a diet allegedly composed of 20 pounds of meat, 20 pounds of bread and 18 pints of wine daily (Harris, 1966; Todd, 1985). However, it was not until the end of the 18th century that the French chemist and biologist Lavoisier figured out that chemical energy released from the breakdown of organic compounds actually fueled muscle contraction. Further, in the mid-1800s, German biochemist Von Liebig argued that because his analyses of muscles failed to show the presence of any fat or carbohydrate, the energy needed for their contraction must come from an explosive breakdown of the protein molecules themselves, resulting in the production and excretion of urea. As a result, he argued that protein was the only true nutrient, providing both the machinery of the body and the fuel for its work (Liebig & Gregory, 1842). About that time, a critical experiment was designed by two Swiss physiologists/chemists Adolf Fick and Johannes Wislicenus to test Liebig’s belief that protein constituted the sole muscle fuel. To do so, they traveled to the base of a mountain in Switzerland with a path to the top that was fairly easy to climb and a hotel at the top. They ate a very low nitrogen diet before and during their experiment, and collected their urine during and for 6 h after their climb (Fick & Wislicenus, 1866). Analysis of the urine samples showed that they had excreted, on average, a quantity of nitrogen equivalent to that contained in 35.0 g protein. They calculated, as best they could, the energy that could have been obtained from the combustion of this quantity of protein and determined it was much less than the work that they had done against gravity in their climbing (Fick & Wislicenus, 1866). Later, Carl Voit, who was trained by Liebig suggested that people with sufficient income would choose a diet that contained an amount of protein they needed to remain healthy and productive. Based on his estimates, the average German workman doing moderate physical work chose to eat 118 g protein/d, and this became his standard (Voit, 1881). About the same time, Atwater found that American workmen were generally better off and ate more protein. He also thought that they worked harder and set a protein intake recommendation at 125 g/d (Atwater, 1887).
Later, Russell Chittenden, a Yale University’s Professor of Physiological Chemistry found some relief from a rheumatic condition by deliberately reducing his general intake of food, and particularly that of meat. Further, he was impressed by having maintained fully both his physical and mental activity; although his intake of protein had not been >40 g/d. Chittenden went on to conduct two longitudinal NB studies of 6 months with US army corpsmen and active university students to show that they were able to maintain their athletic performance and felt better on a 64 g protein/d diet which was about 50% of what was recommended before (Chittenden, 1913). At that time, many other scientists were reluctant to accept Chittenden’s recommendation of such diets as representing “physiological economy,” and argued that the almost universal consumption of high protein diets in prosperous countries showed an important relationship that might not become apparent in short-term trials. Chittenden replied that his critics were reversing cause and effect; people did not become rich because they ate more protein, but ate meat and other more expensive high protein foods because they had already attained an income sufficient to afford them (Carpenter, 2003). Subsequently, considerable studies were conducted in an attempt to determine the fuel source for exercise and by the 1930s it had become clear that a mixture of carbohydrate and fat was the main fuel source for exercise. Further, carbohydrate predominated with intense exercise and as the duration of exercise increased reliance on fat use increased because carbohydrate stores were much more limited. (Carpenter, 1931; Gemmill, 1942). This implied that protein use for exercise was minimal and, therefore, protein was considered unimportant as an exercise fuel. As such, over the following 30 y or so there was little interest in studying the effect of exercise on protein metabolism. However, in the 1970s, reports began to appear demonstrating that nitrogen production tended to increase with prolonged strenuous exercise (Haralambie & Berg, 1976; Refsum & Stromme, 1974) and this brought the potential role of protein as an exercise fuel into the spotlight again. Moreover, these studies underestimated the actual protein use during exercise because both the exercise-induced delay in urinary nitrogen excretion and significance of nitrogen losses via perspiration were not yet appreciated (Lemon & Mullin, 1980). As it became clear that repeated, strenuous, intermittent and/or longer duration exercise could exhaust body carbohydrate stores, it was found that initiation of exercise with low versus high body carbohydrate stores increased nitrogen excretion more than two fold, mainly via sweat nitrogen losses (Lemon & Mullin, 1980). Later, beginning in the 1980s, the use of isotope metabolic tracers become prevalent. These studies demonstrated
that some AA were oxidized during exercise (Lemon & Nagle, 1981; Rennie, et al., 1980; White & Brooks, 1981) and that this oxidation increased as carbohydrate stores become depleted (Wagenmakers, et al., 1991). However, the total quantity of AA oxidized during the exercise is likely no more than about 5-10% of the total energy expended (Lemon, 1998; Tarnopolsky, 2004). As a result, it is now understood that carbohydrate and fat are the major fuel sources for physical exercise. Protein utilization as a fuel source during exercise is not significant. However, this does not mean that the protein requirement of exercise-trained individuals is similar to sedentary individuals because many other factors are important. For example, the post-exercise period is a time when the processes of repair and remodelling in muscle occurs (Hawley, et al., 2011; Koopman, et al., 2007; Moore, et al., 2009a). Of course, this could increase dietary protein requirements considering the increase in muscle mass as well as in mitochondria numbers and oxidative enzymes that occur in regular exercisers. This could especially be true in trained athletes where there is a greater muscle protein synthesis rate compared with breakdown which would imply a positive net protein balance (Damas, et al., 2016).

The debate on whether the dietary protein requirement of the physically active is greater than sedentary individuals continues today and to be resolved will likely require different methodologies. This is not unusual, as indicated in a statement attributed to Dr. Carl Sagan, “scientific debates are often settled not by better arguments but by better instruments and research”. In fact, the use of mass spectrometry, stable isotopes and new research designs to assess protein (or AA) requirements in humans over the past 30 years has provided intriguing research data (Food And Agricultural Organization, 2007; Young, et al., 1985) enabling investigators to more accurately study the kinetics of protein metabolism under a variety of conditions including physical exercise.

2.2 Methodology used in determination of dietary protein requirements

Typically, the determination of protein (or AA) requirements involves the feeding of graded quantities of the test protein (or AA) to participants and looking for a clearly definable change in a relevant biological parameter. Ideally, graded quantities should be fed both above and below
the requirement, as this allows for a more precise determination of the requirement (Pencharz & Ball, 2003). Irrespective of the method used, fundamentally all are a surrogate for measuring protein synthesis which is very difficult to measure directly. As a result, at least theoretically, a similar estimate of requirement should be obtained with all methods (Figure 2.1). However, in practice, the merit of any method with respect to an accurate determination of protein (or AA) requirement depends upon the sensitivity of the response parameter being measured. For example, with nitrogen balance (NB) or the rate of growth (only used in animal for ethical reasons) methods there is a progressive increase in nitrogen balance (or growth rate) in response to the increasing intakes of the test protein (or AA) until the requirement is reached, after which point there is no further increase. Other responses that have been measured in protein (or AA) determination studies include plasma AA concentration, oxidation of a test AA [direct AA oxidation method (DAAO)], or the 24-h balance of the test AA technique (24-h DAAB). Here as graded amounts of the test AA are fed below the requirement there is no change in the response pattern, however once the requirement is reached there is a progressive linear increase in the outcome parameter. With the plasma urea method, the oxidation of an indicator amino acid [indicator amino acid oxidation technique (IAAO)], or the 24-h oxidation of the indicator amino acid methodology (24-h IAAO), the outcome parameter falls as graded quantities of the test protein (or AA) are fed below requirement, until the requirement is reached, after which there is no further change (Figure 2.1).
2.2.1. Nitrogen balance

Nitrogen balance methodology is based on the measurement of the difference between nitrogen intake (food) and nitrogen excretion (urine, faeces, sweat, together with minor losses occurring by other routes [the later are often estimated due to the difficulty of collection]). The requirement of protein (or AA) is determined by measuring NB at varying dietary intakes ranging from deficient to excess, while consuming an otherwise nutritionally complete and adequate diet. The requirement estimate is taken to be the intake which results in nitrogen equilibrium (or the zero balance point) for the sample population of interest (Figure 2.1). One of the first attempts to determine protein (or AA) requirements in humans were the classical NB studies performed by Rose and colleagues between 1943 and 1955 (Rose, 1957) on young, male graduate students. Participants each received mixtures of purified AA plus preparations of cornstarch, sugar, butterfat, corn oil, vitamins and minerals (Rose, 1957). Once NB was established, the specific AA of study would be removed from the diet then added back in a stepwise fashion to determine the amounts that would re-establish balance. The minimum daily requirement was set as the lowest protein (or AA) intake that produced NB in all the subjects and twice that amount was proposed as a safe allowance, i.e., recommended daily intake. However with time, the NB technique has been criticized because of several significant methodological problems (Hegsted, 1976; Millward, 2001; Rand & Young, 1999). Basically, it is difficult to attain exact measurement of intake and output of nitrogen (Hegsted, 1976), to determine the time required for adjustment to variable protein intakes, and/or to measure or account for nitrogen losses through a variety of routes other than urine and faeces (Rand & Young, 1999). The resulting inconsistencies in the balance data have made it difficult to account for several important variables including age, physical activity, and gender (Institute of Medicine, et al., 2005).

In general, the NB technique overestimates intake and underestimates excretion resulting in erroneous positive balances (Fuller & Garlick, 1994; Hegsted, 1976). In addition, following a change in nitrogen intake, the determination of NB must occur after a period of time (at least 7-10 days) to allow physiological steady state to be re-established because complete readjustment in the excretion of nitrogen is not immediate, especially in the adult (Calloway, 1975; Calloway & Margen, 1971). These inherent problems with the NB technique have resulted in research into alternative methods for assessing protein (or AA) adequacy.
2.2.2. **Plasma amino acid concentration**

Plasma free AA concentrations at varying intakes of protein (or AA) have also been investigated as a possible approach for estimating AA requirements in humans (Tontisirin, et al., 1973; Young, et al., 1971). The concept applied here is that the relative plasma concentration of the limiting AA will vary according to dietary supply (Young & Munro, 1973). Earlier studies on animals reported a high degree of correlation between free AA content of the blood and AA in dietary protein (Almquist, 1954) and were suggestive of a relationship between free AA in blood and the requirements of the animal. Consistently, other studies have demonstrated that when an indispensable AA was limiting in the diet there is a corresponding reduction in plasma concentration of that AA (Breuer Jr, et al., 1964; Rose, et al., 1948). Conversely, a dietary indispensable AA excess showed accumulation of that AA in the plasma (Clark, et al., 1963; Scrimshaw, et al., 1966) (Figure 2.1). Therefore, there is adequate evidence of a relationship between dietary AA intake and plasma AA concentrations and it has been postulated that plasma AA concentrations could be used to predict dietary AA needs in animals and humans. Several researchers (Mitchell, et al., 1968; Morrison, et al., 1961; Zimmerman & Scott, 1965) have attempted to establish a plasma AA response curve demonstrating the relationship between plasma AA concentration and graded intakes of single indispensable AA, predicting that the curve would reflect AA requirements for the animal. In human studies, it was found that meal ingestion was associated with an increased concentration in plasma of the limiting dietary AA when meals contained an adequate supply of the AA (Young, et al., 1971). However, below a specific dietary intake quantity, meal ingestion reduced the plasma concentration of the limiting AA below the fasting level. The intake quantity at which the altered plasma AA response occurs has been interpreted as a reflection of an inadequate intake and the minimal physiological requirement for the AA (Tontisirin, et al., 1973; Young & Munro, 1973). While attempting to standardize the plasma AA technique and increase its precision, it was demonstrated in young rats that the point of inflection relating blood concentration to dietary tryptophan intake was more precise when blood was sampled during the active period of nutrient absorption or within six hours of a meal than when sampled after 18 hours (Young & Munro, 1973). In humans, plasma AA concentrations sampled three hours after meals showed the greatest difference to post absorptive concentrations (Young, et al., 1971). The decrease in test AA plasma concentration at
three hours was probably the result of increased AA utilization for hepatic protein synthesis during absorption and the increased uptake of AA due to the actions of insulin released in response to the diet, specifically carbohydrates (Munro, 1970). In the end, it was suggested that both the post absorptive and the fed plasma AA response curve could be used to determine requirements in humans and that three days was sufficient time for metabolic adaptation to a test diet (Young, et al., 1971). However, application of the fasting study protocol has resulted in insensitive plasma responses for lysine (Young, et al., 1972) perhaps because the regulation of AA metabolism in the liver, skeletal muscle and other organs may complicate the relationship between plasma concentration and AA requirement (Ball & Bayley, 1984). Consequently, plasma AA concentration might not provide a very sensitive measure of requirement, because it is the sum of many homeostatic processes controlled by the liver (Ball & Bayley, 1984). Further, only about five percent of the total indispensable AA in the diet appear in the systemic circulation following a meal, suggesting that the liver acts as a buffer to changing dietary AA intakes (Ball & Bayley, 1984; Elwyn, 1970). Importantly, at high dietary AA intakes, the change in plasma concentrations is small relative to the amount of AA digested, oxidized or incorporated into protein (Elwyn, 1970; Reeds & Fuller, 1983) and this might explain why the plasma AA response method has not been used routinely to determine indispensable AA requirements, especially in humans (Ozalp, et al., 1972). Finally, interpretation of the same data by different statistical and non-statistical methods often yields different estimates of requirement because the response curve is usually sigmoidal in shape making the inflection point difficult to ascertain (Robbins, et al., 1979). It is now known that the pattern of changes in plasma AA vary depending on age, the timing of plasma sampling, the length of adaptation to the experimental diet as well as the specific AA being studied so the plasma AA technique is not sensitive enough to be a good protein (or AA) requirement method (Young & Scrimshaw, 1978).

2.2.3. Amino acid oxidation

Amino acid oxidation studies (or direct oxidation) refers to the measurement of the oxidation rate at graded dietary concentrations of that AA. Brookes et al. (Brookes, et al., 1972) were the first to apply this method to determine the lysine requirement in rodents. This technique is based upon the concept that AA in excess of the amounts needed for protein synthesis are oxidized preferentially in the liver. Therefore, when a dietary AA is deficient, most of the AA will be used
efficiently for protein synthesis and oxidation will be remain low and constant (Figure 2.2.1). When the dietary AA increases above the requirement for protein synthesis, increased catabolism of the AA ensues (Ball, 1984; Brookes et al., 1972). The inflection in the oxidation curve has been suggested as the physiological requirement for the test AA (Figure 2.1). Direct evidence for the metabolic significance of the inflection point was illustrated by a study showing that at intakes of lysine below the inflection in the oxidation curve, young rats stopped growing and lost mass, while at lysine intakes above the inflection point, normal growth and mass gain were maintained (Brookes, et al., 1972). Other evidence suggests that direct AA oxidation is an improvement over the measurement of plasma AA from a metabolic perspective. For example, the oxidation of an isotopically labelled AA should provide an estimate of the partition of that AA between oxidation and protein synthesis, because the major portion of AA entering the liver from the portal vein are either oxidized or incorporated into liver protein (Ball & Bayley, 1984). In humans, the most frequent and favoured technique used for oxidation studies is one of continuous isotope infusion which has been described and explored in detail by Waterlow and colleagues (Waterlow, et al., 1978). The continuous infusion of an AA tracer, administered either orally or intravenously, may be either coupled with measurement of end products of nitrogen metabolism or with determination of the isotope (\(^{15}\)N or \(^{13}\)C) enrichment in plasma following administration of a labelled AA. By combining this latter approach with measurements of \(^{13}\)C in expired CO\(_2\) (Matthews, et al., 1980) or \(^{15}\)N in urinary urea or other metabolic or excretory products, the components of whole body AA flux, namely, AA catabolism or oxidation, the incorporation of AA into and their release from tissue protein, can be determined (Figure 2.2).
The criteria for choosing an appropriate AA labelled at its carboxyl carbon for oxidation studies include: 1) it is indispensable; 2) it undergoes no other significant reactions other than oxidation to CO$_2$ and incorporation into protein; 3) the labelled carboxyl CO$_2$ is oxidized irreversibly and its oxidation can be calculated quantitatively by the appearance of label in the breath; and 4) plateau values in both plasma and the breath are reached as quickly as possible, so as to avoid recycling of the label in the body (Wolfe & Chinkes, 2004). Lysine, phenylalanine and leucine are suggested indispensable AA which meet these criteria (Pencharz & Ball, 2003). Using the direct tracer oxidation approach in sedentary young adult men the requirements for the indispensable AA of leucine, valine, lysine and threonine have been determined (Meguid, et al., 1986; Meredith, et al., 1986; Young & Bier, 1987; Zhao, et al., 1986). In these studies, AA flux and oxidation were measured by a constant intravenous infusion of a $^{13}$C-carboxyl labelled AA at...
varying dietary intakes of the specific AA of interest. In general, the results of these studies suggest that the indispensable AA requirements based upon NB methodology (Rose, 1957) have been underestimated by a factor of two or three (Young & Bier, 1987; Young, et al., 1989; Young & Pellett, 1987). Unfortunately, the direct oxidation method is limited because only a few AA can be studied with this approach. Further, there is an increase in the size of the free pool of the test AA with increasing dietary concentrations of that AA which results in a variable dilution of the labelled AA with unlabelled dietary AA. Finally, studies at lower AA intakes are restricted by the amount of isotope infused which has to be included as part of dietary intake (Elango, et al., 2012; Pencharz & Ball, 2003).

2.2.4. **Indicator amino acid oxidation**

To rectify the problems associated with direct oxidation method, a different carbon oxidation model, the indicator amino acid oxidation method (IAAO) was introduced to study adult protein (or AA) requirements (Ball & Bayley, 1984). This method was first developed and validated in growing pigs in comparison with the classical techniques of NB and growth (Kim, et al., 1983). The IAAO model uses an indispensable AA, such as phenylalanine (in the presence of an excess of tyrosine), lysine, or leucine, as the tracer. Importantly, it is independent of the changes in intake of the test protein (or AA) and is based on the concept that when one indispensable AA is deficient for protein synthesis, then all other AA including the indicator AA (usually L-[1-\(^{13}\)C]phenylalanine) are in excess and, therefore, will be oxidized (Pencharz & Ball, 2003).

Primarily this is because excess AA cannot be stored and, therefore, must be partitioned between incorporation into protein or oxidation. With increasing intake of the limiting protein (or AA), oxidation of the indicator AA will decrease, reflecting increasing incorporation into protein synthesis (Kim, et al., 1983). Once the requirement is met for the limiting protein (or AA), there will be no further change in the oxidation of the indicator AA even with increasing intakes of the test AA (Figure 2.1). The inflection point where the oxidation of the indicator AA stops decreasing and reaches a plateau is referred to as the ‘breakpoint’. Consequently, the inflection point is considered a reverse proxy for the whole body protein synthesis where increases in protein (or AA) intake beyond what is provided do not result in further increases in whole body
protein synthesis. The breakpoint, identified with the use of two-phase linear regression analysis, indicates the estimated average requirement (EAR) of the test protein (or AA). In the early animal studies it was shown that the IAAO method requires minimal adaptation (a few hours) to changing test protein (or AA) intakes, which is a huge advantage vs the NB technique (Ball & Bayley, 1986). Further studies in humans confirmed that minimal adaptation was needed (Elango, et al., 2009; Zello, et al., 1990). In addition, the lysine requirement of malnourished Indian school age children was similar to that of well-nourished Canadian children (Elango, et al., 2007; Pillai, et al., 2010), indicating that habitual protein intake of the participants is also not a factor. So, the indicator amino acid oxidation technique is based on the partitioning of the indicator AA either to protein synthesis or to oxidation in which any adaptation occurs primarily at the acyl-t-RNA level, which adapts in less than four hours (Pencharz & Ball, 2003). Based on these studies and the earlier animal work (Ball & Bayley, 1986; Elango, et al., 2009; Kim, et al., 1983; Zello, et al., 1990), it was concluded that a prior 2d adaptation to the level of the test protein/AA intake was sufficient. So at present, the IAAO method is considered as an approach which eliminates most of the limitations encountered in other requirement methods (Food And Agricultural Organization, 2007). However, despite its use in several studies determining protein requirements in sedentary individuals, there is only one report on its use in physically active individuals (Kato, et al., 2016). Therefore, the purpose of this thesis was to determine protein requirement of both strength and endurance exercise-trained individuals using IAAO method.

2.3 Protein requirement for strength and endurance-trained athletes

Exercise has a profound effect on muscle and whole body protein metabolism. The induced response in muscle is affected by the type of exercise (endurance vs strength) affecting myofibrillar or mitochondrial and sarcoplasmic protein subfractions (Atherton & Smith, 2012; Wilkinson, et al., 2008). In healthy adult skeletal muscle proteins have a daily turnover rates of about 1.2% in a dynamic equilibrium such that muscle protein breakdown in the fasted state (negative balance) is balanced out with an increase in muscle protein synthesis in the fed state. The two principal determinants of adult skeletal muscle maintenance are physical activity and nutrient availability. The anabolic effects of nutrition are principally driven by the transfer and
incorporation of amino acids from dietary protein sources, into skeletal muscle proteins. The purpose of this is to compensate for muscle protein that is lost in fasted (post-absorptive) periods due to, for example, amino acid oxidation and/or carbon donation for liver gluconeogenesis (Wackerhage & Rennie, 2006). These dynamic “fasted-loss/fed-gain” cycles throughout the day is the determinant of the whole body protein maintenance in balance. Subsequently, it has been shown that the anabolic effect of feeding in the form of an increased protein synthesis is transient and saturates by a bolus intake of about 10 g essential AA (Cuthbertson, et al., 2005) equivalent to ~20 g protein (Moore, et al., 2009). However, this transient increase in protein synthesis is compensated by a similar adaptive response of protein breakdown hence the result would be a net balance with no protein mass gained.

With an acute bout of exercise in untrained individuals the response of an increased muscle protein synthesis is prolonged beyond that can be accomplished just by feeding. This is due to a greater sensitivity of muscle protein to protein/AA intake (Burd, et al., 2011). It is reported that dependant on the training status of the individual increase in muscle protein synthesis can last up to 24 to 48 h post-exercise (Atherton & Smith, 2012). However, as an athlete becomes more trained the law of diminishing returns applies in that training shortens the duration of the anabolic response perhaps to 24 h duration following exercise session (Tang, et al., 2008). Further, recently it has been shown that in exercise-trained individuals the resultant increase in muscle hypertrophy is due to a discordant increase in muscle protein synthesis relative to a decreased muscle protein breakdown (Damas, et al., 2016). Without post-exercise protein supplementation, protein synthesis is limited because of the decreased availability of AA.

Further, it has been shown that without post-exercise protein/AA provision there is a prolonged effect of muscle protein breakdown relative to protein synthesis and this would lead to a maladaptation (Biolo, et al., 1995). However, increasing dietary protein/AA availability after exercise enhances both the magnitude and duration of the increase in muscle protein synthesis (Pennings, et al., 2011). Consideration of whole body protein synthesis is also important. Provision of 10 to 16 g of protein after an acute bout of moderate endurance exercise for ~ 60 min has been shown to enhance muscle protein synthesis and net muscle protein balance despite a negative whole body protein balance (Levenhagen, et al., 2002; Lunn, et al., 2012).
Today, dietary protein requirements may be the most debated macronutrient among both exercise-trained individuals and scientists and it has been that way for many years. In fact, numerous reviews have been published on this topic from a variety of perspectives (Lemon & Nagle, 1981; Lemon, 1996, 1997, 1998, 2000; Phillips, 2004, 2012; Phillips & Van Loon, 2011). Generally, there are two schools of thought - one suggesting that regular participation in exercise or sport increases protein requirements and another stating that the requirement of exercise-trained individuals are no different from sedentary individuals. The methodology used in defining protein requirement in exercise-trained individuals has been based on NB method primarily (Friedman & Lemon, 1989; Lemon, et al., 1992; Tarnopolsky, et al., 1992; Tarnopolsky, et al., 1988). Here the requirement is defined as the minimum amount necessary to maintain NB and prevent deficiency symptoms (Institute of Medicine, et al., 2005). However, this is problematic because it has been shown that nitrogen equilibrium can be maintained even on a less than adequate protein (or AA) intake through “accommodation” (increased nitrogen utilization efficiency and lower whole body protein synthesis) (Butterfield, 1987; Butterfield & Calloway, 1984; Young, et al., 1987; Young, et al., 1989; Young & Marchini, 1990). Further, application of NB as an end point for protein requirement in exercise-trained individuals may not be suitable (Phillips, 2012) because exercise-trained individuals, whether strength or endurance, aim to consume adequate amounts of dietary protein to induce a positive protein balance via increased protein synthesis rates. Based on earlier stable isotope studies (Young, et al., 1987; Young & Bier, 1987c; Young, et al., 1989; Young & Marchini, 1990) assessing both protein and AA requirements, four different states of protein metabolism have been identified: 1) “protein deficiency” defined as the maximal reduction in protein synthesis to all but the essential organs, 2) “accommodation” where NB is achieved with a decrease in physiological relevant processes, 3) “adaptation” where optimal growth, inter-organ AA exchange, and immune function are present and 4) “excess” which is characterized by AA oxidization for energy and nitrogen excretion via urea, resulting in no further stimulation of protein synthesis (Young, et al., 1981).

When a protein intake close to the current RDA is combined with strength or endurance training accommodation can occur through increased nitrogen utilization efficiency and/or decreased whole body protein synthesis rates which may not be conducive to optimal training-induced adaptation (Butterfield & Calloway, 1984; Campbell, et al., 1995; Campbell & Leidy, 2007; Tarnopolsky, et al., 1992; Torun, et al., 1977). Consequently, it has been suggested that stable
isotope tracer methodology would be a more accurate technique for the determination of protein requirement than NB (Lemon, et al., 1992; Tarnopolsky, et al., 1992; Young & Marchini, 1990). Based on the discussion above on methodologies, the IAAO method is probably the most suited one to be used to determine protein (or AA) requirement because it eliminates many of the shortcomings of the other methods. As such, the purpose of this thesis was to determine dietary protein requirements of ST and ET using the IAAO technique.
2.4 References


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Chapter 3.

Indicator Amino Acid Derived Estimate of Dietary Protein Requirement for Strength-Trained Athletes at Rest is Several Fold Greater than the Current RDA
3.1 Abstract

Background: Despite a number of studies indicating increased dietary protein needs in strength-trained individuals (ST) using the nitrogen balance (NB) technique, the Institute of Medicine (2005) has concluded, based largely on methodological concerns, that “no additional dietary protein is suggested for healthy adults undertaking resistance or endurance exercise”.

Objective: The objective of this study was to determine the dietary protein requirement of healthy young ST men (≥3 y training experience) at rest on a non-training day by measuring the oxidation of ingested L-[1-\(^{13}\)C] phenylalanine to \(^{13}\)CO\(_2\) in response to graded intakes of protein (Indicator Amino Acid Oxidation [IAAO] Technique).

Method: Eight men (22.5±1.7y; 83.9±11.6kg; 13.0±6.3% body fat) were studied on a non-training day, each on several occasions (4-8 times) with protein intakes ranging from 0.1 to 3.5 g•kg\(^{-1}\)•d\(^{-1}\) for a total of 42 experiments. The diets provided energy at 1.5 times each individual’s measured resting energy expenditure and were isoenergetic across all treatments. Protein was fed as an amino acid (AA) mixture on the basis of the protein pattern in egg, except for phenylalanine and tyrosine, which were maintained constant across all protein intake quantities. For two days prior to the study day, the participants accommodated to a dietary protein of 1.5 g•kg\(^{-1}\)•d\(^{-1}\). The mean dietary protein requirement was determined by applying a mixed-effects change-point regression analysis to F\(^{13}\)CO\(_2\) (label tracer oxidation in \(^{13}\)CO\(_2\) breath), which identified a breakpoint in the F\(^{13}\)CO\(_2\) in response to graded amounts of protein.

Results: The estimated average protein requirement (EAR) and upper 95% CI (approximating the recommended dietary allowance (RDA)) for these young ST men were 1.7 and 2.2 g•kg\(^{-1}\)•d\(^{-1}\), respectively.

Conclusion: These IAAO data suggest that the protein EAR and RDA for ST men at rest on a non-training day exceed the current recommendations by ~2.6 fold.

Key Words: protein requirement, strength-trained individuals, indicator amino acid oxidation, stable isotope
3.2 Introduction

Historically, the dietary protein requirement has been determined using nitrogen balance (NB) data; however, this measurement technique has been criticized widely because of a variety of methodological concerns (Fuller & Garlick, 1994; Millward, 2001; Pencharz & Ball, 2003; Rand & Young, 1999). Two of the main concerns are the use of a linear regression line for analyzing nonlinear data (Rand & Young, 1999; Zello, et al., 1995) and an over/underestimation of nitrogen intake and excretion, respectively (Hegsted, 1976). Specifically, it has been suggested that applying a single linear regression line with a greater residual error, is not a good fit for either NB or oxidation data points (Zello, et al., 1995). As such, data analysis that locates the breakpoint more accurately in NB and/or in oxidation studies is one key to determining accurate dietary AA or protein requirements. Interestingly, a bilinear regression model has been used on the NB results of 28 published studies in which repeated measurements were made within the same individuals and the reported breakpoint in the data for either zero NB or oxidation of L-[1-13C] phenylalanine using the indicator amino acid oxidation technique (IAAO) was at a protein intake of 0.93 g·kg⁻¹·d⁻¹ (Humayun, et al., 2007). This estimate exceeds the current requirement by >50%. In addition, the repeated 7 to 10 d adaptation periods required to produce accurate NB data at each of the several different protein intakes are not really practical. Moreover, the available NB results on ST are limited and quite variable (Lemon, et al., 1992; Tarnopolsky, et al., 1992; Tarnopolsky, et al., 1988). As a result, the current dietary protein recommendation for ST varies widely from the RDA of 0.85 g·kg⁻¹·d⁻¹ established by Institute of Medicine (Institute of Medicine, et al., 2005) to as much as 1.7 g·kg⁻¹·d⁻¹ (Rodriguez, et al., 2009). Interestingly, the habitual protein intake of ST has been reported to be ~2-4 g·kg⁻¹·d⁻¹ depending on training phase (Faber & Benade, 1987; Helms, et al., 2014; Steen, 1991). This might be important as over the years of trial and error, these individuals might have figured out, as an observation, the amount of protein that provides the best end result. Further, it is possible that NB may be achievable at low protein intakes for the brief study durations often used because of a more efficient AA utilization, reduced turnover rates, and/or accommodation (Pellet, 1990; Young, et al., 1987; Young & Marchini, 1990). Likely, this scenario would be unfavorable for ST attempting to hypertrophy or even simply to maintain their enlarged muscle mass. Finally, even very positive NB (3.8 to 20 g·d⁻¹) with protein intakes of 1.8 to 2.7 g·kg⁻¹·d⁻¹ observed in ST individuals do
not result in the expected fat-free mass accrual (Lemon, et al., 1992; Tarnopolsky, et al., 1988). Of course, the major goal of ST is to increase lean body mass which requires that muscle protein synthesis exceed protein breakdown over time, hence a positive net protein synthesis. Therefore, it seems reasonable to assume that the optimal method to determine the dietary protein requirement for ST must involve an assessment of protein turnover.

One such method is the indicator amino acid oxidation (IAAO) technique, which is based on the concept that when dietary protein is inadequate, the oxidation of all AA, including the indicator AA, will be substantial. With increasing dietary protein, oxidation of the indicator AA will decrease because more AA are being incorporated into body protein. Once the dietary requirement is met, there is no further change in the oxidation of the indicator AA and the resulting inflection or ‘breakpoint’ is thought to be the requirement (Pencharz & Ball, 2003). According to the Institute of Medicine, the IAAO technique is an acceptable method to assess protein or AA requirements (Institute of Medicine, et al., 2005).

The purpose of our study was to quantify the daily dietary protein requirement of ST men at rest on a non-training day using the IAAO technique. We hypothesized that protein requirement of ST men is greater than both RDA and the results determined using NB method.

3.3 Subjects and Methods

Participants

Potential candidates were invited for an interview via postings at the Western University campus athletic and recreation centre. During the interviews each candidate was informed of the study procedures and any who participated in our study signed an informed consent statement previously approved by Western University’s Health Sciences Research Ethics Board. The inclusion criteria were: 1) healthy men between 18 to 40 y of age, 2) at least 3 y of weight-training experience, ≥4 d\(^{-1}\)•wk\(^{-1}\),~1 h\(^{-1}\).d\(^{-1}\), 3) body mass stable (<4 kg mass gain or loss within the past 6 mo), 4) nonsmoker, 5) <2 alcoholic drinks\(^{-1}\)•d\(^{-1}\), 6) nonmedication user (including anabolic steroids), and 7) without allergies to milk or milk products (because Boost\(^\text{®}\) meal replacement
and whey protein isolate were given as part of the experimental diet). All participants passed the physical activity readiness questionnaire (PAR-Q) health survey (Thomas, et al., 1992) and all were screened for muscularity using published values of Mr. USA winners during the pre-steroid era (1939-1959) (Kouri, et al., 1995). Further, to ensure that only participants near their respective maximal attainable muscularity were selected, an Index of Muscularity (IOM) (%) was calculated using the FFM (kg) of each exercise-trained individuals over the FFM (kg) of past Mr. USA winners, normalized for the height of each participant (Index of Muscularity (%) = FFM ÷ [height² × (25.4 - (6.1 × (1.8 - height)))]) × 100 (Kouri, et al., 1995). Only those with an IOM ≥90% were selected. All participants engaged in strength training primarily with minimal aerobic activity (~20 min of walking•wk⁻¹) in their training routine.

Study Design

This study used the minimally invasive IAAO technique (Bross, et al., 1998) as described previously in several studies (Humayun, et al., 2007; Rafii, et al., 2015; Stephens, et al., 2015; Tang, et al., 2014). For the pre-study assessment and to prevent any acute effect of exercise on the measurements, participants abstained from training for 48-h prior to visiting the laboratory. During this initial visit, which followed a 12-h overnight fast, both resting energy expenditure (REE) and body composition [fat-free mass (FFM) and fat mass] were measured using open-circuit indirect calorimetry (Vmax Legacy, Sensor Medics, CA, USA), and air displacement plethysmography with a BodPod® (Life Measurements, Concord, CA), respectively.

Each dietary protein quantity given was studied over a 3-d period (two adaptation days followed by an IAAO study day (Elango, et al., 2009)). Participants did not train on the two days prior to the study day in order to eliminate any acute effect of exercise on measuring protein requirement. During the adaptation days, participants received Boost® (Nestlé) meal replacement as a maintenance diet supplemented with Polycose® (Abbot Nutrition) and lactose-free, gluten-free whey protein isolate (Kaizen Protein) providing 1.5 g protein•kg⁻¹•d⁻¹ and 1.7 × REE energy. On the third day (the study day), participants arrived at the laboratory after a 12-h fast and were assigned randomly to receive test protein intakes ranging from 0.1 to 3.5 g•kg⁻¹•d⁻¹ and 1.5 REE energy (lesser energy here due to the largely sedentary measurement day). Four participants
were each tested at 4 protein intakes and single participants at each of 5, 6, 7, and 8 protein intakes for a total of 42 IAAO studies. Each 3-d study period was separated by at least one week.

**Study Diets**

The adaptation/study diets (described above) provided all of the participant’s macronutrient needs on the basis of the current DRI. During the two adaptation days, the daily diet was consumed as four equal meals and participants did not consume any other food items except one cup of clear tea or coffee and water ad libitum. Participants were also provided a multivitamin supplement (Centrum®, Wyeth Consumer Health Care) and fibre (RestoraLax®, Bayer) daily for the duration of all studies.

On the third or IAAO study day, participants arrived at the laboratory where they consumed eight hourly isoenergetic meals, each meal representing one-twelfth of the daily energy/protein requirement. The study day diet consisted of a protein- and AA-free powder (PDF1, Mead Johnson), flavored drink crystals (Tang and Kool-Aid, Kraft Foods), grape seed oil, a crystalline AA mixture (Ajinomoto Amino Science LLC) patterned after egg protein (Table 3.1) and protein-free cookies. Energy intake was set at 1.5 × REE and the carbohydrate content of the diets was adjusted based on the protein to keep the diets isoenergetic.

**Tracer Protocol**

An oral priming doses of 0.176 mg NaH\(^{13}\)CO\(_3\)•kg\(^{-1}\) body mass (99 atom percent excess, Cambridge Isotope Laboratories) and 0.66 mg L-[\(^{1-13}\)C] phenylalanine•kg\(^{-1}\) started with the fifth hourly meal. In addition, a dose of L-[\(^{1-13}\)C] phenylalanine (1.2 mg•kg\(^{-1}•h^{-1}\)) was started with the fifth meal and continued hourly for the remaining 3 h of the study. The quantity of L-[\(^{1-13}\)C] phenylalanine supplied during the last 4 h of the study was subtracted from the diet to provide a total intake of 25 mg phenylalanine•kg\(^{-1}•d^{-1}\) and tyrosine was provided at 40 mg•kg\(^{-1}•d^{-1}\) to ensure an excess of tyrosine (Shiman & Gray, 1998; Zello, et al., 1990).
Table 3.1 Amino acid composition of reference protein and test protein intakes

<table>
<thead>
<tr>
<th></th>
<th>Reference protein, mg·g⁻¹</th>
<th>Test protein intake, g·kg⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1</td>
<td>1.0</td>
</tr>
<tr>
<td>L-Alanine</td>
<td></td>
<td>61.5</td>
</tr>
<tr>
<td>L-Arginine-HCL³</td>
<td>75.1</td>
<td>7.51</td>
</tr>
<tr>
<td>L-Asparagine</td>
<td>33.3</td>
<td>3.33</td>
</tr>
<tr>
<td>L-Aspartic acid</td>
<td>33.3</td>
<td>3.33</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>22.1</td>
<td>2.21</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>56.6</td>
<td>5.66</td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>56.6</td>
<td>5.66</td>
</tr>
<tr>
<td>Glycine</td>
<td>33.3</td>
<td>3.33</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>22.7</td>
<td>2.27</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>62.8</td>
<td>6.28</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>83.3</td>
<td>8.33</td>
</tr>
<tr>
<td>L-Lysine-HCL³</td>
<td>75.7</td>
<td>7.57</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>29.6</td>
<td>2.96</td>
</tr>
<tr>
<td>L-Phenylalanine⁴</td>
<td>30.0</td>
<td>25.0</td>
</tr>
<tr>
<td>L-Proline</td>
<td>41.9</td>
<td>4.19</td>
</tr>
<tr>
<td>L-Serine</td>
<td>83.9</td>
<td>8.39</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>47.1</td>
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<tr>
<td>L-Tryptophan</td>
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<td>1.56</td>
</tr>
<tr>
<td>L-Tyrosine⁵</td>
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<td>4.00</td>
</tr>
<tr>
<td>L-Valine</td>
<td>70.3</td>
<td>7.03</td>
</tr>
</tbody>
</table>

¹participants received several test protein quantities ranging from 0.1 to 3.5 g protein·kg⁻¹·d⁻¹.
²represents the egg protein composition.
³actual amounts of amino acids were as follows: 62.1 mg arginine·g⁻¹ and 60.6 mg lysine·g⁻¹.
⁴L-Phenylalanine intake was kept constant at 25.0 mg·kg⁻¹·d⁻¹.
⁵L-Tyrosine intake was kept constant at 40.0 mg·kg⁻¹·d⁻¹.

Sample Collection and Analysis

During each study day, three baseline breath and urine samples were collected at 45, 30, and 15 min before the tracer protocol began and five total breath and urine samples were collected one every 30 min beginning at 2.5 h after administration of the tracer (L-[1-¹³C] phenylalanine) in order to establish that an isotopic steady state was attained. Breath samples were collected in disposable Exetainer tubes (Labco) with a collection mechanism (Easy-Sampler, Quintron) that permitted the removal of dead-space air. Breath samples were stored at room temperature, and urine samples were stored at −20 ºC until analysis. During each study day, the rate of carbon
dioxide production (VCO$_2$) was measured immediately after the fifth meal for a period of 20 min with an indirect calorimeter (Vmax Legacy, Sensor Medics, CA, USA).

Expired $^{13}$CO$_2$ enrichment was measured with a continuous-flow isotope ratio mass spectrometer (CF-IRMS 20/20 isotope analyzer, PDZ Europa). Enrichments were expressed as atom percent excess compared with a reference standard of compressed CO$_2$. Urinary L-[1-$^{13}$C] phenylalanine enrichment was analyzed by an API 4000 triple quadrupole mass spectrometer (Applied Biosystems/MDS Scieix) in positive electrospray ionization mode (Rafii, et al., 2015). Isotopic enrichment was expressed as mol percent excess and calculated from peak area ratios at isotopic steady state both at baseline and plateau.

**Estimation of Isotope Kinetics**

L-[1-$^{13}$C] phenylalanine kinetics were calculated with use of the stochastic models described previously (Matthews, et al., 1980). Isotopic steady state in the tracer enrichment at baseline and plateau was represented as unchanging values of L-[1-$^{13}$C] phenylalanine in urine and $^{13}$CO$_2$ in breath.

Phenylalanine flux ($\mu$mol$\cdot$kg$^{-1}$$\cdot$h$^{-1}$) was calculated from the dilution of the orally administered L-[1-$^{13}$C] phenylalanine into the metabolic pool (at steady state) by using the enrichment of L-[1-$^{13}$C] phenylalanine in urine. The rate of appearance of $^{13}$CO$_2$ in breath ($F^{13}$CO$_2$ $\mu$mol$\cdot$kg$^{-1}$$\cdot$h$^{-1}$) after the oxidation of ingested L-[1-$^{13}$C] phenylalanine was calculated according to the model of Matthews et al. (Matthews, et al., 1980) using a factor of 0.82 to account for carbon dioxide retained in the body’s bicarbonate pool (Hoerr, et al., 1989).

**3.4 Statistical Analysis**

All results are reported as means±SD. Statistical analyses were performed by using SAS (Version 9.2.1; SAS Institute Inc). Significance was set at $P \leq 0.05$. Participants were assigned randomly to differing protein intakes with protein quantity as the independent variable. The
effect of protein intake on phenylalanine flux, oxidation, and F\textsuperscript{13}CO\textsubscript{2} was tested by using a mixed linear model (PROC MIXED) with subject as a random variable. The mean protein requirement was estimated by applying a nonlinear mixed-effects model (PROC NLMIXED; SAS Institute) to the F\textsuperscript{13}CO\textsubscript{2} data (Rafii, et al., 2015). Observations within subjects were regarded as statistically dependent. Confidence intervals (CI) were obtained by following the standard asymptotic theory of the maximal likelihood estimation. The model minimizing the Akaike information criterion was regarded as the model with the best fit (Hayamizu, et al., 2011). The following statistical model was used, accounting for correlations within observations from the same subject: \( Y_{id} = \beta_0 + b_i + \beta_1 I(x_{id} > x_{cp})(x_{id} - x_{cp}) + \epsilon_{id}, \)

where \( Y_{id} = F^{13}CO_2 \) or phenylalanine oxidation at the dose of the protein of \( i \), \( x_{id} \) is the dose amount of the test protein intake of the \( i \)-th participant, \( \epsilon_{id} \) are random errors that are independently normally distributed with a mean of 0 and variance of \( \sigma^2 \), \( \beta_0 \) is the left line intercept, \( b_i \) is the random intercept that incorporates within-subject correlation, \( \beta_1 \) is the left line slope, \( x_{cp} \) is the breakpoint, and the slope for \( x_{id} \) is 0 for \( x_{id} \) more than breakpoint.

3.5 Results

Participant Characteristics

Eight ST men (≥3 y of consistent strength training experience, ≥4 d•wk\textsuperscript{-1}, ~1 h•d\textsuperscript{-1} with minimal aerobic activity in their training, ~20 min•wk\textsuperscript{-1}) participated in the study. All subjects were non-competing natural strength/bodybuilding trained individuals. The normalized IOM for the respective height of each participant was ≥90% (95.9 ± 5.2%) that of past Mr. USA winners (Table 3.2) or ~16-20 kg more FFM than healthy young non-strength-trained individuals of similar height in a previous study (Humayun, et al., 2007). Habitual protein intake based on 3-d dietary record analysis was 2.4±0.8 g•kg\textsuperscript{-1}•d\textsuperscript{-1} which is about three times the current RDA.
Table 3.2 Physical Characteristics and daily energy and macronutrient intake of the strength-trained men studied¹

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>22.5±1.7</td>
</tr>
<tr>
<td>Mass, kg</td>
<td>83.9±11.6</td>
</tr>
<tr>
<td>Height, m</td>
<td>1.74±0.1</td>
</tr>
<tr>
<td>Body fat</td>
<td>13.0 ±6.3</td>
</tr>
<tr>
<td>FFM, kg</td>
<td>72.4±5.5</td>
</tr>
<tr>
<td>FFMI, kg•m⁻²</td>
<td>24.0±1.3</td>
</tr>
<tr>
<td>Index of muscularity (%)³</td>
<td>95.9±5.2</td>
</tr>
<tr>
<td>REE⁴, kcal•kg⁻¹•d⁻¹</td>
<td>22.3±2.2</td>
</tr>
<tr>
<td>Energy intake⁵, kcal•kg⁻¹•d⁻¹</td>
<td>37.0±7.0</td>
</tr>
<tr>
<td>Carbohydrate intake⁵, g•kg⁻¹•d⁻¹</td>
<td>3.9±1.6</td>
</tr>
<tr>
<td>Protein intake⁵, g•kg⁻¹•d⁻¹</td>
<td>2.4±0.8</td>
</tr>
<tr>
<td>Fat intake⁵, g•kg⁻¹•d⁻¹</td>
<td>1.3±0.5</td>
</tr>
</tbody>
</table>

¹All values are means±SD, n= 8.
²Body fat was measured using Bod Pod® (Life Measurements, Concord, CA).
³Index of Muscularity (%) was calculated from FFM (kg) of each exercise-trained individuals over FFM (kg) of past Mr. USA winners (FFMI = 25.4 kg•m⁻²) and subsequently normalized for the height of each participant: Index of muscularity (%) = FFM ÷[height² × (25.4 - (6.1 × (1.8 - height))))] ×100 (Kouri, et al., 1995).
⁴Resting energy expenditure measured by open circuit indirect calorimetry (Vmax Legacy, Sensor Medics, CA, USA).
⁵Energy intake and macronutrient breakdown were based on a 3-d dietary record analysis.

**Phenylalanine Flux**

Phenylalanine flux was not affected within each exercise-trained individuals by the different protein intakes (P=0.35) as required by the IAAO method (Table 3.3). This provides evidence that the precursor pool for the IAAO did not change significantly with increasing test protein
intakes, and suggests that the changes in oxidation were inversely proportional to whole-body protein synthesis. Whole body phenylalanine flux was 67.4±3.8 µmol•kg⁻¹•h⁻¹.

Table 3.3 Protein intakes and phenylalanine flux

<table>
<thead>
<tr>
<th>Subject</th>
<th>Test protein intakes g•kg⁻¹•d⁻¹</th>
<th>Phenylalanine flux µmol•kg⁻¹•h⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.1, 1.6, 2.0, 2.3, 3.2</td>
<td>59±6</td>
</tr>
<tr>
<td>2</td>
<td>0.5, 1.0, 1.1, 1.5, 1.8, 2.1, 2.6, 3.3</td>
<td>63±9</td>
</tr>
<tr>
<td>3</td>
<td>0.7, 1.0, 1.6, 2.2</td>
<td>77±12</td>
</tr>
<tr>
<td>4</td>
<td>0.8, 1.2, 1.7, 2.0, 2.5, 2.9, 3.5</td>
<td>60±12</td>
</tr>
<tr>
<td>5</td>
<td>0.9, 1.7, 2.7, 3.4</td>
<td>72±9</td>
</tr>
<tr>
<td>6</td>
<td>0.9, 1.1, 1.4, 1.6, 2.2, 3.0</td>
<td>68±10</td>
</tr>
<tr>
<td>7</td>
<td>1.3, 1.9, 2.4, 3.1</td>
<td>61±5</td>
</tr>
<tr>
<td>8</td>
<td>1.3, 1.8, 2.8, 3.3</td>
<td>58±8</td>
</tr>
<tr>
<td>All</td>
<td></td>
<td>67.4±3.8</td>
</tr>
</tbody>
</table>

¹Values are means±SD. No significant differences (P=0.35) in phenylalanine flux were observed within each subject across all test protein intakes (0.1 to 3.5 g•kg⁻¹•d⁻¹). Each subject completed a minimum of 4 test intakes for a total of 42 studies.

**L-[1⁻¹³C] phenylalanine**

Nonlinear mixed-effects change-point regression analysis of the F¹³CO₂ data resulted in a breakpoint at a protein intake of 1.7 g•kg⁻¹•d⁻¹, i.e., the rate of¹³CO₂ released from the oxidation of L-[1⁻¹³C] phenylalanine (F¹³CO₂) declined in response to increasing protein intakes up to 1.7 g•kg⁻¹•d⁻¹ (Figure 3.1), such that additional increases in protein intake did not result in changes in F¹³CO₂ values. This indicates that the incorporation of the phenylalanine for protein synthesis plateaued at a protein intake of 1.7 g•kg⁻¹•d⁻¹ (r² = 0.67) and suggests this is mean dietary requirement of these strength exercise-trained individuals. The upper 95% CI of the breakpoint was at 2.2 g•kg⁻¹•d⁻¹, suggesting the RDA for protein of strength-trained individuals should be near this intake. The lower CI was 1.2 g•kg⁻¹•d⁻¹.
Figure 3.1 Protein intake and production of $^{13}$CO$_2$ from phenylalanine oxidation ($F^{13}$CO$_2$) in young strength trained men ($n = 42$). Individual values for each exercise-trained individual are represented by different symbols. The breakpoint represents the estimated mean protein requirement. A mixed-effects change-point regression analysis identified a breakpoint and upper 95% CI for the relation between protein intake and phenylalanine oxidation to be 1.7 ($r^2 = 0.67$) and 2.2 g·kg$^{-1}$·d$^{-1}$, respectively.

3.6 Discussion

Typically, ST report much greater protein intakes than their inactive peers and previous NB studies with ST (Lemon, et al., 1992; Tarnopolsky, et al., 1992) have measured a protein requirement and RDA of about 1.4 and 1.7 g·kg$^{-1}$·d$^{-1}$, respectively. Further, a meta-analysis of 680 ST (>6wk) young (23±3y) as well as older (62±6y) individuals concluded that receiving a supplement of 50±32 g protein per day in addition to a regular dietary intake of ~1.2 g·kg$^{-1}$·d$^{-1}$ (daily protein intake of ~1.8 g·kg$^{-1}$·d$^{-1}$) increased skeletal muscle mass and strength gains (Cermak, et al., 2012). Moreover, recently Snijders et al (Snijders, et al., 2015) confirmed that 3 months of strength training together with a total daily protein intake of 1.9 (nightly protein supplementation before sleep) compared with 1.3 g·kg$^{-1}$·d$^{-1}$ resulted in increased skeletal muscle
mass, strength, and muscle fibre size in young men (22±1 y). Our current study is the first to
determine the protein requirement in ST men using the IAAO technique and the results indicate
that the estimated protein requirement and RDA are 1.7 and 2.2 g•kg⁻¹•d⁻¹, respectively (Figure
1), which is about 23% greater than the results reported previously for ST men using the NB
method (Lemon, et al., 1992; Tarnopolsky, et al., 1992). Interestingly, this increase is consistent
with published reports of the protein requirement for sedentary individuals using IAAO, in that
those data also exceeded the published NB protein requirement set by the Institute of Medicine
by about 30–40% (Elango, et al., 2011; Humayun, et al., 2007; Stephens, et al., 2015; Tang, et
al., 2014).

In this study for the two adaptation days a protein intake of 1.5 g•kg⁻¹•d⁻¹ was used. Although
lower relative to participant’s habitual protein intake (2.4±0.8 g•kg⁻¹•d⁻¹), this selection was
based the protein requirement determined with previous NB studies (Lemon, et al., 1992;
Tarnopolsky, et al., 1992) and in line with the recommended guidelines of the Academy of
Nutrition and Dietetics, Dietitians of Canada and the American College of Sport Medicine
(Thomas, et al., 2016). Importantly, habitual protein/AA intake levels have been shown not to be
a factor in determination of protein/AA requirement in IAAO technique (Elango, et al., 2009;
Pillai, et al., 2010; Zello, et al., 1990). As discussed previously, inaccuracies inherent in the NB
method due to overestimation of intake and underestimation of losses, slow physiological urea
pool adaptation to altered protein intake, confounding effects of energy intake, true nitrogen
accretion below the limits of detection, and loss of nitrogen could explain a protein requirement
underestimation (Tarnopolsky, et al., 1992). However, the regression model used is also a factor
because it has been shown that when repeated measures of NB data from different protein
intakes on the same participants are analyzed using a biphase linear regression model, the
estimates of protein requirement by NB and IAAO technique become similar (Humayun, et al.,
2007). The young men in those studies, although of similar height and age to our current ST
participants, were sedentary (Table 3.4) (Humayun, et al., 2007) and, therefore, not surprisingly,
had a much lower body mass (~16 kg less FFM mass). Of course, to accrue FFM, a chronic net
positive protein and energy balance is necessary and is consistent with the observed 1.8 times
greater protein requirement for our ST men (Table 3.4). Further, whole body phenylalanine flux
in the fed state at rest was about 15.2% or 1.1 times greater \((P= 0.02)\) in the ST men compared with their sedentary counterparts.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Strength trained men(^2)</th>
<th>Sedentary young men(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>22.5±1.7</td>
<td>26.8±2.0</td>
</tr>
<tr>
<td>(N)</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Height, m</td>
<td>1.7±0.1</td>
<td>1.7±2.6</td>
</tr>
<tr>
<td>Mass, kg</td>
<td>83.9±11.6(^*)</td>
<td>69.6±3.7</td>
</tr>
<tr>
<td>FFM, kg</td>
<td>72.4±5.5(^*)</td>
<td>56.4±1.9</td>
</tr>
<tr>
<td>Phenylalanine flux, (\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{BM} \cdot \text{h}^{-1})</td>
<td>67.4±3.8 (^*)</td>
<td>58.5±14</td>
</tr>
<tr>
<td>Phenylalanine flux, (\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{FFM} \cdot \text{h}^{-1})</td>
<td>76.3±11.7</td>
<td>71.9±18.7</td>
</tr>
<tr>
<td>Protein requirement (g \cdot \text{kg}^{-1} \cdot \text{BM} \cdot \text{d}^{-1})</td>
<td>1.72</td>
<td>0.93</td>
</tr>
<tr>
<td>Protein requirement (g \cdot \text{kg}^{-1} \cdot \text{FFM} \cdot \text{d}^{-1})</td>
<td>2.0</td>
<td>1.1</td>
</tr>
<tr>
<td>Protein recommendation (g \cdot \text{kg}^{-1} \cdot \text{BM} \cdot \text{d}^{-1})</td>
<td>2.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Protein recommendation (g \cdot \text{kg}^{-1} \cdot \text{FFM} \cdot \text{d}^{-1})</td>
<td>2.5</td>
<td>1.5</td>
</tr>
</tbody>
</table>

\(^1\)values are means ± SD, comparisons were performed by \(t\) test. BM, body mass; FFM, Fat-Free Mass; IAAO, Indicator Amino Acid Oxidation.
\(^2\)current study.
\(^3\)data from (24).
\(*\) Different from Sedentary, \(P<0.05\).

However, when expressed based on FFM, the flux values between ST and young sedentary men become statistically non-significant (6.1% greater in ST). In contrast, despite different methodology, participant training experience and protein intakes used, there are studies showing no change or even a reduction in whole body protein flux as a result of ST (Hartman, et al., 2006; Tipton, et al., 1996). It is known that muscle protein synthesis can be elevated for 24-48 h following exercise (Burd, et al., 2011; Phillips, et al., 1997) and this might explain the variable results because whole body flux was measured <24 h following a ST session in these earlier studies. Moreover, the time course and magnitude of any increase in muscle protein flux and synthesis can vary depending on the training status of the individual (Tang, et al., 2008). It appears that in exercise-trained individuals, a discordant decreased muscle protein breakdown response relative to muscle protein synthesis to ST is the driving force for the expected training induced hypertrophy (Damas, et al., 2016). Also, very recently using IAAO technique it has been
reported that greater protein intake is required after an acute bout of endurance exercise than at rest to maximize whole body protein synthesis in endurance trained rats (Kato, et al., 2016). To avoid this important potential confounder which could result in an overestimation of protein requirement, we measured whole body protein flux and requirement 48h following the previous bout of ST. Of course, protein “requirement” is traditionally defined in a resting steady state when whole body protein synthesis in fed state is matched with a similar rate of fasted state whole body protein breakdown at a balance. While we think measurement of protein requirement with exercise on study day is of interest, we decided to eliminate acute effect of a bout of exercise in our measurement. Very simply, we were interested in the effect of chronic training on protein requirements not the acute effects of strength exercise.

Compared to a previous IAAO study on young sedentary men (Humayun, et al., 2007), the 1.1 fold increase in whole body protein flux in this study is less than the observed 1.8 fold measured increase in protein requirement (Table 3.4). Given that the muscle contribution to the whole body protein flux is estimated at 30% (Wagenmakers, 1999), it is possible that there could be a concomitant accommodative response, perhaps via a reduction in non-muscle tissues protein synthesis and flux. Whether this possibility is correct requires further investigation.

All previous studies of the protein requirement for ST were completed using the NB technique. Also, those participants had vastly different strength training experience (novice to elite) so it is difficult to compare results (Lemon, et al., 1992; Tarnopolsky, et al., 1992; Tarnopolsky, et al., 1988) with our present data because likely training experience, training intensity, and muscularity, are all critical variables relative to protein requirements (Lemon, 1997, 2000). For example, NB studies on novice (≤1 y training experience) (Lemon, et al., 1992; Tarnopolsky, et al., 1992) and elite (≥3 y training experience) ST men (Tarnopolsky, et al., 1988) observed protein requirements of 1.4 and 0.82 g•kg\(^{-1}\)•d\(^{-1}\), respectively. This 71% greater protein need in novices compared with elite ST has been attributed, at least in part, to a greater rate of muscle mass accrual in novices (Tarnopolsky, et al., 1992). To control for this variable, the men in our study were selected based on their training experience (≥3 y training experience) and, as mentioned, were close to their maximal natural, i.e., without steroids, muscularity (90-100% ) based on published data of pre-steroid era Mr. USA winners (Kouri, et al., 1995). While these exercise-trained individuals could be close to their respective maximal muscularity, it cannot be
ruled out with certainty that they were not adding more FFM because a true marker of maximal muscularity gauge in exercise-trained individuals does not exist. It needs to be appreciated that as an exercising individual becomes more trained, the law of diminishing returns applies in that their FFM gain would become minimal and slow and certainly less than 4 kg FFM gain in 6 mo which was the criterion used in this study. A comparison of the current ST IAAO results to the previous NB data of elite ST-trained individuals (Tarnopolsky, et al., 1988) indicates a 2.1 times greater dietary protein requirement with the IAAO technique. This difference could be explained, at least partially, by inaccuracies in nitrogen loss quantification in the NB studies. For example, the unrealistic positive observed nitrogen balance (~12 to 20 g N•d⁻¹) at a protein intake of 2.8 g•kg⁻¹•d⁻¹ (Tarnopolsky, et al., 1988) should produce ~300 to 500 g of lean mass gain per day which, of course, was not observed. As mentioned, determining dietary protein requirements with NB is problematic and especially so for ST. The Institute of Medicine defines protein requirement, based on NB data, to be the minimal estimate to replace losses and prevent nutrient deficiency (Institute of Medicine, et al., 2005). Obviously protein requirement for these exercise-trained individuals can be defined as a minimum intake necessary to maximize whole body protein synthesis (anabolism) for all the repair and remodelling processes necessary for hypertrophy (growth). Of course, it is clear that the NB method underestimates the protein requirement due to its methodological flaws and, as alluded to above (Fuller & Garlick, 1994; Institute of Medicine, et al., 2005; Millward, 2001; Rand & Young, 1999), perhaps even more so with ST (Phillips, 2004, 2012; Phillips & Van Loon, 2011). The main adaptive response of strength training is muscle hypertrophy, which requires muscle protein synthesis to exceed muscle protein breakdown (Lemon, 1997; Phillips, 2004). This necessitates an increase in feeding so that both the net protein and energy balances remain positive chronically. Based on earlier stable isotope studies (Young, et al., 1987; Young & Bier, 1987; Young, et al., 1989; Young & Marchini, 1990) assessing both protein and AA requirements, four different states of protein metabolism have been identified: 1) “protein deficiency” defined as the maximal reduction in protein synthesis to all but the essential organs, 2) “accommodation” where NB is achieved with a decrease in physiological relevant processes, 3) “adaptation” where optimal growth, inter-organ AA exchange, and immune function are present and 4) “excess” which is characterized by AA oxidization for energy and nitrogen excretion via urea, resulting in no further stimulation of protein synthesis (Young, et al., 1981). It is known that when a protein
intake close to the current RDA is combined with strength training, accommodation results through increased nitrogen utilization efficiency and lower whole body protein synthesis rates rather than adaptation (Campbell, et al., 1995; Campbell & Leidy, 2007; Tarnopolsky, et al., 1992; Torun, et al., 1977). Consequently, it has been suggested that stable isotope tracer methodology would be a more accurate technique for the determination of protein requirement than NB (Lemon, et al., 1992; Tarnopolsky, et al., 1992; Young & Marchini, 1990). Further, because adaptation is required for the maximal training response in exercise-trained individuals, optimal protein intake for this population would have to be at a value that corresponds to a plateau in whole body protein synthesis, i.e., the breakpoint in oxidation using the IAAO technique to induce growth or the maintenance of FFM.

In the present study, postprandial whole body protein synthesis was measured by providing participants small hourly aliquots of a liquid meal supplemented with some protein-free cookies to induce a mild state of hyperaminoacidemia and hyperinsulinemia to stimulate protein synthesis and inhibit protein degradation (Rennie, et al., 2004). This approach to establishing a postprandial state has been used previously to measure fed-state leucine kinetics and was shown to provide reasonable estimates of 24-h leucine oxidation (Campbell, et al., 1995; El-Khoury, et al., 1994; Tipton, et al., 2003). Although this constant feeding is necessary at maintaining isotopic equilibrium for the accurate evaluation of steady state protein/AA kinetics, some studies suggest this model might underestimate the anabolic effect of feeding (El-Khoury, et al., 1995; Raguso, et al., 1999). However, the positive leucine balances in these studies suggest that individuals were gaining FFM, which was not the case. On the contrary, there is considerable evidence in young adults that feeding pattern of protein (pulse vs. spread pattern) has no influence in whole body net protein balance (Arnal, et al., 2000; Moore, et al., 2012). In addition, in a couple of recent reports (Min, et al., 2013; Tian, et al., 2011) using IAAO method, whole food (intact protein) and normal feeding pattern, protein requirement estimates were similar to the one determined using AA mixture and hourly feeding (Humayun, et al., 2007). Further, comparison between fed-state measurement of AA requirement by IAAO and 24h IAAO balance technique (both fed and fasted state) demonstrate similar estimates (Elango, et al., 2012). Interestingly the fundamental differences between the two methods have been the period of adaptation, and measurements during fed-fasted conditions.
In our study, oxidation of L-[1\textsuperscript{13}C] phenylalanine at each protein intake was measured as a reverse proxy for the whole body protein synthesis. This relationship has been confirmed previously both in animal and human studies (Ball & Bayley, 1986; Rafii, et al., 2008). In other words, as dietary protein intake increases, the rate of L-[1\textsuperscript{13}C] phenylalanine oxidation decreases, eventually reaching a plateau at the requirement; here 1.7 g•kg\textsuperscript{-1}•d\textsuperscript{-1} (the estimated RDA would be ~2.2 g•kg\textsuperscript{-1}•d\textsuperscript{-1}, Figure 3.1).

In summary, we report here for the first time an IAAO determined dietary protein recommendation for ST men, studied under conditions to eliminate any acute exercise effects, that exceeds the current NB derived estimations for exercise-trained individuals as well as the current EAR and RDA by ~1.3 and ~2.6 fold, respectively. Importantly, criticisms of this methodology (Fukagawa, 2014; Millward & Jackson, 2012) have been addressed previously (Rafii, et al., 2015; Tang, et al., 2014), yet still one might question how the results of a short term IAAO study can be translated into the long term desired adaptations in exercise-trained individuals. Although such data will need to come from future longitudinal strength training experiments, there is evidence that enhanced performance and greater increases in muscle mass occur among strength-trained participants with greater than RDA protein intakes (Cermak, et al., 2012; Snijders, et al., 2015). In conclusion, our IAAO data are consistent with earlier NB findings suggesting increased dietary protein needs with ST but, in addition, indicate that protein needs are even greater. Consequently, based on these data, we believe that, at least in the case of strength training, the conclusion of the Institute of Medicine (Institute of Medicine, et al., 2005) that “no additional dietary protein is suggested for healthy adults undertaking resistance or endurance exercise” needs to be reassessed.

3.7 Acknowledgements

AB, PWR, GC-M and PBP designed the study; PWR and GC-M supervised research; AB conducted experiments, AB and MR analyzed study samples; AB analyzed data; AB and PWR wrote the paper. All authors read and approved the final manuscript.
3.8 References


Chapter 4.

Indicator Amino Acid Derived Estimate of Dietary Protein Requirement of Endurance-Trained Athletes 24h Post-Exercise Exceeds the Current RDA
4.1 Abstract

**Background:** Despite a number of studies indicating increased dietary protein needs in Endurance-trained individuals (ET) using the nitrogen balance technique (NB), the Institute of Medicine (2005) has concluded, based largely on methodological concerns, that “no additional dietary protein is suggested for healthy adults undertaking resistance or endurance exercise”.

**Objective:** The objective of this study was to determine the dietary protein requirement of healthy young ET men (≥1 y training experience) 24 h post exercise by measuring the oxidation of ingested L-[1-13C] phenylalanine to 13CO₂ in response to graded intakes of protein (Indicator Amino Acid Oxidation (IAAO) Technique).

**Method:** Eight men (26.6±5.8y; 68.2±5.6kg; 9.8±5.0% body fat, 64.1±3.7 VO2max) were studied 24 h post-exercise, each on several occasions (3-9 times) with protein intakes ranging from 0.3 to 3.5 g•kg⁻¹•d⁻¹ for a total of 43 experiments. The diets provided energy at 1.5 times each individual’s measured resting energy expenditure and were isoenergetic across all treatments. Protein was fed as an amino acid (AA) mixture on the basis of the protein pattern in egg, except for phenylalanine and tyrosine, which were maintained constant across all protein intake quantities. For two days prior to the study day, the participants accommodated to a dietary protein of 1.5 g•kg⁻¹•d⁻¹. The mean dietary protein requirement was determined by applying a mixed-effects change-point regression analysis to F¹³CO₂ (label tracer oxidation in ¹³CO₂ breath), which identified a breakpoint in the F¹³CO₂ in response to graded amounts of protein.

**Results:** The estimated average protein requirement (EAR) and upper 95% CI (approximating the recommended dietary allowance (RDA)) for these young ET men were 2.0 and 2.6 g•kg⁻¹•d⁻¹, respectively.

**Conclusion:** These IAAO data suggest that the protein EAR and RDA for ET men exceed current recommendations by ~3 fold.

**Key Words:** protein requirement, strength exercise-trained individuals, indicator amino acid oxidation, stable isotope
4.2 Introduction

Current dietary protein recommendations are based on the nitrogen balance (NB) technique, which defines requirement as minimum intake necessary to maintain nitrogen equilibrium (Institute of Medicine, et al., 2005). Despite reports from NB studies on endurance-trained individuals (ET) indicating protein intake recommendations between 1.2-1.6 g•kg⁻¹•d⁻¹ (Friedman & Lemon, 1989; Lemon, et al., 1997; Phillips, et al., 1993; Tarnopolsky, 2004; Tarnopolsky, et al., 1988), the Institute of Medicine (Institute of Medicine, et al., 2005) does not acknowledge these recommendations due to methodological concerns. Although a tool that has provided a significant amount of data, NB measurements have several inherent problems (Millward, 2001; Rand & Young, 1999; Tome & Bos, 2000; Young, et al., 1989). This is evident in NB data used for protein requirement determination in ET (Friedman & Lemon, 1989; Meredith, et al., 1989; Tarnopolsky, et al., 1988), where at greater protein intakes NB often remains unrealistically positive. Equally concerning is an accommodative NB response at lower protein intakes via an increased N reutilization which would underestimate what is required for optimal function of all protein-requiring processes (Young, et al., 1989). Nitrogen balance at lower protein intakes may be achieved through a compromise in some physiologically relevant processes such as lesser enzyme activity upregulation, decreased capillarization, and/or reduced mitochondrial biogenesis after endurance exercise training (Tarnopolsky, 2004). Further, application of NB as an end point for the protein requirement determination in exercise-trained individuals may not be suitable (Phillips, 2012) as an exercise-trained individual whether strength or endurance aims to consume optimal amounts of dietary protein to induce a positive protein balance via increased protein synthesis rates. As such, a better method for determination of protein requirement in exercise-trained individuals should be able to define an intake which can maximize protein synthesis for repair and remodelling of muscle proteins necessary for an enhanced training-induced adaptive response. One such method is the indicator amino acid oxidation (IAAO) technique, which is based on the concept that when dietary protein is inadequate the oxidation of all AA, including the indicator AA, will be substantial. With increasing dietary protein, oxidation of the indicator AA will decrease because more AA are being incorporated into body protein. Once the dietary requirement is met, there is no further change in the oxidation of the indicator AA and the resulting inflection or ‘breakpoint’ is thought
to be the requirement (Pencharz & Ball, 2003). According to the Institute of Medicine, the IAAO technique is an acceptable method to assess protein or AA requirements (Institute of Medicine, et al., 2005). So far, there are several reports on protein requirement determination in non-exercise-trained individuals using IAAO (Elango, et al., 2011; Humayun, et al., 2007; Rafii, et al., 2016; Rafii, et al., 2015; Tang, et al., 2014), however protein requirement of ET using IAAO on a non-training day at rest has not been reported yet. Therefore, the purpose of this study was to quantify the daily dietary protein requirement of ET men 24h post-exercise using the IAAO technique.

4.3 Subjects and Methods

Participants

Potential candidates were invited for an interview via postings at the Western University campus and community athletic and recreation centres. During the interviews each candidate was informed of the study procedures and any who participated in our study signed an informed consent statement previously approved by Western University’s Health Sciences Research Ethics Board. The inclusion criteria were: 1) healthy men between 18 to 40 y of age, 2) ≥1 y of endurance-training experience, ≥6 d•wk⁻¹, ~1 – 1.5 h•d⁻¹, 3) body mass stable (<4 kg mass gain or loss within the past 6 mo), 4) nonsmoker, 5) <2 alcoholic drinks•d⁻¹, 6) nonmedication user (including anabolic steroids), and 7) without allergies to milk or milk products (because Boost® meal replacement and whey protein isolate were given as part of the experimental diet). All participants passed the physical activity readiness questionnaire (PAR-Q) health survey (Thomas, et al., 1992). As part of baseline testing, to determine participant’s maximal aerobic capacity, every exercise-trained individuals performed a VO₂max test on a treadmill. Briefly, after a 5-min warm-up on the treadmill (Desmo Pro, Woodway USA, Waukesha, Wis., USA) breath-by-breath gas analysis (Vmax Legacy, Sensormedics, Yorba Linda, Calif., USA) together with a progressive incremental-slope treadmill test (starting at 0% grade and speed of 11 -12 km/h) was conducted to determine VO₂max. The slope started at 0% grade and was increased by 2% every minute until volitional exhaustion. The duration of the test varied from 6 to 12 min. Oxygen uptake was measured continuously throughout the test. Heart rate was monitored and recorded
throughout using a Polar RST200TM heart rate monitor (Polar Electro Inc., Lachine, Que., Canada). VO$_{2\text{max}}$ was taken as the greatest value averaged over a 30-s collection period. All the participants achieved a plateau in oxygen consumption and attained a maximal HR within 10 beats of the age-predicted maximum. Exercise-trained individuals were primarily endurance training with 1-2 d•wk$^{-1}$ of mobility, flexibility, and strength-training.

**Study Design**

The minimally invasive IAAO technique was used (Bross, et al., 1998) to determine daily dietary protein requirement of ET 24h post-exercise as described in several previous studies (Elango, et al., 2011; M. A. Humayun, et al., 2007; Rafii, et al., 2016; Rafii, et al., 2015; Stephens, et al., 2015; Tang, et al., 2014). For the pre-study assessment and to prevent any acute effect of exercise in the measurements, participants abstained from training for 48h prior to visiting the laboratory. During the initial visit, which followed a 12-h overnight fast, both resting energy expenditure (REE) and body composition [fat-free mass (FFM) and fat mass] were measured using open-circuit indirect calorimetry (Vmax Legacy, Sensor Medics, CA, USA), and air displacement plethysmography with a BodPod® (Life Measurements, Concord, CA), respectively. Each dietary protein quantity given was studied over a 3-d period (two adaptation days followed by an IAAO study day (Elango, et al., 2009)). During the adaptation days, participants received Boost® (Nestlé) meal replacement as a maintenance diet supplemented with Polycose® (Abbot Nutrition) and lactose-free, gluten-free whey protein isolate (Kaizen Protein) providing 1.5 g protein•kg$^{-1}$•d$^{-1}$ and 1.7 × REE energy. Exercise-trained individuals were allowed to train on the two adaptation days. They followed a consistent training schedule for the two days. On the third day (the study day), participants arrived after a 12-h fast and were assigned randomly to receive test protein intakes ranging from 0.3 to 3.5 g•kg$^{-1}$•d$^{-1}$ and 1.5 REE energy (lesser energy here due to the largely sedentary measurement day). Three and two participants were each tested at 4 and 6 protein intakes and one participant at each of 3, 7, and 9 protein intakes for a total of 43 IAAO studies. Each 3-d study period was separated by at least one week.
Study Diets

The adaptation/study diets (described above) provided all of the participant’s macronutrient needs on the basis of the current DRI. During the two adaptation days, the daily diet was consumed as four equal meals and participants did not consume any other food items except one cup of clear tea or coffee and water ad libitum. Macronutrients of the diet for the two adaptation days were composed of 7.5, 1.5 and 0.7 g•kg⁻¹•d⁻¹ of carbohydrate, protein and fat, respectively which complied with the current recommendation for ET (Burke, et al., 2011). Participants were also provided a multivitamin supplement (Centrum®, Wyeth Consumer Health Care) and fibre (RestoraLax®, Bayer) daily for the duration of all studies.

On the third or IAAO study day, participants arrived at the laboratory where they consumed eight hourly isoenergetic meals, each meal representing one-twelfth of the daily energy requirement. The study day diet consisted of a protein- and AA-free powder (PDF1, Mead Johnson), flavored drink crystals (Tang and Kool-Aid, Kraft Foods), grape seed oil, a crystalline AA mixture (Ajinomoto Amino Science LLC) patterned after egg protein (Table 4.1) and protein-free cookies. Energy intake was set at 1.5 × REE and the carbohydrate content of the diets was adjusted based on the protein to keep the diets isoenergetic.

Tracer Protocol

An oral priming doses of 0.176 mg NaH¹³CO₃•kg⁻¹ body mass (99 atom percent excess, Cambridge Isotope Laboratories) and 0.66 mg L-[¹³C] phenylalanine•kg⁻¹ started with the fifth hourly meal. In addition, a dose of L-[¹³C] phenylalanine (1.2 mg•kg⁻¹•h⁻¹) was started with the fifth meal and continued hourly for the remaining 3 h of the study. The quantity of L-[¹³C] phenylalanine supplied during the last 4 h of the study was subtracted from the diet to provide a total intake of 25 mg phenylalanine•kg⁻¹•d⁻¹ and tyrosine was provided at 40 mg•kg⁻¹•d⁻¹ to ensure an excess of tyrosine (Shiman & Gray, 1998; Zello, et al., 1990).
Table 4.1  Amino acid composition of reference protein and test protein intakes

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Reference protein, mg•g⁻¹</th>
<th>Test protein intake, g•kg⁻¹</th>
<th>0.1</th>
<th>1.0</th>
<th>1.5</th>
<th>2.0</th>
<th>2.5</th>
<th>3.0</th>
<th>3.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Alanine</td>
<td>61.5</td>
<td>6.15</td>
<td>61.5</td>
<td>92.3</td>
<td>123</td>
<td>153</td>
<td>184</td>
<td>215</td>
<td></td>
</tr>
<tr>
<td>L-Arginine-HCL³</td>
<td>75.1</td>
<td>7.51</td>
<td>75.1</td>
<td>113</td>
<td>150</td>
<td>187</td>
<td>225</td>
<td>263</td>
<td></td>
</tr>
<tr>
<td>L-Asparagine</td>
<td>33.3</td>
<td>3.33</td>
<td>33.3</td>
<td>50.0</td>
<td>66.6</td>
<td>83</td>
<td>99.9</td>
<td>116</td>
<td></td>
</tr>
<tr>
<td>L-Aspartic acid</td>
<td>33.3</td>
<td>3.33</td>
<td>33.3</td>
<td>50.0</td>
<td>66.6</td>
<td>83.2</td>
<td>99.9</td>
<td>116</td>
<td></td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>22.1</td>
<td>2.21</td>
<td>22.1</td>
<td>33.2</td>
<td>44.2</td>
<td>55.2</td>
<td>66.3</td>
<td>77.3</td>
<td></td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>56.6</td>
<td>5.66</td>
<td>56.6</td>
<td>84.9</td>
<td>113</td>
<td>141</td>
<td>169</td>
<td>198</td>
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</tr>
<tr>
<td>L-Asparagine-HCL⁴</td>
<td>75.7</td>
<td>7.57</td>
<td>75.7</td>
<td>114</td>
<td>151</td>
<td>189</td>
<td>227</td>
<td>265</td>
<td></td>
</tr>
<tr>
<td>L-Methionine</td>
<td>29.6</td>
<td>2.96</td>
<td>29.6</td>
<td>44.4</td>
<td>59.2</td>
<td>74</td>
<td>88.8</td>
<td>103</td>
<td></td>
</tr>
<tr>
<td>L-Phenylalanine⁴</td>
<td>30.0</td>
<td>25.0</td>
<td>25.0</td>
<td>25.0</td>
<td>25.0</td>
<td>25.0</td>
<td>25.0</td>
<td>25.0</td>
<td></td>
</tr>
<tr>
<td>L-Proline</td>
<td>41.9</td>
<td>4.19</td>
<td>41.9</td>
<td>62.9</td>
<td>83.8</td>
<td>104</td>
<td>125</td>
<td>146</td>
<td></td>
</tr>
<tr>
<td>L-Serine</td>
<td>83.9</td>
<td>8.39</td>
<td>83.9</td>
<td>126</td>
<td>168</td>
<td>209</td>
<td>251</td>
<td>293</td>
<td></td>
</tr>
<tr>
<td>L-Threonine</td>
<td>47.1</td>
<td>4.71</td>
<td>47.1</td>
<td>70.7</td>
<td>94.2</td>
<td>117</td>
<td>141</td>
<td>165</td>
<td></td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>15.6</td>
<td>1.56</td>
<td>15.6</td>
<td>23.4</td>
<td>31.2</td>
<td>39</td>
<td>47</td>
<td>54.6</td>
<td></td>
</tr>
<tr>
<td>L-Tyrosine⁵</td>
<td>40.0</td>
<td>40.0</td>
<td>40.0</td>
<td>40.0</td>
<td>40.0</td>
<td>40.0</td>
<td>40.0</td>
<td>40.0</td>
<td></td>
</tr>
<tr>
<td>L-Valine</td>
<td>70.3</td>
<td>7.03</td>
<td>70.3</td>
<td>105</td>
<td>141</td>
<td>175</td>
<td>211</td>
<td>246</td>
<td></td>
</tr>
</tbody>
</table>

¹Participants received several test protein quantities ranging from 0.1 to 3.5 g protein•kg⁻¹•d⁻¹.
²Represents the egg protein composition.
³Actual amounts of amino acids were as follows: 62.1 mg arginine•g⁻¹ and 60.6 mg lysine•g⁻¹.
⁴L-Phenylalanine intake was kept constant at 25.0 mg•kg⁻¹•d⁻¹.
⁵L-Tyrosine intake was kept constant at 40.0 mg•kg⁻¹•d⁻¹.

Sample Collection and Analysis

During each study day, three baseline breath and urine samples were collected at 45, 30, and 15 min before the tracer protocol began and five total breath and urine samples were collected one every 30 min beginning at 2.5 h after administration of the tracer (L-[1-¹³C] phenylalanine) in order to establish that an isotopic steady state was attained. Breath samples were collected in disposable Exetainer tubes (Labco) with a collection mechanism (Easy-Sampler, Quintron) that permitted the removal of dead-space air. Breath samples were stored at room temperature, and
urine samples were stored at –20 ºC until analysis. During each study day, the rate of carbon dioxide production (VCO₂) was measured immediately after the fifth meal for a period of 20 min with an indirect calorimeter (Vmax Legacy, Sensor Medics, CA, USA).

Expired ¹³CO₂ enrichment was measured with a continuous-flow isotope ratio mass spectrometer (CF-IRMS 20/20 isotope analyzer, PDZ Europa). Enrichments were expressed as atom percent excess compared with a reference standard of compressed CO₂. Urinary L-[¹³C] phenylalanine enrichment was analyzed by an API 4000 triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex) in positive electrospray ionization mode (Rafii, et al., 2015). Isotopic enrichment was expressed as mol percent excess and calculated from peak area ratios at isotopic steady state both at baseline and plateau.

**Estimation of Isotope Kinetics**

L-[¹³C] phenylalanine kinetics were calculated with use of the stochastic models described previously (Matthews, et al., 1980). Isotopic steady state in the tracer enrichment at baseline and plateau was represented as unchanging values of L-[¹³C] phenylalanine in urine and ¹³CO₂ in breath.

Phenylalanine flux (µmol·kg⁻¹·h⁻¹) was calculated from the dilution of the orally administered L-[¹³C] phenylalanine into the metabolic pool (at steady state) by using the enrichment of L-[¹³C] phenylalanine in urine. The rate of appearance of ¹³CO₂ in breath (F¹³CO₂ µmol·kg⁻¹·h⁻¹) after the oxidation of ingested L-[¹³C] phenylalanine was calculated according to the model of Matthews et al. (Matthews, et al., 1980) using a factor of 0.82 to account for carbon dioxide retained in the body’s bicarbonate pool (Hoerr, et al., 1989).

**4.4 Statistical Analysis**

All results are reported as means±SD. Statistical analyses were performed by using SAS (Version 9.2.1; SAS Institute Inc). Significance was set at P≤ 0.05. Participants were assigned randomly to differing protein intakes with protein quantity as the independent variable. The effect of protein intake on phenylalanine flux, oxidation, and F¹³CO₂ was tested by using a mixed linear model (PROC MIXED) with subject as a random variable. The mean protein
requirement was estimated by applying a nonlinear mixed-effects model (PROC NLMIXED; SAS Institute) to the F\textsuperscript{13}CO\textsubscript{2} data (Rafii, et al., 2015). Observations within subjects were regarded as statistically dependent. Confidence intervals (CI) were obtained by following the standard asymptotic theory of the maximal likelihood estimation. The model minimizing the Akaike information criterion was regarded as the model with the best fit (Hayamizu, et al., 2011). The following statistical model was used, accounting for correlations within observations from the same subject: 

\[ Y_{id} = \beta_0 + b_i + \beta_1 I(x_{id} > x_{cp})(x_{id} - x_{cp}) + e_{id}, \]

where \( Y_{id} = F^{13}CO_2 \) or phenylalanine oxidation at the dose of the protein of \( i \), \( x_{id} \) is the dose amount of the test protein intake of the \( i \)-th participant, \( e_{id} \) are random errors that are independently normally distributed with a mean of 0 and variance of \( \sigma^2 \), \( \beta_0 \) is the left line intercept, \( b_i \) is the random intercept that incorporates within-subject correlation, \( \beta_1 \) is the left line slope, \( x_{cp} \) is the breakpoint, and the slope for \( x_{id} \) is 0 for \( x_{id} \) more than breakpoint.

4.5 Results

Participant Characteristics

Eight ET men (≥1 y of consistent endurance training experience, ≥6 d•wk\(^{-1}\), ~1- 1.5 h•d\(^{-1}\) with minimal strength-training in their routine, ~1-2 d•wk\(^{-1}\)) participated in the study. Of the 8 participants 4 were du-athletes (training for running and cycling), 1 marathon and 3 cross country runners with a VO\textsubscript{2max} of 64.1±3.7 mL•kg\(^{-1}\)•min\(^{-1}\) (Table 4.2). Their habitual protein intake based on 3-d dietary record analysis was 2.1±0.9g•kg\(^{-1}\)•d\(^{-1}\) which is about 2.6 times the RDA.

Phenylalanine Flux

Phenylalanine flux was not affected within each exercise-trained individual by the different protein intakes (\( P=0.15 \)) as required by the IAAO method (Table 4.3). This provides evidence that the precursor pool for the IAAO did not change significantly with increasing test protein
intakes, and suggests that the changes in oxidation were inversely proportional to whole-body protein synthesis. Whole body phenylalanine flux was 62.6±8.7 μmol•kg⁻¹•h⁻¹.

Table 4.2  Physical Characteristics and daily energy and macronutrient intake of the endurance-trained men studied

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>26.6±5.8</td>
</tr>
<tr>
<td>Mass, kg</td>
<td>68.2±5.6</td>
</tr>
<tr>
<td>Height, m</td>
<td>1.8±0.1</td>
</tr>
<tr>
<td>Body fat, %</td>
<td>9.8 ±5.0</td>
</tr>
<tr>
<td>FFM, kg</td>
<td>61.2±6.0</td>
</tr>
<tr>
<td>VO₂ max, ml•kg⁻¹•min⁻¹</td>
<td>64.1±3.7</td>
</tr>
<tr>
<td>REE³, kcal•kg⁻¹•d⁻¹</td>
<td>24.9±3.1</td>
</tr>
<tr>
<td>Energy intake⁴, kcal•kg⁻¹•d⁻¹</td>
<td>40.7±3.9</td>
</tr>
<tr>
<td>Carbohydrate intake⁴, g•kg⁻¹•d⁻¹</td>
<td>5.1±0.3</td>
</tr>
<tr>
<td>Protein intake⁴, g•kg⁻¹•d⁻¹</td>
<td>2.1±0.9</td>
</tr>
<tr>
<td>Fat intake⁴, g•kg⁻¹•d⁻¹</td>
<td>1.3±0.3</td>
</tr>
</tbody>
</table>

¹All values are means±SD, n= 8.
²Body fat was measured using Bod Pod® (Life Measurements, Concord, CA).
³Resting energy expenditure measured by open circuit indirect calorimetry (Vmax Legacy, Sensor Medics, CA, USA).
⁴Energy intake and macronutrient breakdown were based on a 3-d dietary record analysis.

**L-[1-¹³C] phenylalanine**

Nonlinear mixed-effects change-point regression analysis of the F¹³CO₂ data resulted in a breakpoint at a protein intake of 2.0 g•kg⁻¹•d⁻¹, i.e., the rate of ¹³CO₂ released from the oxidation of L-[1-¹³C] phenylalanine (F¹³CO₂) declined in response to increasing protein intakes up to 2.0 g•kg⁻¹•d⁻¹ (Figure 4.1), such that additional increases in protein intake did not result in changes in F¹³CO₂ values. This indicates that the incorporation of the phenylalanine for protein synthesis plateaued at a protein intake of 2.0 g•kg⁻¹•d⁻¹ (r² = 0.50) and suggests this is mean dietary requirement of these endurance exercise-trained individuals. The upper 95% CI of the breakpoint was at 2.6 g•kg⁻¹•d⁻¹, suggesting the RDA for protein of endurance exercise-trained individuals should be near this intake. The lower CI was 1.4 g•kg⁻¹•d⁻¹.
Table 4.3  Protein intakes and phenylalanine flux$^1$

<table>
<thead>
<tr>
<th>Subject</th>
<th>Test protein intakes</th>
<th>Phenylalanine flux $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5, 0.9, 1.2, 1.7, 2.1, 2.4</td>
<td>53±4</td>
</tr>
<tr>
<td>2</td>
<td>0.9, 1.3, 1.5, 1.9, 2.3, 2.8, 3.5</td>
<td>65±11</td>
</tr>
<tr>
<td>3</td>
<td>0.3, 1.1, 1.6, 1.6, 2.3, 1.9, 2.9, 3.3</td>
<td>64±09</td>
</tr>
<tr>
<td>4</td>
<td>0.8, 1.0, 1.5, 1.9</td>
<td>68±07</td>
</tr>
<tr>
<td>5</td>
<td>0.6, 1.0, 1.4, 1.7, 2.0, 2.5</td>
<td>62±04</td>
</tr>
<tr>
<td>6</td>
<td>0.8, 1.3, 1.7</td>
<td>55±07</td>
</tr>
<tr>
<td>7</td>
<td>1.1, 1.8, 2.4, 3.0</td>
<td>70±06</td>
</tr>
<tr>
<td>8</td>
<td>0.5, 1.2, 1.7, 2.0</td>
<td>65±10</td>
</tr>
<tr>
<td>All</td>
<td></td>
<td>62.6±8.7</td>
</tr>
</tbody>
</table>

$^1$Values are means±SD. No significant differences ($P=0.15$) in phenylalanine flux were observed within each subject across all test protein intakes (0.3 to 3.5 g·kg$^{-1}$·d$^{-1}$). Each subject completed a minimum of 3 test intakes for a total of 43 studies.

Figure 4.1  Protein intake and production of $^{13}$CO$_2$ from phenylalanine oxidation ($F^{13}$CO$_2$) in young endurance trained men ($n=43$). Individual values for each exercise-trained individual are represented by different symbols. The breakpoint represents the estimated mean protein requirement. A mixed-effects change-point regression analysis identified a breakpoint and upper 95% CI for the relation between protein intake and phenylalanine oxidation to be 2.0 ($r^2=0.50$) and 2.6 g·kg$^{-1}$·d$^{-1}$, respectively.
4.6 Discussion

A review of several studies indicates that the habitual protein intake of ET (1.8±0.4 g·kg\(^{-1}\)·d\(^{-1}\)) exceeds the RDA (Tarnopolsky, 2004). Similarly, our ET had a daily protein intake (2.1±0.9 g·kg\(^{-1}\)·d\(^{-1}\)) in excess of the RDA. Previous NB studies with ET have measured a protein requirement anywhere from 0.94 to 1.6 g·kg\(^{-1}\)·d\(^{-1}\) (Friedman & Lemon, 1989; Meredith, et al., 1989; Phillips, et al., 1993; Tarnopolsky, et al., 1988). This wide range of estimates has been attributed partially to varying training experience (intensity, duration, and volume of training) and nutritional status (Lemon, 1998, 2000; Tarnopolsky, 2004). In our study the protein requirement and RDA of these ET men using the IAAO technique was 2.0 and 2.6 g·kg\(^{-1}\)·d\(^{-1}\), respectively (Figure 4.1) which is about 25 to 50 % greater than previously reported estimates using NB technique (Friedman & Lemon, 1989; Meredith, et al., 1989; Tarnopolsky, 2004; Tarnopolsky, et al., 1988). Interestingly, a similar trend has been reported previously for sedentary individuals using IAAO, in that the protein requirement also exceeded the published NB protein requirements set by the Institute of Medicine by about 30–40% (Elango, et al., 2011; Humayun, et al., 2007; Rafii, et al., 2016; Rafii, et al., 2015; Stephens, et al., 2015; Tang, et al., 2014). Inaccuracies inherent in the NB method due to overestimation of intake and underestimation of losses, slow physiological urea pool adaptation to altered protein intake, confounding effects of energy intake, true nitrogen accretion below the limits of detection, and loss of nitrogen as molecular N\(_2\) could explain a protein requirement underestimation (Millward, 2001; Tarnopolsky, et al., 1992). However, the regression model used is also an important factor because it has been shown that when repeated measures of NB data from different protein intakes on the same participants are analyzed using a biphasic linear regression model, the estimates of protein requirement by NB increases so that both methods produce similar estimations (Humayun, et al., 2007).

A comparison between the EAR estimate of our study and an IAAO study on sedentary young men revealed a ~2.1 times greater protein requirement in ET (Table 4.4). Also, although non-significant (\(P=0.16\)), whole body phenylalanine flux at rest was 7.0% greater in ET compared to sedentary young men (Humayun, et al., 2007). However, when expressed based on FFM, there was no difference between the flux values of ET compared with their sedentary counterparts. Importantly, all our measurements were carried out 24h post-exercise session of each participant.
This could be critical as there are reports showing an increase of 17 to 22% in mixed muscle protein synthesis for up to 48-h after the previous endurance exercise session with 4-wk to 4-mo of endurance training, respectively (Pikosky, et al., 2006; Short, et al., 2004). However, any effect of this exercising muscle protein synthesis increase post exercise on whole body protein flux is by no means clear because it could be compensated for by a down-regulation of protein synthesis in less active tissues (Devlin, et al., 1990). Consequently resting whole body flux rates in ET may be less than expected (Lamont, et al., 1999; Short, et al., 2004). Indeed, previous studies have reported a non-significant change in resting whole body protein flux in trained vs untrained individuals (Devlin, et al., 1990; Gaine, et al., 2005; Short, et al., 2004).

Table 4.4 Comparison between young endurance-trained men (current study) and young healthy sedentary men who participated in another protein requirement study\(^3\) conducted with IAAO method\(^1\)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Endurance-trained young men(^2)</th>
<th>Sedentary young men(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>26.6±5.8</td>
<td>26.8±2.0</td>
</tr>
<tr>
<td>(N)</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Height, m</td>
<td>1.8±0.1</td>
<td>1.7±2.6</td>
</tr>
<tr>
<td>Mass, kg</td>
<td>68.2±5.6</td>
<td>69.6±3.7</td>
</tr>
<tr>
<td>FFM, kg</td>
<td>61.2±6.0</td>
<td>56.4±1.9</td>
</tr>
<tr>
<td>Phenylalanine flux, (\mu\text{mol\cdot kg}^{-1}\text{BM\cdot h}^{-1})</td>
<td>62.6±8.7</td>
<td>58.5±14</td>
</tr>
<tr>
<td>Phenylalanine flux, (\mu\text{mol\cdot kg}^{-1}\text{FFM\cdot h}^{-1})</td>
<td>70.2±9.0</td>
<td>71.9±18.7</td>
</tr>
<tr>
<td>Protein requirement (\text{g\cdot kg}^{-1}\text{BM\cdot d}^{-1})</td>
<td>2.0</td>
<td>0.93</td>
</tr>
<tr>
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<td>2.2</td>
<td>1.1</td>
</tr>
<tr>
<td>Protein recommendation (\text{g\cdot kg}^{-1}\text{BM\cdot d}^{-1})</td>
<td>2.6</td>
<td>1.2</td>
</tr>
<tr>
<td>Protein recommendation (\text{g\cdot kg}^{-1}\text{FFM\cdot d}^{-1})</td>
<td>2.9</td>
<td>1.5</td>
</tr>
</tbody>
</table>

\(^1\)Values are means ± SD, comparisons were performed by \(t\) test. BM, body mass; FFM, Fat-Free Mass; IAAO, Indicator Amino Acid Oxidation.

\(^2\)Current study.

\(^3\)Data from (24).

* Different from Sedentary, \(P<0.05\).

The greater protein requirement observed in our ET could be a result of an increased AA oxidation (alanine, asparagines, aspartate, glutamate, lysine) (Lamont, et al., 2001; Smith &
Rennie, 1996), especially for the branched chain AA (leucine, isoleucine and valine) (BCAA) during exercise (Lamont, et al., 1999; Lemon, et al., 1982; Phillips, et al., 1993; Rennie, et al., 1980), greater mitochondrial biogenesis (Hawley, 2002; Holloszy & Coyle, 1984), and/or upregulated repair and remodelling processes in the muscles used in training (e.g., increased aerobic enzymes, capillaries, hemoglobin, and myoglobin) (Harber, et al., 2010; Mascher, et al., 2011; Pikosky, et al., 2006; Short, et al., 2004). In fact, previous studies have shown that oxidation of AA can contribute up to ~5-15% of total energy requirement during exercise dependent on exercise duration, intensity and carbohydrate availability (Bowtell, et al., 1998, 2000; Davies, et al., 1982; Kasperek & Snider, 1987; Lamont, et al., 1999, 2001; Lemon & Mullin, 1980; Rennie, et al., 2006; Wagenmakers, et al., 1991). An increased activation of branched chain oxo-acid dehydrogenase enzyme (BCOAD), the rate limiting enzyme in BCAA oxidation during exercise, is related to decreases in the ratio of ATP/ADP, pH, and muscle glycogen (Howarth, et al., 2010; Kasperek & Snider, 1987; Wagenmakers, et al., 1991; Wagenmakers, et al., 1989). Furthermore, although endurance training has been shown to lower the relative maximal activity of BCOAD and BCAA oxidation during exercise (McKenzie, et al., 2000), whole body oxidation rates remain significantly elevated above rest (Bowtell, et al., 2000). Therefore, an endurance exercise-trained individual who has up-regulated mitochondrial potential to a large degree would also have an increased total BCOAD capacity and this together with a more frequent rigorous training program, increases leucine oxidation/protein requirements (Moore, et al., 2014; Tarnopolsky, 2004). Also, It has been suggested that AA participation in exchange reactions in the tricarboxylic acid cycle could also increase their net use (Gibala, et al., 1998; Gibala, et al., 1997).

Physical activity at varying intensities causes catabolism of AA (Rennie, et al., 1981). Furthermore, exercise promotes conversion of AA to glucose via gluconeogenic pathways, and as a result, the rate of urea production is stimulated (Rennie, 1996). The AA utilized during exercise are derived from increased proteolysis and/or decreased protein synthesis (Rennie, et al., 1981; Rennie, 1996). The decrease in protein synthesis is related to the diversion of AA and energy away from the events supporting protein synthesis toward the events supporting muscle contraction. Therefore, it is after exercise that rates of muscle protein synthesis have the potential to be accelerated beyond the rates of proteolysis, allowing protein balance to become positive.
Without post-exercise protein supplementation, protein synthesis is limited because of the decreased availability of AA. Provision of 10 to 16 g of protein after an acute bout of moderate endurance exercise for ~ 60 min has been shown to enhance muscle protein synthesis and net muscle protein balance despite a negative whole body protein balance (Levenhagen, et al., 2002; Lunn, et al., 2012).

Consideration of whole body protein synthesis is also important as the damage and repair of splanchnic tissues such as hypoxia-mediated small intestinal injury which is a result of a redistribution of blood away from splanchnic tissue to exercising muscles can be significant (van Wijck, et al., 2012; Van Wijck, et al., 2011). Consequently, symptoms such as nausea, vomiting, abdominal angina, and bloody diarrhea are experienced by up to 70% of ET (Peters, et al., 1999; Ter Steege, et al., 2008). Following the ingestion of protein some AA consumed in diet are retained in the splanchnic area which can function as a labile pool of essential AA (Soeters, et al., 2001). First-pass splanchnic extraction of AA accounts for a large portion (20-90%) of ingested AA (Matthews, et al., 1993a, 1993b). The splanchnic tissue has a high protein turnover rate and is therefore capable of rapidly retaining AA for protein synthesis, then releasing those AA during exercise to be used in muscle (Deutz & Wolfe, 2013). Because splanchnic protein breakdown is increased during exercise (Wasserman, et al., 1991), it is possible that splanchnic extraction of AA and protein synthesis is enhanced afterwards, as replacement (Rennie, 1996; Rennie & Tipton, 2000). Similarly, less appreciated is the role of the immune response in the repair process for an enhanced training induced adaptation. It has been shown that protein/AA are important fuel substrates for proper immune function (Li, et al., 2007; Newsholme & Parry-Billings, 1990). Optimal immune response post-exercise as part of repair and regeneration processes (Tidball & Villalta, 2010) and to prevent training-induced (Gleeson, 2007) suppression of immunocompetence (Witard, et al., 2012) might translate into an increased protein need in ET. Moreover, recent studies indicate that when carbohydrate intakes are suboptimal (≤6 g•kg⁻¹•d⁻¹), greater protein intake (≥1.5 g•kg⁻¹•d⁻¹) is effective in restoring markers of immune function to a normal values (Witard, et al., 2014; Witard, et al., 2012). Collectively, these studies point to a greater dietary protein/AA need of ET to support not only the greater rates of whole body protein synthesis (including splanchnic tissues) but also the less appreciated need of one’s immune response.
Relative to protein requirement determination for ET, the optimal protein intake for this population would have to be at a value that results in adaptation rather than accommodation (Pellet, 1990; Phillips, 2012). It is suggested that nitrogen equilibrium can be maintained through accommodation (increased nitrogen utilization efficiency and lower whole body protein synthesis) (Young, et al., 1989; Young & Marchini, 1990) even when an insufficient protein intake is combined with endurance training (Butterfield, 1987; Butterfield & Calloway, 1984; Gontzea, et al., 1962; M.A. Tarnopolsky, et al., 1988). Consequently, it has been suggested that stable isotope tracer methodology would be a more accurate technique for the determination of protein requirement than NB (Lemon, et al., 1992; Tarnopolsky, et al., 1992; Young & Marchini, 1990). For an ET, the adaptation response would correspond to a plateau in increased whole body protein synthesis including all relevant repair and remodelling processes as discussed above. As such, in our study, oxidation of L-[1-13C] phenylalanine at each protein intake was measured as a reverse proxy for the whole body protein synthesis. This relationship has been confirmed previously both in animal and human studies (Ball & Bayley, 1986; Rafii, et al., 2008). In other words, as dietary protein intake increases, the rate of L-[1-13C] phenylalanine oxidation decreases, eventually reaching a plateau (maximal whole body protein synthesis response) at the requirement; here 2.0 g•kg\(^{-1}\)•d\(^{-1}\) (the estimated RDA would be ~2.6 g•kg\(^{-1}\)•d\(^{-1}\)). Interestingly, this estimated requirement level is similar to the habitual protein intake of our ET participants (Table 4.2). It comprises ~18\% of their total daily energy intake which is within an acceptable macronutrient distribution range of 10-35\% of total daily energy intake as defined by the Institute of Medicine (Institute of Medicine, et al., 2005). However, macronutrient intakes expressed this way are quite limited because the absolute intake can vary greatly if energy intakes differ.

Criticisms of IAAO methodology (Fukagawa, 2014; Millward & Jackson, 2012) have been addressed previously (Rafii, et al., 2016; Rafii, et al., 2015; Tang, et al., 2014) and it is generally accepted as valid (Institute of Medicine, et al., 2005). In an ideal world the optimal method for determination of protein intake for endurance exercise-trained individuals would come from longitudinal studies of endurance training with treatment groups assigned to different amounts of protein intakes in order to determine maximal improvements in physiologic adaptation. Unfortunately such studies are very difficult to conduct due to numerous variables (genetic
capacity, exercise intensity and duration, state of training, energy balance, sex, and type of protein ingested) that could influence the requirement (Tarnopolsky, 2004). Further, such experiments are very labour intensive. It has been suggested that meeting protein need is unlikely to be a concern for exercise-trained individuals who consume adequate energy, however this was based on the NB derived estimation of 1.6 g•kg	extsuperscript{-1}•d	extsuperscript{-1} for ET (Phillips, 2012; Phillips & Van Loon, 2011; Tarnopolsky, 2004) which is a 30% underestimation based on our data. Also, from a practical standpoint it needs to be appreciated that both training program and nutritional status of ET is variable, including any combination of prolonged constant load, repeated high intensity intervals or concurrent training. Furthermore, nutrition-wise “train low, compete high”, referring to CHO availability has become a popular concept practiced among many ET. Hence, considering these factors it seems reasonable that an increase in dietary protein intake is necessary for greater glycogen synthesis rates (Beelen, et al., 2010; van Loon, Saris, Kruijshoop, et al., 2000; van Loon, Saris, Verhagen, et al., 2000), to attenuate muscle mass loss (Haakonssen, et al., 2013; Tipton & Witard, 2007) and to enhance whole body (Howarth, et al., 2010) as well as muscle protein synthesis (Coffey, et al., 2011) to improve athletic performance.

4.7 Acknowledgements

AB, PWR, GC-M and PBP designed the study; PWR and GC-M supervised research; AB conducted experiments, AB and MR analyzed study samples; AB analyzed data; AB and PWR wrote the paper. All authors read and approved the final manuscript.
4.8 References


*Clin exp pharmacol physiol,* 29(3), 218-222.


Chapter 5.
Protein requirement in athletes: An Update.
5.1 Abstract

Based on recent stable isotope studies regarding the effect of protein nutrition in endurance (ET) and strength-trained (ST) individuals, the protein requirement for these individuals should be defined as an amount needed to induce a maximal protein synthetic response at the whole body level for all the protein requiring processes. Although previous nitrogen balance (NB) experiments have shown an increase in protein requirement of exercise-trained individuals, results are confounded mainly due to methodological problems involved in underestimation of protein requirement. Indicator amino acid oxidation method (IAAO), a robust and sensitive stable isotope oxidation method, has been used recently for the determination of protein requirement in humans. This method is based on the concept that when one indispensable amino acid is deficient for protein synthesis, then all other AA, including the indicator, will be considered excess and oxidized. With increasing intakes of protein, oxidation of the indicator will decrease, reflecting its increasing incorporation into protein. Once the requirement for the protein is met there will be no further change in the indicator oxidation. In this method the oxidation of the indicator (an indispensable AA e.g., L-[1\textsuperscript{-13}C] phenylalanine) is a reverse proxy for the whole body protein synthesis and the requirement (breakpoint) is defined as a protein intake quantity that maximizes the incorporation of indicator into protein synthetic processes by minimizing its oxidation. Studies from this dissertation using the IAAO have shown a protein requirement of 1.7 and 2.0 g·kg\textsuperscript{-1}·d\textsuperscript{-1} for ST and ET, respectively. These values are 25 to 30% greater than NB derived estimates of similar exercise-trained individuals. We believe that the response criterion used in IAAO determination of protein requirement is best suited for exercise-trained individuals as optimum training-induced adaptation cannot occur unless the needs of all protein requiring processes at the whole body level are met. Consequently, dietary protein requirements for exercise-trained individuals need to be reassessed.

5.2 Introduction

For many years, protein intake has been one of, if not the most, debated nutrient requirement question among both exercise-trained individuals and scientists. From the early studies concluding protein was the main fuel source for muscle contraction (Liebig & Gregory, 1842; Voit, 1881) to later studies discounting it as an insignificant nutrient for exercise-trained
individuals (Carpenter, 1931; Gemmill, 1942), the debate about specific recommendations continues. The current protein recommendations for adult humans are based on nitrogen balance (NB) derived estimates established by the Institute of Medicine (Institute of Medicine, et al., 2005) which defines the protein requirement and the RDA of 0.6 and 0.8 g\(\text{kg}^{-1}\cdot\text{d}^{-1}\), respectively. Further, due largely to methodological concerns (Lemon, et al., 1992; Tarnopolsky, 2004; Mark A Tarnopolsky, et al., 1988), the Institute of Medicine has concluded that “no additional dietary protein is suggested for healthy adults undertaking resistance or endurance exercise” (Institute of Medicine, et al., 2005). In contrast, appreciating the greater need for exercise-trained individuals based on NB derived estimates, the American Dietetic Association, the Dieticians of Canada and the American College of Sport Medicine have collectively recommended 1.2-1.6 and 1.4-1.7 g\(\text{kg}^{-1}\cdot\text{d}^{-1}\) daily protein intake for endurance (ET) and strength (ST) trained individuals, respectively (Rodriguez, et al., 2009). The problem is that, despite having contributed a vast amount of information, the NB method suffers from many methodological drawbacks discussed in detail elsewhere (Millward, 1998; Millward, 2001). Clearly, other techniques are needed to settle this debate.

In fact, mass spectrometry, stable isotopes and research designs to determine protein and amino acid (AA) requirements in humans have evolved over the past 30 years (Food And Agricultural Organization, 2007; Young, et al., 1985). This has enabled the use of newer stable isotope carbon oxidation methods to access the kinetics of protein metabolism. These methods have proven to be more rapid and sensitive to changes in protein (or AA) intake and have resulted in the identification of requirement values ~20-30% greater than those based on nitrogen balance at least for sedentary adults (Arentson-Lantz, et al., 2015; Pencharz & Ball, 2003). Recently, the indicator amino acid oxidation method (IAAO), a carbon oxidation method judged valid by the Institute of Medicine (Food And Agricultural Organization, 2007; Institute of Medicine, et al., 2005), has been used to determine protein requirements of humans at different ages (Elango, et al., 2011; M. A. Humayun, et al., 2007; Rafii, et al., 2016; Rafii, et al., 2015; Stephens, et al., 2015; Tang, et al., 2014). The studies within this dissertation have for the first time used IAAO to determine protein requirement in ET and ST and report it to be about 25 to 30% greater than previous NB derived estimates (Lemon, et al., 1992; Tarnopolsky, 2004; Tarnopolsky, et al., 1992; M.A. Tarnopolsky, et al., 1988).
5.3 Indicator amino acid oxidation method vs Nitrogen balance

In general, determination of protein (or AA) requirements involves feeding of graded quantities of the test protein (or AA) to the participants and looking for a clearly definable change in a relevant biological parameter. Therefore, theoretically, a similar estimate of requirements should be obtained irrespective of the method used (Pencharz & Ball, 2003). However, the merit of any method for an accurate determination of protein requirement depends on the sensitivity of its response parameter that is being measured. Classically protein requirement in humans is measured with the NB method (nitrogen in minus nitrogen out). Although a useful tool, its application in recent years has greatly diminished due to the many problems inherent in it (Food And Agricultural Organization, 2007; Millward, 2001). Two of the main concerns are the use of a linear regression line for analyzing a nonlinear response (Rand & Young, 1999; Zello, et al., 1995) and an over/underestimation of nitrogen intake and excretion, respectively (Hegsted, 1976). Further, NB is achievable at low protein intakes for the brief study durations often used because of a combination of more efficient AA utilization, reduced turnover rates, and/or accommodation (Pellet, 1990; Young, et al., 1987; Young & Marchini, 1990). For example, it has been suggested that applying a single linear regression line with a greater residual error, is not a good fit for either NB or oxidation data points (Zello, et al., 1995). As such, data analysis that locates the breakpoint more accurately in NB and/or in oxidation studies is one key to determining accurate dietary AA or protein requirements. Interestingly, a bilinear regression model has been used on the NB results of the published studies in which repeated measurements were made within the same individuals and the reported breakpoint in the data for either zero NB or oxidation of L-[1-13C] phenylalanine using IAAO was at a protein intake of 0.93 g. kg\(^{-1}\).d\(^{-1}\) (M.A. Humayun, et al., 2007) which exceeds the current requirement by >50%. However, the repeated 7 to 10 d adaptation periods required to produce accurate NB data for every participant at each of the several different protein intakes are not really practical.

Methods using stable isotopes and carbon oxidation to study protein (or AA) have proven to be more rapid and sensitive to changes in protein (or AA) intakes (Young, et al., 1987; Young, et al., 1985). The IAAO relies on stable isotopes to measure AA oxidation, but it differs from other carbon oxidation/balance designs in that the oxidation of indicator (an indispensable AA e.g., L-[1-13C] phenylalanine) is measured rather than the test AA. The cellular partitioning of the
indicator AA either to protein synthesis or oxidation in response to different protein (or AA) intakes is *rapid* and any adaptation occurs primarily at the acyl-t-RNA level. This critical because it adapts in less than four hours meaning than the long adaptation periods to changing intakes needed with the NB method can be avoided (Pencharz & Ball, 2003; Zello, et al., 1995). When dietary protein (or AA) intake is inadequate the oxidation of all other AA, including the indicator AA, will be substantial. With increasing dietary protein, oxidation of the indicator AA will decrease because more AA are being incorporated into body protein. Once the dietary requirement is met, there is no further change in the oxidation of the indicator AA and the resulting inflection or ‘breakpoint’ is thought to be the requirement (Elango, et al., 2012; Pencharz & Ball, 2003) (Figure 5.1).

![Diagram](image.png)

Figure 5.1. Pattern of indicator amino acid oxidation (IAAO) observed due to increasing intake of limiting amino acid/protein intake. Adapted from (Elango, et al., 2012).

The oxidation of $^{13}$C-labeled phenylalanine as an indicator in response to changes in protein (or AA) intake is a more accurate reverse proxy for whole body protein synthesis in both humans and animals than nitrogen response (Ball & Bayley, 1986; Rafii, et al., 2008). Two of the main criticisms of this method include whether the short adaptation period to the test protein (or AA) intake on the study day is appropriate and whether the measurements made during the fed-only state are representative, i.e., the fasting period within each day is not assessed. Studies both in animal and humans have shown that the oxidation of phenylalanine as a result of 8h to 10d of
adaptation to a wide range of lysine or protein intake was not different (Elango, et al., 2009; Moehn, et al., 2004) so it is clear that with the IAAO method the very long 7-10d adaptations to diet change required for the NB method are unnecessary. In addition, habitual protein intake of the participants is also not a factor with the IAAO method because the lysine requirement of malnourished Indian school age children is the same as that of well-nourished Canadian children (Elango, et al., 2007; Pillai, et al., 2010). Further, there were no systematic differences in measurement of the AA requirement when the results of 24h indicator balance method were compared with those of the IAAO method (Elango, et al., 2012) indicating that measures during the fed state are representative of a 24 hour period.

5.4 Protein requirement in endurance and strength trained individuals

Although NB derived estimates of protein requirement in ET and ST trained individuals point to a greater protein need (1.2- 1.7 g•kg\(^{-1}\)•d\(^{-1}\)) vs sedentary individuals, using NB as a response variable to measure protein requirement in exercise-trained individuals is not ideal for several reasons (Phillips, 2004, 2012; Phillips & Van Loon, 2011; Tarnopolsky, 2004). For example, NB studies on exercise-trained individuals have shown that with greater than RDA protein intakes NB is unrealistically positive (Friedman & Lemon, 1989; Lemon, et al., 1992; M.A. Tarnopolsky, et al., 1988). Moreover, this increased nitrogen retention within the short duration of these studies (10-15d) does not result in any increase in fat free mass and, therefore, was dismissed by some as an artefact. Further, based on the past evidence, it appears that NB may be achieved at lower protein intakes in exercise-trained individuals but at a cost of reduced protein synthesis rates in other tissues (Devlin, et al., 1990; Tarnopolsky, 2004; Tarnopolsky, et al., 1992; Young, et al., 1987), perhaps due to accommodation.

In fact, based on earlier stable isotope studies (Young, et al., 1987; Young & Bier, 1987; Young, et al., 1989; Young & Marchini, 1990) assessing both protein and AA requirements, four different states of protein metabolism were identified: 1) “protein deficiency” defined as the maximal reduction in protein synthesis to all but the essential organs, 2) “accommodation” where NB is achieved with a decrease in physiological relevant processes, 3) “adaptation” where optimal growth, inter-organ AA exchange, and immune function are present and 4) “excess” which is characterized by AA oxidization for energy and nitrogen excretion via urea, resulting in
no further stimulation of protein synthesis (Young, et al., 1981). Importantly it is known that when a protein intake close to the RDA is combined with exercise, accommodation results through increased nitrogen utilization efficiency and lower whole body protein synthesis rates rather than adaptation (Campbell, et al., 1995; Campbell & Leidy, 2007; Tarnopolsky, et al., 1992; Torun, et al., 1977). This would affect an exercise-trained individuals adversely. It well known that exercise at varying intensities promotes muscle damage and AA catabolism (Rennie, et al., 1981). The AA utilized during exercise are derived from increased proteolysis and decreased protein synthesis (Rennie, et al., 1981; Rennie, 1996). The decrease in protein synthesis is likely related to the diversion of AA and energy away from the events supporting protein synthesis (anabolism) toward the events supporting muscle breakdown (catabolism). Therefore, it is after exercise that rates of muscle protein synthesis have the potential to be accelerated beyond the rates of proteolysis, allowing protein balance to become positive (Devlin, et al., 1990; Rennie, 1996). Without post-exercise dietary protein supplementation, the protein synthesis rate would be suboptimal because of the decreased availability of AA. Consistent with this are data showing that provision of 10 to 16 g of protein after an acute 60 min bout of moderate endurance exercise enhances muscle protein synthesis and net muscle protein balance despite a negative whole body protein balance (Levenhagen, et al., 2002; Lunn, et al., 2012). Similarly, a protein intake of about 20-25g after an acute bout of strength exercise can result in a maximum increase in muscle protein synthesis (Moore, et al., 2009; Pennings, et al., 2012). Of course, maximizing whole body protein synthesis for exercise-trained individuals is important. This is exactly what IAAO method measures, as it determines an intake of protein (or AA) which results in a plateau of whole body protein synthesis including all relevant repair and remodelling processes. It should be emphasized that these processes are not only limited to muscles because ligaments, tendons, bones, as well as the gut (van Wijck, et al., 2012; Van Wijck, et al., 2011; Wasserman, et al., 1991) and immune system (Newsholme & Parry-Billings, 1990; Tidball & Villalta, 2010) are also affected by exercise and their need for repair and recovery needs be taken into consideration.

We have measured the protein requirement in ET and ST trained exercise-trained individuals using IAAO method. The break point (EAR; Estimated Average Requirement) for ET and ST were 2.0 and 1.7 g•kg⁻¹•d⁻¹ with RDA of 2.6 and 2.2 g•kg⁻¹•d⁻¹, respectively. Our EAR results in
comparison to previous NB studies on ET (Friedman & Lemon, 1989; Meredith, et al., 1989; Tarnopolsky, 2004; M.A. Tarnopolsky, et al., 1988) and ST (Lemon, et al., 1992; Tarnopolsky, et al., 1992) are on average about 50-70% greater (Table 5.1). This follows the same trend observed when the protein requirement measured using NB in sedentary young men (SED) were compared with the results of IAAO (M. A. Humayun, et al., 2007). A cross-sectional comparison between our studies on ET and ST and an IAAO study on SED young men (M. A. Humayun, et al., 2007) revealed a 2.1 and 1.8 fold greater protein requirement in ET and ST, respectively with no significant differences for age or height between any groups (Table 5.2).

Table 5.1 Comparison of studies measured protein requirement (EAR) in endurance and strength trained athletes using nitrogen balance or indicator amino acid oxidation method

<table>
<thead>
<tr>
<th>Nitrogen balance</th>
<th>Indicator amino acid oxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Endurance Athletes</strong></td>
<td><strong>Endurance Athletes</strong></td>
</tr>
<tr>
<td>1.4 g•kg(^{-1})•d(^{-1})</td>
<td></td>
</tr>
<tr>
<td>1.4 g•kg(^{-1})•d(^{-1})</td>
<td>2.0 g•kg(^{-1})•d(^{-1})</td>
</tr>
<tr>
<td>0.94 g•kg(^{-1})•d(^{-1})</td>
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</tr>
<tr>
<td><strong>Strength Athletes</strong></td>
<td><strong>Strength Athletes</strong></td>
</tr>
<tr>
<td>0.82 g•kg(^{-1})•d(^{-1})</td>
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</tr>
<tr>
<td>1.4 g•kg(^{-1})•d(^{-1})</td>
<td>1.72 g•kg(^{-1})•d(^{-1})</td>
</tr>
<tr>
<td>1.4-1.5 g•kg(^{-1})•d(^{-1})</td>
<td></td>
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</tbody>
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90
Table 5.2 Physical characteristics, daily energy and macronutrient intake and protein requirement of sedentary, strength- and endurance trained young men\(^1,2\)

<table>
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<th>Characteristics</th>
<th>Sedentary</th>
<th>Strength trained</th>
<th>Endurance trained</th>
</tr>
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<tbody>
<tr>
<td>Age, y</td>
<td>22.5±1.7</td>
<td>22.5±1.7</td>
<td>26.6±5.8</td>
</tr>
<tr>
<td>Mass, kg</td>
<td>69.6±10.3</td>
<td>83.9±11.6*</td>
<td>67.9±5.7</td>
</tr>
<tr>
<td>Height, m</td>
<td>1.7±0.1</td>
<td>1.74±0.1</td>
<td>1.8±0.1</td>
</tr>
<tr>
<td>Body fat(^3), %</td>
<td>18.3±5.8</td>
<td>13.0 ±6.3</td>
<td>9.8 ±5.0*</td>
</tr>
<tr>
<td>FFM, kg</td>
<td>56.4±5.5</td>
<td>72.4±5.5*</td>
<td>61.2±6.0</td>
</tr>
<tr>
<td>REE(^4), kcal•kg(^{-1})•d(^{-1})</td>
<td>19.8±3.1</td>
<td>22.3±2.2</td>
<td>24.9±3.1</td>
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<tr>
<td>Energy intake(^5), kcal•kg(^{-1})•d(^{-1})</td>
<td>-</td>
<td>37.0±7.0</td>
<td>40.7±3.9</td>
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<td>Carbohydrate intake(^5), g•kg(^{-1})•d(^{-1})</td>
<td>-</td>
<td>3.9±1.6</td>
<td>5.1±0.3</td>
</tr>
<tr>
<td>Protein intake(^5), g•kg(^{-1})•d(^{-1})</td>
<td>-</td>
<td>2.4±0.8</td>
<td>2.1±0.9</td>
</tr>
<tr>
<td>Fat intake(^5), g•kg(^{-1})•d(^{-1})</td>
<td>-</td>
<td>1.3±0.5</td>
<td>1.3±0.3</td>
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<td>Phenylalanine flux, μmol•BM(^{-1})•h(^{-1})</td>
<td>58.5±14</td>
<td>67.4±3.8*</td>
<td>62.6±8.7</td>
</tr>
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<td></td>
<td>71.9±19</td>
<td>76.3±11.7*</td>
<td>70.2±9.0</td>
</tr>
<tr>
<td>Protein requirement, g•kg(^{-1})•BM•d(^{-1})</td>
<td>0.93</td>
<td>1.72</td>
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\(^1\)All values are means±SD, n= 8, comparisons were performed by t test. BM, Body Mass; FFM, Fat-Free Mass.
\(^2\)Protein requirements were measured by indicator amino acid oxidation technique.
\(^3\)Body fat was measured using Bod Pod\(^\circledR\) (Life Measurements, Concord, CA) except for sedentary men where bioelectrical impedance was used.
\(^4\)Resting energy expenditure measured by open circuit indirect calorimetry (Vmax Legacy, Sensor Medics, CA, USA).
\(^5\)Energy intake and macronutrient breakdown were based on a 3-d dietary record analysis.

*\(^p<0.05\).

Among the three groups compared, flux values at rest were 15.2 and 7% greater in ST and ET compared with SED but only flux value of ST was significantly different from that of ET (\(p<0.05\)). Greater variation in results reported in flux values of SED prevented detection of a statistical significance between this group and any of the other two. Greater flux in ST and ET is consistent with previous reports demonstrating an increase in flux within the range of 15 - 21% in both ST and ET (Pikosky, et al., 2006; Short, et al., 2004; Tarnopolsky, et al., 1992; Welle, et al., 1995) which might indicate a greater adaptive remodelling at the whole body level in exercise-trained individuals. Compared with SED, the expected greater protein requirement and flux of ST could be mainly due to the maintenance and remodelling of 16 kg greater fat free...
mass accrued in these trained individuals. While the same cannot be said of ET, a greater protein requirement in these exercise-trained individuals has been suggested (Tarnopolsky, 2004) to be involved in metabolic adaptations relevant to the increased amount of oxidative enzymes, mitochondria biogenesis, capillary bed formation, as well as hemoglobin and myoglobin synthesis induced by regular aerobic training (Tarnopolsky, 2004). Indeed, as has been explained within the concept of the adaptive metabolic demand model (Millward, 2003), there could be a long term adaptive response to protein requirement that is relatively insensitive to short-term training and dietary manipulation but can change over the long term. Therefore, we studied individuals who were exercise trained for more than a year to eliminate any possible acute training induced changes in their metabolic demand and protein requirement. Although, the definitive answer to the required protein for optimal adaptation in exercise trained individuals will need to come from longitudinal studies, current findings with IAAO method points to a greater protein requirement in those trained.
5.5 References


Liebig, J., & Gregory, W. (1842). Animal chemistry, or organic chemistry in its application to physiology and pathology: JSTOR.


Chapter 6.

Discussion
6.1 General discussion

Habitual protein intakes of ET and ST are reported to be several times greater than the RDA (Lemon, 2000; Tarnopolsky, 2004). Although questionable on methodological grounds, protein estimates derived using NB method on exercise-trained individuals point to a requirement of 1.4 to 1.7 g\(\text{kg}^{-1}\text{d}^{-1}\) (Lemon, et al., 1992; Tarnopolsky, 2004), i.e., several fold greater than RDA. However, the Institute of Medicine does not recognize these estimates as valid and recommends no additional dietary protein for exercising individuals compared with sedentary adults. Several longitudinal studies have shown that protein intakes closer to RDA result in increased nitrogen utilization efficiency and decreased whole body protein synthesis rates which may not be conducive to optimal training-induced adaptation (Butterfield & Calloway, 1984; Campbell, et al., 1995; Campbell & Leidy, 2007; Tarnopolsky, et al., 1992; Torun, et al., 1977). It is reasonable to assume that the dietary protein needed to maximize whole body protein synthesis in order to repair and remodel would be increased in exercise trained vs sedentary individuals. In fact, recent reports have shown greater training-induced adaptation as a result of protein intakes of about twice RDA (Cermak, et al., 2012; Snijders, et al., 2015). However, studies on measuring protein requirement of exercise-trained individuals have not been an active area recently due to a lack of a better methodology. Recently, the IAAO method has been proven to be an accurate and sensitive method to determine the protein requirement and it has been used for sedentary individuals (Rafii, et al., 2016; Rafii, et al., 2015). The objective of this dissertation was to determine the protein requirement in ET and ST using IAAO method and to compare the results with values reported on sedentary young men using the same methodology.

Our results indicate a break point (EAR; Estimated Average Requirement) for ET and ST of 2.0 and 1.7 g\(\text{kg}^{-1}\text{d}^{-1}\) with RDA of 2.6 and 2.2 g\(\text{kg}^{-1}\text{d}^{-1}\), respectively. These results are about 50-70% greater in comparison to previous NB studies on ET (Friedman & Lemon, 1989; Tarnopolsky, 2004; Tarnopolsky, et al., 1988) and ST (Lemon, et al., 1992; Tarnopolsky, et al., 1992). This greater need in the exercise trained follows the same trend observed when protein requirement measured using NB in sedentary young men (SED) are compared with the results of IAAO (Humayun, et al., 2007). A cross-sectional comparison between our studies on ET and ST and an IAAO study on SED young men (Humayun, et al., 2007) revealed a 2.1 and 1.8 fold
greater protein requirement in ET and ST, respectively with no significant differences for age or height between any groups.

Among the three groups compared, flux values at rest were 15.2 and 7% greater in ST and ET compared with SED but only flux values of ST were significantly different from those of ET ($p<0.05$). This is similar to the previous reports reporting an increase in flux within the range of 15 to 21% in both ST and ET (Pikosky, et al., 2006; Short, et al., 2004; Tarnopolsky, et al., 1992; Welle, et al., 1995) which might refer to a greater adaptive remodelling at the whole body level in those exercise trained. Compared with SED, the expected greater protein requirement of ST could be due to the maintenance and remodelling of 16 kg greater fat free mass accrued in these trained individuals. While the same cannot be said of ET, their greater requirement has been suggested to be due to the metabolic adaptations relevant to the increased quantities of oxidative enzymes, mitochondria, capillaries, hemoglobin and myoglobin induced via regular aerobic training (Tarnopolsky, 2004).

Whether protein requirement in exercise trained women would be similar is unclear. Previous studies have demonstrated that women oxidize proportionately more lipid and less carbohydrate than do men during endurance exercise (Phillips, et al., 1993; Tarnopolsky, et al., 1990; Tarnopolsky, 2004). Further stable isotope tracer studies have reported less leucine oxidation during exercise in women compared to men (Lamont, et al., 2001, 2003; McKenzie, et al., 2000). Therefore, it may be that exercise trained women might have lower protein requirement compared to men. We studied a single female endurance runner using IAAO method as a pilot study (at 4 protein intakes from 0.5-2.0 g•kg$^{-1}$) and, much more data are needed, it appears that her protein requirement might be lower than the male exercise-trained individuals studied (Figure 6.1).

### 6.2 Summary/Conclusion

Our IAAO derived break point (EAR; Estimated Average Requirement) for ET and ST were 2.0 and 1.7 g•kg$^{-1}$•d$^{-1}$ with RDA of 2.6 and 2.2 g•kg$^{-1}$•d$^{-1}$, respectively. These estimates are 2.7- 3.2 fold greater than RDA for sedentary young men. These results are clear evidence for greater dietary protein needs of exercise trained men on a non-training day. The protein requirements
for exercise trained women might not be as great; however, more data are required to document this.

6.3 Future studies

Of course, many different training methods are used routinely by exercise-trained individuals depending on their respective sports. These could result in both different adaptations and protein requirements. Consequently, future studies need to evaluate protein (or AA) requirements of different targeted athletic populations, (e.g., ball sports, swimmers, sprinters, etc) to get a better idea how various chronic exercise regimes affect dietary protein need. Our studies focused on strength and endurance trained men only. In addition, there is a dearth of studies investigating protein (or AA) requirements in exercise trained women and these are needed because some evidence suggests protein needs might be lower in women. Finally, controlled longitudinal nutrition/training studies with differing protein intakes as well as assessments of performance and body composition are required to confirm the results from these short-term oxidation studies.

Figure 6.1 Protein intake and production of $^{13}\text{CO}_2$ from phenylalanine oxidation ($F^{13}\text{CO}_2$) in a young endurance trained woman ($n = 4$).
6.4 References


Appendices

Study Day Protocol

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Chapter 5: Figure 5.1 (Elango R, Ball RO, Pencharz PB. Recent advances in determining protein and amino acid requirements in humans. Br J Nutr 2012;108:S22-S30.)
Western University Health Science Research Ethics Board
HSREB Full Board Initial Approval Notice

Principal Investigator: [Redacted]
Department & Institution: Health Sciences/Kinesiology, Western University

Review Type: Full Board
HSREB File Number: 100916
Study Title: Measuring protein requirement using the indicator amino acid oxidation method in healthy young strength and endurance exercise trained individuals
Sponsor:

HSREB Initial Approval Date: November 19, 2015
HSREB Expiry Date: November 19, 2016

Documents Approved and/or Received for Information:

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The Western University Health Science Research Ethics Board (HSREB) has reviewed and approved the above named study, as of the HSREB Initial Approval Date noted above.

HSREB approval for this study remains valid until the HSREB Expiry Date noted above, conditional to timely submission and acceptance of HSREB Continuing Ethics Review.

The Western University HSREB operates in compliance with the Tri-Council Policy Statement Ethical Conduct for Research Involving Humans (TCPS2), the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use Guideline for Good Clinical Practice Practices (ICH E6 R1), the Ontario Personal Health Information Protection Act (PHIPA, 2004), Part 4 of the Natural Health Product Regulations, Health Canada Medical Device Regulations and Part C, Division 5, of the Food and Drug Regulations of Health Canada.

Members of the HSREB who are named as Investigators in research studies do not participate in discussions related to, nor vote on such studies when they are presented to the REB.

The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

Ethics Office to Contact for Further Information: Erika Boulé, Nicole Kajikia, Grace Kelly, Meera Mathiak, Vikki Trask

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