AN ABSTRACT OF THE THESIS OF

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James E. Leklem

The purpose of this study was to examine the effect of extreme exercise on vitamin B-6 metabolism and urea nitrogen. Nine men and five women completed two 5-day trials; Trial 1 (T1) included a 50-km ultramarathon on day 4 and during Trial 2 (T2) subjects were "inactive" on day 4. During both trials, subjects consumed a diet providing men 2.0 and women 1.5 mg of vitamin B-6. With the exception of the ultramarathon, T1 activity was replicated during T2. Twenty four-hour urine collections were completed and blood was drawn pre-race (pre), mid-race (mid), post-race (post) and 60 minutes post race (P-60). On the inactive, day blood was drawn at the same intervals. Plasma was analyzed for pyridoxal 5'-phosphate (PLP), pyridoxal, 4-pyridoxic acid (4-PA), urea nitrogen (PUN), creatinine, albumin, glucose, and lactate concentration and alkaline phosphatase activity. Urine was analyzed for 4PA, creatinine, and total urinary nitrogen (TUN).

During T1, compared to pre, plasma PLP concentration increased 17% at mid, decreased 5% by post, and 19% by P-60. During T2, plasma PLP concentration decreased 13% pre to P-60. During T1, plasma 4-PA concentration increased 135% and the percent dietary vitamin B-6 that was excreted as urinary 4-PA the day of the ultramarathon was higher than that

excreted the day before and the day after. During T1, from pre to post mean PUN concentration increased 36.9%, and the average rate of increase from pre to mid, mid to post, and post to P60 was 0.5, 1.75, and 2 mg/dL/hour, respectively. During T1 on days 3, 4, and 5, 88%, 100%, and 95% of nitrogen intake was excreted in the urine compared to 86%, 83%, and 84% for the same days during T2. The day of the ultramarathon, 24-hour TUN excretion was 2 g higher than the previous day.

Extreme exercise of greater than six hours initially increases the plasma concentration of PLP but ultimately results in a significant decrease in plasma PLP, an increase in plasma 4-PA, and an increase in percent of dietary vitamin B-6 (as 4-PA) excreted in the urine. Additionally, the rate of change in PUN increases as duration increases.

The Effect of a 50-km Ultramarathon on Vitamin B-6 Metabolism and Plasma and Urinary Urea Nitrogen

by

Ann Grediagin

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Head of Department of Nutrition and Food Management

Dean of Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Ann Grediagin, Author

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DEDICATION

In memory of my mother, Nancy Earle Grediagin, who passed to me her love of learning and taught me I could do or be anything I wanted.

The Effect of a 50-km Ultramarathon on Vitamin B-6 Metabolism and Plasma and Urinary Urea Nitrogen

INTRODUCTION

Vitamin B-6, in its active coenzyme form 5'-phosphate (PLP), is involved in over 100 different enzymatic reactions (Sauberlich, 1985). Related to energy metabolism, PLP is essential for the breakdown of glycogen and amino acids into glucose and for transamination of amino acids prior to their oxidation for energy. Without PLP, the body would not be able to access glycogen stores or metabolize amino acids.

Endurance events create an extreme metabolic challenge to the body. To sustain performance, such events may require over 1000 calories per hour over extended periods of time. The body's challenge is to maintain blood glucose while being deprived of adequate calories, a situation not unlike acute starvation. Initially, the primary fuel is glucose derived from stored glycogen. However, as these stores are depleted, metabolism shifts to an increased reliance on fat and amino acids to provide blood glucose and energy.

The limits of human endurance are continually being challenged. One hundred-mile events are not uncommon and some cover several hundred miles and span several days or weeks. Not surprisingly, one of the key limiting factors in such events is fuel availability and/or accessibility and competitors frequently succumb to advertisements for products that claim to maintain energy levels and improve performance. Because of its role in fuel metabolism, there has been a boon of "performance" products fortified with vitamin B-6 claiming to "enhance energy production". Unfortunately, quite often it is unknown how much, or if, exercise increases the body's nutritional requirements and the requirement for vitamin B-6 is no exception. Science is often a slow, methodical process and no single study can answer such a

question. Researchers agree that vitamin B-6 metabolism is altered by exercise. However, the significance of the alterations and their impact on vitamin B-6 requirement, is still hotly debated.

Purpose

Most research on endurance events less than two hours shows that plasma vitamin B-6 levels increase during the event and then return to normal within an hour. However, in a recent study on ultramarathon subjects (average duration of exercise 6 hours and 15 minutes), during the event, plasma PLP levels decreased to below baseline values (Leonard and Leklem, 2000). Furthermore, in the hour following the event, the levels continued to decline instead of returning to the baseline values as expected.

The purpose of this study was to examine the effect of an ultramarathon (31 miles with a 12,800 foot elevation change) on vitamin B-6 metabolism and plasma and urinary urea nitrogen. This was accomplished by measuring plasma and urine indices of vitamin B-6 and urea metabolism. As a basis for comparison, subjects served as their own controls during an identical "inactive" trial. Additionally, to control for the effect of diet on plasma and urine metabolites, the subjects consumed a controlled diet during both the ultramarathon and rest trial.

Hypotheses

The following hypothesis will be tested:

1. Compared to control (inactive) conditions, running an ultramarathon will cause significant changes in plasma pyridoxal 5'-phosphate, 4-pyridoxic acid (4-PA), and urea concentration during and after the event.
2. Compared to control (inactive) conditions, running an ultramarathon will cause urinary 4-PA and urea excretion to increase.

Objectives

The purpose of this project was to examine the effect of extreme endurance exercise on vitamin B-6 metabolism and plasma and urinary urea nitrogen. Specific objectives are to:

1. Determine if running longer than six hours results in significant changes in plasma PLP, pyridoxal (PL), 4-PA, and urea, and to determine if the magnitude and direction of the change is the same during the first three hours of exercise compared to the second three hours.

2. Determine how much of the change in plasma variables is attributable to foods consumed during the event and/or to normal daily fluctuations.

3. Determine if the amount of dietary vitamin B-6 excreted as urinary 4-PA, and dietary nitrogen as urinary urea nitrogen, is greater the day of the ultramarathon compared to the day before or the day after.

4. Determine if the change in plasma PLP is correlated with the change in the plasma concentration of albumin, glucose, lactate, and/or urea and/or alkaline phosphatase activity.

LITERATURE REVIEW

Vitamin B-6

History

For the past 70 years scientists have investigated the role of vitamin B-6 in health and disease. Vitamin B-6 was discovered in the 1930s as the result of nutritional studies with rats fed vitamin-free diets. Paul Gyorgy coined the term in 1934 (Gyorgy, 1934), and isolation of pure crystalline vitamin B-6 was accomplished in 1938 (Lepkovsky, 1938). By 1939, the chemical structure of the compound had been identified as 3-hydroxy-4, 5-dihydroxymethyl-2-methylpyridine. This compound was named pyridoxine because of its structural similarity to pyridine (Gyorgy and Eckhardt, 1939).

Laboratory synthesis of vitamin B-6 allowed for more detailed research on microorganisms and animals. During the course of investigation, it was noted that some organisms had greater growth activities than predicted. In 1942, Snell and co-workers concluded that another form(s) of pyridoxine must be present (Snell et al, 1942). This compound was initially called pseudopyridoxine and was subsequently isolated, synthesized, and found to be two compounds: pyridoxal and pyridoxamine. (Snell, 1944b).

The discovery and identification of pyridoxal 5'-phosphate (PLP) led to the recognition that vitamin B-6 was physiologically active as a coenzyme. In the 1940s, Esmond Snell and his colleagues discovered the key to the function of PLP (Rabinowitz and Snell, 1947). They noted that all known PLP requiring reactions could be catalyzed by pyridoxal and metallic ions (Al^{3+} or Cu^{2+}) in the *absence* of the enzyme. The reaction rates were much lower but the model provided an opportunity to detail the Schiff base reaction between pyridoxal and the amino acid group. The identification of PLPs role in Schiff base reactions opened up a whole new

appreciation of this remarkably versatile coenzyme and its precursors. By the 1960s it was clear that there were three natural forms of vitamin B-6: pyridoxine (PN), pyridoxal (PL), and pyridoxamine (PM) and that all were interrelated and could be converted to PLP (Gyorgy, 1971).

Structure and Chemistry

Since Gyorgy first applied the name "vitamin B-6" there has been confusion about what the name applies to. The term "vitamin B-6" refers not just to one compound, but to all biologically active forms of vitamin B-6. These include three basic forms and their phosphorylated counterparts as listed in Table 1.1.

Table 1.1. Biologically active forms of vitamin B-6 in humans

Basic Form	Phosphorylated form
Pyridoxal (PL)	Pyridoxal 5'-phosphate (PLP)
Pyridoxine (PN)	Pyridoxine 5'-phosphate (PNP)
Pyridoxamine (PM)	Pyridoxamine 5'-phosphate (PMP)

These six forms are commonly referred to as B-6 vitamers, and PL, PN, PM, PNP, and PMP are able to be metabolized to PLP as illustrated in Figure 1.1. The major excretory product is the irreversible end product 4-pyridoxic acid (4-PA), although humans have been reported to excrete substantial amounts of 5-pyridoxic acid (5-PA) during periods of high dose PN supplementation (Mahuren, 1991). B-6 vitamers are soluble in water and minimally soluble in organic solvents. The free forms are considered to be relatively labile with pH playing a major role. B-6 vitamers are heat-stable under acidic conditions and heat-liable under alkaline conditions. (Leklem, 1991). In aqueous solution, the forms are light sensitive in alkaline conditions but relatively stable under acidic conditions (Ang, 1979).

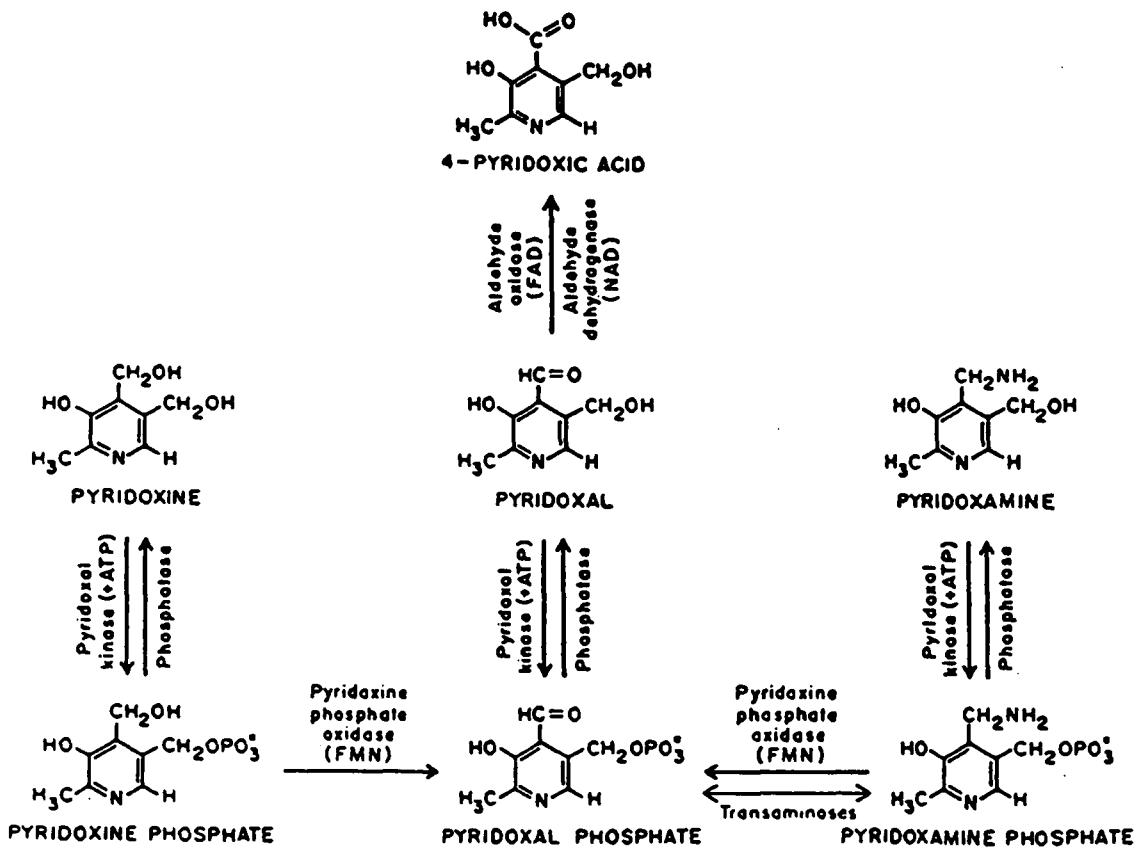

Figure 1.1. Metabolic interconversions of the B-6 vitamers (Adapted from Leklem, 1988)

The physiologically active coenzyme form of vitamin B-6 is PLP and is bound to enzymes by formation of a Schiff base with the ε-amino group of a lysine residue (Leussing, 1986). In most instances, PLP participates in enzymatic reactions by forming a Schiff base with an amino group of the substrate. Figure 1.2 depicts this formation.

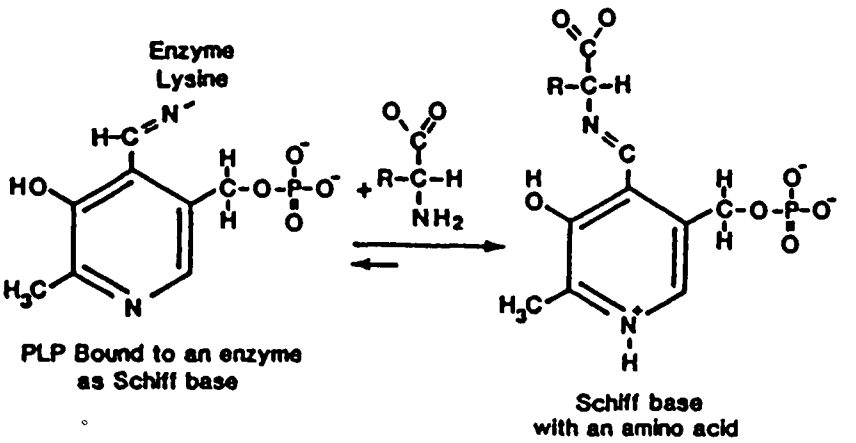

Figure 1.2. Schiff base formation between pyridoxal 5'-phosphate and an amino acid.

PLP is well suited for Schiff base formation because of the unique combination of structural features detailed in Table 1.2. Structurally, the most important catalytic feature of PLP is the electrophylic nitrogen of the pyridine ring.

Table 1.2. Function and effect of PLP moieties[1].

Chemical Moiety	Function/Effect
Protonated pyridine nitrogen	◆ Electrophylic, aids in delocalizing the negative charge. ◆ Regulates the pK_a of the 3-hydroxal group.
Phenoxide Oxygen	◆ Contributes to the stability of the ammonium Schiff base. ◆ Facilitates expulsion of a nucleophile at the 4-C position.
5'-Phosphate	◆ Serves as an anchor for the coenzyme in a polar pocket directed away from the aldimine. ◆ Prevents hemiacetal formation and the drain of electron density from the ring.
2-Methyl group	◆ Brings the pK_a of the ring pyridine proton closer to the biological range.

[1] Adapted from Leklem, 1988.

Absorption and Transport

Vitamin B-6 enters the body via the gastrointestinal tract and is either absorbed or excreted in the feces. All B-6 vitamers are absorbed in their nonphosphorylated forms (Henderson, 1985) and transported to the liver for conversion to PLP (Lumeng et al, 1974a). The overall pathway of absorption, transport, and metabolism of vitamin B-6 is shown in Figure 1.3.

Absorption of vitamin B-6 has been studied most extensively in the rat. In this animal, PN, PM, and PL cross the intestinal lumen by nonsaturable passive diffusion (Middleton, 1977). Absorption of the phosphorylated forms is very limited and the majority are hydrolyzed intraluminally by gastrointestinal phosphatase then either rephosphorylated or transported in free form via the blood (Henderson, 1985). Human absorption of vitamin B-6 is thought to be similar with most of the process occurring in the jejunum (Brian and Booth, 1964).

Once in the circulation, PLP and PL normally account for greater than 90% of the 0.2–0.4 μmol of circulating vitamin B-6 (Leklem, 1996). The majority is carried in the plasma (0.12–0.24 μmol) and the remainder in the erythrocytes. PLP is transported in the plasma bound tightly to albumin and in the erythrocyte bound to hemoglobin. PL is transported in free form or can bind loosely to albumin or may be taken up by the erythrocyte and either bound to hemoglobin or converted to PLP. PN can also be taken up and transported by the erythrocytes, where some PN can be converted to PL, with possible further conversion to PLP (Mehansho and Henderson, 1980). Through the uptake of PL and PN, followed by partial conversion to PLP, erythrocytes transport both PL and PLP, but it is unclear how available the PL/PLP is to other tissues.

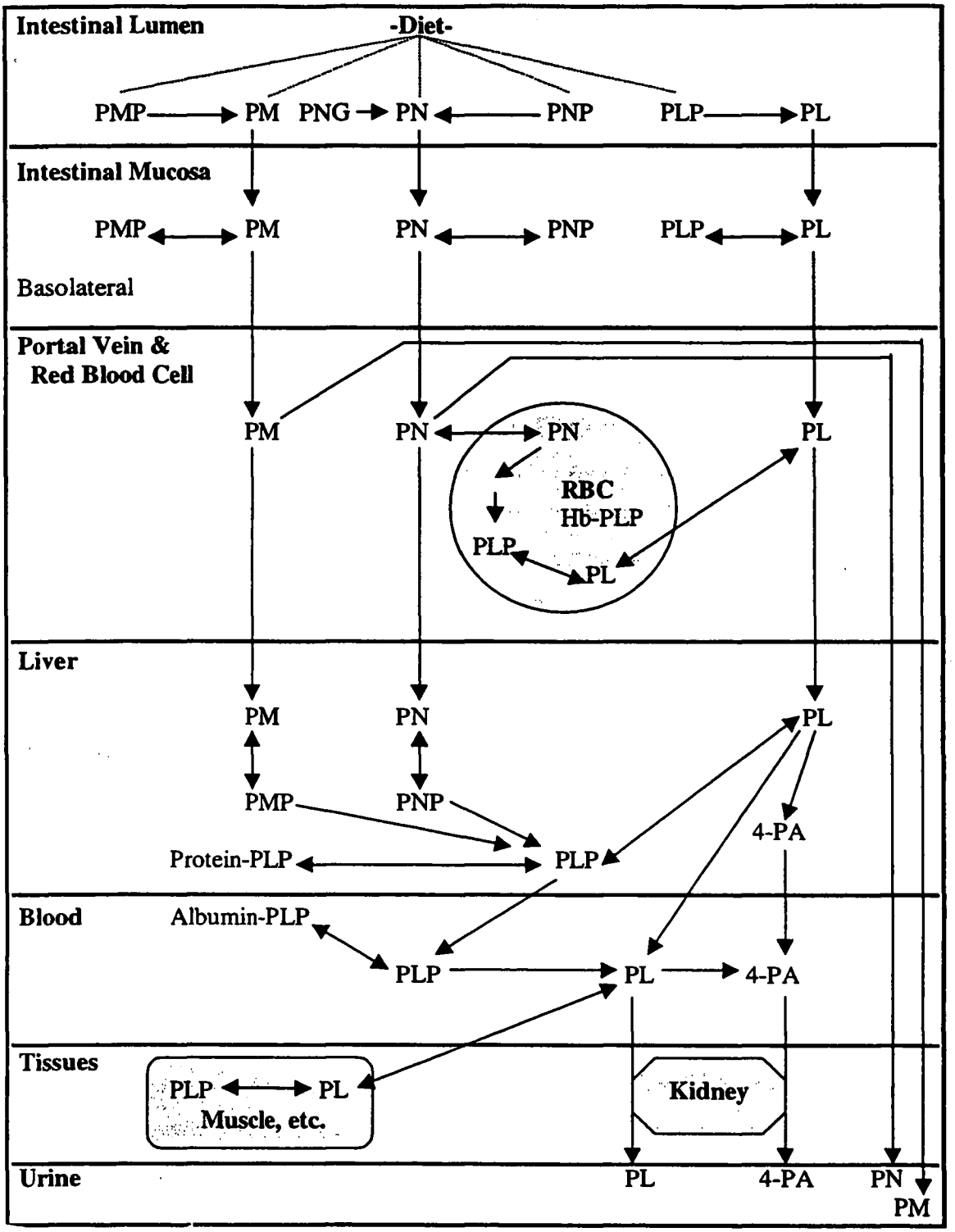

Figure 1.3. Overview of vitamin B-6 transport, metabolism, and excretion (Adapted from Leklem, 1996). PNG = pyridoxine-5'-β-D-glucoside, see Table 1.1 for other abbreviations.

Metabolism

Several organs and tissues are involved in the metabolism and interconversion of B-6 vitamers. Based on tissue distribution of pyridoxal kinase, the liver, spleen, brain, and kidneys are possible sources of PLP in the plasma (Lumeng et al, 1974a). However, most research supports the claim that though extra-hepatic tissues can take up PN and PM and convert them to their respective phosphorylated forms, they cannot convert appreciable amounts to PLP. To determine the tissue origin of PLP Lumeng et al (1974a) carried out organ ablation studies in dogs. He found that following pyridoxine or pyridoxal loading, only when the liver was intact was there a significant increase in plasma PLP. This study provided evidence that the liver is the principal source of plasma PLP.

Liver

The liver serves a central role in vitamin B-6 metabolism and is the major source of plasma PLP (Lumeng et al, 1974a), which it synthesizes from the other vitamers. Essentially all tissues have PL kinase, but few have significant amounts of PNP and PMP oxidase. Thus, the liver is responsible for converting dietary PN and PL to PL (via PLP), which the other tissues take up from circulation and convert to PLP (Merrill and Henderson, 1990; Leklem, 1991). Free forms of vitamin B-6 enter the hepatocytes by diffusion and are then metabolically trapped by phosphorylation (Merrill et al, 1984) and loosely bound to macromolecules in the cytosol (Lui et al, 1981). Although the exact mechanism is unknown, it appears that the liver possesses a unique transport mechanism which allows efflux of PLP from the cytosol into the plasma (Lumeng et al, 1974a) in sufficient quantities to meet peripheral tissue requirements. The final function of the liver is to convert PLP and PL to the dead-end catabolite 4-PA, which is then excreted in the urine.

Table 1.3. Activity of Liver Enzymes[1]

Enzyme	Conversion Catalyzed	Activity[2] nmol/min/g
Pyridoxal Kinase	PL → PLP	11.2
	PM → PMP	3.0
	PN → PNP	3.0
Pyridoxamine/Pyridoxine Oxidase	PMP → PLP	2.4
	PNP → PLP	0.4
Phosphatase	PLP → PL	0.1–2.0
	PMP → PM	
	PNP → PN	
Pyridoxal Oxidase	PL → 4-PA	16.5

[1] Substrate concentration of 10 μM.
[2] Merrill and Henderson, 1990 and Merrill et al, 1984

Liver enzyme kinetics are complex and vitamin B-6 metabolism is affected by numerous factors including vitamer concentration, pH, and mineral concentration. Using human liver biopsies, Merrill et al (1984) determined the activities of the kinase, oxidase, and phosphatase enzymes involved in hepatic vitamin B-6 metabolism (Table 1.3). Key findings from this work are that the rates of phosphorylation of PL and PL are much greater than for dephosphorylation, the rate of oxidation of PL to 4-PA is similar to that of phosphorylation, and the phosphorylation of PN and PM is somewhat slower than the conversion of PNP and PMP to PLP (Figure 1.4). Other interesting findings include the fact that phosphatase activity was optimal at pH 9, and kinase activity was stimulated by Zn^{2+}, and, to a lesser extent, Mg^{2+}, and activity increased as the pH went from 7 to 5.8. In contrast, in a study on human erythrocytes, Fonda (1992) purified a membrane acid phosphatase specific to vitamin B-6 that had optimum activity at pH 6–6.5 and was inhibited by Zn^{2+}.

Because PLP is highly reactive with other enzymes, accumulation in the liver must be regulated. This is accomplished in part by PLP inhibiting pyridoxamine and pyridoxine oxidase thus decreasing the conversion of PMP and PNP to PLP. Additionally, once PLP is formed it is quickly bound to a protein (usually albumin) and released from the liver. If PLP does

accumulate in the liver, phosphatase activity increases and there is an increased conversion of PLP to PL which in turn is rapidly converted to 4-PA by pyridoxal oxidase (activity 16.5 nmol/min/g) rather than rephosphorylation by pyridoxal kinase (activity 11.2 nmol/min/g). The goal of liver enzyme activity is to convert dietary vitamers to the physiologically active PLP and release it to circulation for uptake by other tissues. In humans urinary excretion of vitamin B-6 is minimal and does not usually exceed 6–12% of intake (Miller and Edwards, 1981). The major form of excretion is urinary 4-PA, which accounts for 40–60% of daily intake (Wozenski et al, 1980).

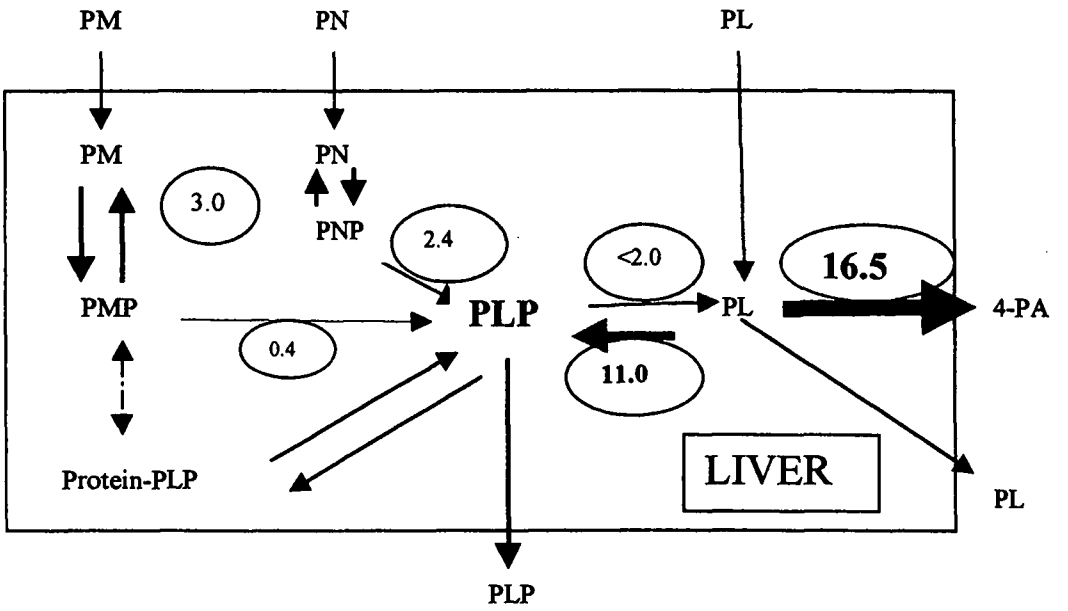

Figure 1.4. Metabolism of vitamin B-6 by the human liver and the estimated activity of the major enzymes involved in metabolism. The rates are nmol/min per gram of liver at the physiological pH with a substrate concentration of 10μM. (Merrill and Henderson, 1990).

To evaluate liver enzyme kinetics in vivo, Wozenski et al (1980) studied the short term (every hour for 5 hours) effect of equimolar (19.5 μmol) doses of PN, PM and PL on plasma PLP and urinary 4-PA. They found that PL caused the greatest increase in total vitamin B-6, but the change in PLP concentration over eight hours was significantly lower compared to the PN or

PM doses. Additionally, the PL dose was associated with higher urinary excretion of 4-PA. These observations suggest that hepatic pyridoxal oxidase activity was greater than pyridoxal kinase activity, and therefore, with the PL dose, less PL remained for conversion to PLP. This is in agreement with the enzyme activities reported by Merrill et al(1990).

Erythrocytes

The role of the erythrocyte in vitamin B-6 metabolism remains controversial. However, the fact that erythrocytes possess unique capabilities is irrefutable. Both PN and PL are taken up by simple diffusion and, because the erythrocyte has both kinase and oxidase activity, both can be converted to PLP (Mehansho and Henderson, 1980). There is also some evidence that PLP can be transported into the erythrocyte as an intact molecule. Lumeng et al (1974a) added PLP to a suspension of red blood cells and incubated them. After two hours, 22% of the added PLP was found in the erythrocytes, 38% was found in the external medium, and the 40% that was not recovered had presumably undergone hydrolysis by membrane-associated phosphatase. In contrast, in the same experiment when phosphatase activity was inhibited, 18% of the added PLP was recovered from within the erythrocyte and 77% was recovered in the external media. In the same study Lumeng also looked at PLP efflux *out* of the erythrocyte and found whereas PLP could not exit, PL could.

In total, erythrocytes contain 0.08–0.2 μmol of PLP and PL combined, which is less than 0.002% of total body content. The erythrocyte PLP is bound tightly to the β-chain of hemoglobin and PL forms a looser bond on the α-chain (Benesch et al, 1969). Ink et al (1982) found that PL accumulates rapidly in human erythrocytes and is not affected by PLP concentration. Furthermore, they found that the PL-hemoglobin bond is stronger than the PL-albumin bond. Because of this, erythrocyte PL concentration is generally 4–5 times higher than the plasma concentration (Leklem, 1988). The physiological reason behind this concentrative

uptake of PL is unclear and it has yet to be determined if the hemoglobin bound PL serves as a source of PL to other tissues.

Plasma

All B-6 vitamers are present in the plasma in varying concentrations and are in dynamic equilibrium between synthesis, cellular extraction, and degradation. Under normal conditions, the vitamin B-6 concentration of the plasma is 0.12–0.24 μmol and is less than 0.002% of total body stores. Of the total plasma vitamin B-6, PLP accounts for 60–70%, PL for 21–27%, PN for 5–17%, PM for 2%, and the remaining phosphorylated forms make up less than 1% (Leklem and Shultz, 1983; Lee and Leklem, 1985).

In the plasma, PLP is almost totally bound to protein, pyridoxal is partially bound, and pyridoxine is not bound at all (Anderson et al, 1974). The capacity of human serum proteins to bind PLP is in excess of 4000 μmol/L, which far exceeds normal plasma PLP concentration. While bound to albumin, PLP is protected from hydrolysis. With the possible exception of red blood cells, PLP must be released from albumin and dephosphorylated to PL by alkaline phosphatase before cells can take it up. The exact mechanism of this reaction is unknown. However, studies by Lumeng et al (1974a) show that hydrolysis occurs even when the phosphatase is separated from the albumin-bound PLP by a semipermeable membrane. This indicates that a small amount of free PLP normally exists in equilibrium with the albumin-PLP complex.

Plasma PLP concentration is affected acutely by supplementation. Lumeng et al (1974a) provided a 25-mg pyridoxine supplement daily and found a four- to five- fold increase in plasma PLP that plateaued in four days. When the supplement was discontinued plasma concentration decreased by 4–6 μmol/L per day for five days and was still slightly above pre-supplement concentration. Ubbink et al (1987) studied the metabolism of single oral doses of 10, 25, 50, and 100 mg of PN on plasma PLP and PL concentration 3, 24, 32, and 48 hours post-

dose. They found that three hours after the 10-mg supplement, the plasma PLP concentration still exceeded the PL concentration. However, with the higher doses, at three hours plasma PL concentration far exceeded PLP concentration. From 24 to 48 hours, a steady decline in plasma PLP and PL concentration was observed and in all cases PL concentration was again less than PLP. This rapid clearing of PL from circulation lead Ubbink to conclude that plasma PL is the immediate source of PLP. However, another explanation for Ubbink's findings is that plasma PL concentration decreased because of the increased hepatic conversion to 4-PA and 5-PA.

Phosphatase Activity

Central to the metabolism of vitamin B-6 are phosphatases. The term "alkaline phosphatase" is commonly applied to a mixture of isoenzymes contributed by bone, liver, and intestine that are substrate non-specific (Eastman and Bixler, 1977). Non-specific means they are capable of hydrolyzing many phosphate esters regardless of their organic moiety. Alkaline and acid phosphatases are found in the plasma, bound to cell membranes, and in almost all cellular organelles. Their concentration varies depending on the tissue. Intestinal and tissue-nonspecific alkaline phosphatases are credited with most PLP and PMP hydrolysis reactions (Fonda, 1992). However, acid phosphatases with broad substrate specificity that hydrolyze PLP have been purified from liver, kidney, bone, neutrophils, and neurons (Fonda, 1992; Wilson et al, 1983). Much of the evidence regarding the role of phosphatases in B-6 metabolism comes from studying disorders. Subjects with alcoholism or liver disease often have high levels of plasma alkaline phosphatase, an increased exertion of urinary 4-PA and low plasma PLP. Conversely, in patients with hypophosphatasia, plasma alkaline phosphatase activities are low and PLP levels high (Whyte et al 1985).

Because PLP does not cross cell membranes to any great degree, knowledge of the presence or absence of vitamin B-6 specific phosphatase enzymes in various tissues is essential to understanding vitamin B-6 regulation. Currently it is unknown how many tissues contain

PLP specific enzymes and/or how their activity is regulated. However, evidence is accumulating. In 1980, Kyaw partially purified an acid phosphatase specific for PLP from mouse liver and Wislon (1983) isolated both acid and alkaline pyridoxal phosphatases from different intracellular organelles in human neutrophils. Similarly, Fonda (1992) purified an acid phosphatase specific to vitamin B-6 from human erythrocytes and found it catalyzed the dephosphorylation with activity being greatest for PLP then 4-PA phosphate then PNP and finally PMP. Further, the phosphatase required Mg^{2+} for activity and was inhibited by Zn^{2+}.

Storage and Excretion (urine, sweat, feces)

Vitamin B-6 is water soluble and the common perception is that the body's store of B-6 is one "big" aqueous pool at equilibrium with all surrounding tissues. This perception is inaccurate at best. Concentrative uptake and retention by tissue occurs because phosphorylation of PL effectively traps it in the cell and also maintains cytosolic PL concentration at low levels which favors further uptake.

Several attempts have been made at identifying body pools of vitamin B-6. Initially, Johansson et al (1966) proposed a two-compartment model in which a large pool with slow turnover was in equilibrium with a small pool with rapid turnover and Shane (1978) refined this model by determining turnover to be 0.5 days and 25–33 days, respectively. More recently, Coburn (1990, 1996) used tracer studies and enzyme activities to model vitamin B-6 metabolism and found that even 75 pools were not sufficient. Human vitamin B-6 pools estimated through muscle biopsies (Coburn et al, 1988) indicate that the total body pool of vitamin B-6 is around 1000 μmol, of which 750–800 μmol is in the muscle, 50–100 μmol is in the liver, and 100–200 μmol is in other body tissues or pools (Table 1.4). This estimate is much higher than an earlier one by Johansson et al (1966) who used an equilibrium model and a single oral dose of labeled pyridoxine to estimate the total body pool to be between 100 and 200 μmol. In the Johansson study, it appears that the labeled pyridoxine did not move freely into all tissues

and was in equilibrium with only 10–20% of the total body pool. Males typically have more muscle mass than females, therefore their pool is greater (917 μmol) compared to females (850 μmol), but per gram of muscle there is no gender difference in muscle vitamin B-6 content (Coburn et al, 1988).

Table 1.4. Vitamin B-6 Content of Body Pools[1]

Pool	Total Amount (μmol)
Muscle	800–1000
Liver	18–24
Other tissues	15 nmol/g
Blood Plasma	0.12–0.24
Erythrocyte	0.08–0.20

[1]Based on data from Coburn, 1988 and Leklem, 1991.

Regulation of vitamin B-6 stores is complex. Lumeng et al (1974a) found the short term distribution of intravenously administered PLP was twice that accounted for in the plasma volume and speculated the plasma PLP was in equilibration with protein bound PLP in an interstitial pool and/or bound to the walls of the vascular system. Equilibrium between the plasma and muscle takes much longer. In animals it takes over 30 days of supplementation for muscle content of B-6 to plateau, however, plasma levels plateau after 2–4 days. Similarly, when supplementation ceases plasma levels of PLP will decrease but muscle concentrations are maintained. In humans, Lui provided a 25 mg PN supplement (by IV) for 28 days, and found within 10 days of discontinuation plasma PLP concentration decreased by 70% but remained three times higher than presupplementation levels for over two months.

McArdle's Syndrome, a disease characterized by myophosphorylase deficiency, provides a unique opportunity to quantify the relationship between muscle glycogen phosphorylase and PLP. Haller et al (1983) studied three patients with McArdle's syndrome

and found their total muscle pyridoxine concentration was less than 22% of subjects without McArdle's syndrome. Despite such low levels of muscle pyridoxine the patients showed no evidence of pyridoxine "deficiency". The fact that muscle pyridoxine concentration was low, but patients did not exhibit deficiency symptoms, suggests the decrease in muscle PLP concentration represents the specific loss of PLP bound to the phosphorylase enzyme and does not reflect overall vitamin B-6 status. Further, the degree of decrease supports the estimate that phosphorylase bound PLP accounts for 75–80% of the total pyridoxine in normal human muscle (Krebs and Fischer, 1964; Coburn et al, 1988). In contrast, in the liver only 10% of its total vitamin B-6 content is accounted for in the PLP-phosphorylase complex (Lumeng et al, 1978).

Because of the large amount of PLP in the muscle, Krebs and Fisher (1964) hypothesized that muscle phosphorylase acts as a vitamin B-6 reservoir. Black et al (1977) studied the reservoir theory and found that the muscle tissue of rats fed a diet high in vitamin B-6 had high concentrations of vitamin B-6 and glycogen phosphorylase. In further work, they found that a diet deficient in vitamin B-6 did not decrease muscle PLP concentration but a calorie deficient diet did (Black et al, 1978). This lead to the conclusion that "phosphorylase may function as an adjunct to adipose tissue necessary for the animal to efficiently meet the exigencies of starvation". In humans, there is limited evidence that muscle may serve as a vitamin B-6 reservoir. Strenuous exercise creates a metabolic state thought to be similar to acute caloric deficit (Lemon and Nagle, 1981) where the requirement for gluconeogenesis is increased; thus, the hepatic PLP requirement is increased. Exercise studies show that plasma PLP concentration increases during exercise (Leklem and Shultz, 1983; Manore et al, 1987) and the theory is that as muscle glycogen is mobilized, PLP is released from glycogen phosphorylase, hydrolyzed to PL, then released into circulation where it is carried to the liver and rapidly converted to PLP (Leklem and Shultz, 1983). The mechanism for the release and

hydrolysis of PLP could be the low pH that is characteristic of exercising muscle or a neural or hormonal change induced by exercise.

Biochemical Functions

Vitamin B-6 is involved in numerous enzymatic reactions that can grouped by type of reaction. The six primary types of enzymatic reactions requiring PLP are listed in Table 1.5.

Table 1.5. Six primary types of enzyme reactions catalyzed by PLP.

Type of Reaction	Definition/Example	Enzymes
Aminotransferase (transaminase)	Transfer of an amino group (NH_2) to a keto acid. For example, the amino group transfer from glutamate to pyruvate yields alanine and α-ketoglutarate.	Alanine aminotransferase Aspartate aminotransferase
Decarboxylation	Removal of –COOH from a compound. For example, decarboxylation of tryptophan yields tryptamine and CO_2.	Tryptophan decarboxylase Tyrosine decarboxylase
Decarboxylation with C–C bond formation	Decarboxylation followed by C–C joining of 2 moieties. For example in heme synthesis, glycine and succinyl-CoA join to form δ-aminolevulinic acid and CO_2.	δ-aminolevulinic synthase Serine plamitoyltransferase
Side Chain Cleavage	Cleavage of a side chain. For example the cleavage of cystathionine into cysteine and α-ketobutyrate.	Cystathionase Serine hydroxy-methyltransferase
Dehydratase	Catalyze removal of water. For instance, dehydration precedes deamination of serine and threonine.	L-serine dehydratase
Racemization	Interconversion of D and L amino acids.	Bacteria only

The common denominator for all these reactions is the unique ability of PLP to participate in Schiff base formation, usually with the substrate. The notable exception to this is PLP's role with glycogen phosphorylase where the Schiff base interaction is with the enzyme

and not the glycogen substrate. Therefore, with one exception, PLP is involved only with substrates that contain nitrogen. Schiff-base formation ability is critical for several functions including gluconeogenesis, the immune system, niacin formation, erythrocyte formation, lipid metabolism, nervous system function, and hormone modulation. The cellular process PLP impacts are listed in Table 1.6 and a brief discussion of each system follows.

Table 1.6. Cellular processes affected by PLP[1].

System	Cellular process or enzyme effected
Gluconeogenesis	Glycogen phosphorylase and transamination
Immune function	1-carbon metabolism and hormone modulation
Niacin formation	Tryptophan metabolism
Erythrocyte formation	Heme synthesis, transamination, O_2 affinity
Nervous system	Neurotransmitter synthesis, lipid metabolism
Hormone modulation	Binding of PLP to hormone receptor

[1] Adapted from Leklem, 1991.

Gluconeogenesis

Gluconeogenesis is the process of "creating" glucose and is critical to the maintenance of blood glucose levels. In a fasted resting state, glycogen and amino acids are the two primary glucose-forming substrates and PLP is required for the conversion of both. It appears that when there is an increased need for new glucose, muscle stores of vitamin B-6 may be mobilized to meet increased need for gluconeogenesis. Such a need may arise from purposeful caloric restriction or from intense bouts of strenuous exercise which the body may sense as acute starvation. As previously stated, in animals, a vitamin B-6 deficiency causes a decrease in blood glucose due to decreased liver alanine and aspartate aminotransferase activity (Angel, 1980). However, the same has not been demonstrated in humans. Rose et al (1975) studied the effect of a low (0.2 mg) vitamin B-6 diet on the blood glucose levels of women and found no alteration in fasting blood glucose levels. However, they did find an impaired glucose tolerance indicating some perturbation of glucose metabolism.

Immune Function

Both animal and human studies show an effect of vitamin B-6 on the immune system (Meydani et al, 1991; Chandra and Puri, 1985). Although the pathway has not been clearly identified, it is thought that PLP affects 1-carbon metabolism (via serine transhydroxymethylase) and thus affects the synthesis of nucleic acids, a process important to maintaining immune function (Schirch and Mason, 1963).

Studies in humans demonstrate variable effects of vitamin B-6 status on immune response. Talbott et al (1987) investigated the effect of a 50-mg PN supplementation on lymphocyte responsiveness in healthy elderly persons. After eight weeks, they found an improvement in lymphocyte function. Specifically, they found an increase in the percentage of T3+ and T4+ cells suggesting that PN may have influenced the differentiation of immature T cells to mature T cells. Meydani et al (1991) studied the elderly and found that an induced vitamin B-6 deficiency significantly decreased the percentage and total number of lymphocytes, mitogenic responses of peripheral blood lymphocytes to T- and B-cell mitogens, and impaired interleukin-2 production. However, following repletion with 2.9 and 1.9 mg/day PN for men and women, respectively, the response returned to baseline values. In a contrasting study, van den Berg et al (1988) induced a marginal vitamin B-6 deficiency (< 30% recommended intake for 11 weeks) and found it did not affect immune response.

Niacin Formation

Niacin is a water soluble vitamin whose requirement by humans is partially met by the PLP dependent hepatic conversion of tryptophan to niacin. To determine dietary adequacy of niacin, the convention is to consider 60 mg of tryptophan equivalent to 1 mg niacin. In the conversion, there is one enzymatic step (kynureniase) that requires PLP. Therefore, one would expect a deficiency in PLP to impact niacin formation. However, Leklem et al (1975) found that four weeks on a low vitamin B-6 intake (< 0.2 mg/day) had only a moderate negative effect

on the amount of tryptophan converted to niacin. It is unclear what the long-term consequences of a continued sub-optimal intake would be on niacin formation and status. This unique relationship between vitamin B-6 and tryptophan is the basis of the tryptophan load test, which challenges the ability of the body to process tryptophan through this PL-dependent pathway, and is a common indirect measure of vitamin B-6 status.

Erythrocyte Formation and Function

PLP is essential for the formation and function of the erythrocyte. During erythrocyte synthesis, PLP is required by δ-aminolevulinic acid synthetase which catalyzes the condensation of glycine and succinyl-CoA to form the heme precursor δ-aminolevulinic acid (Kikuchi et al, 1958). In humans, a deficiency in vitamin B-6 can lead to hypochromic, microcytic anemia that resolves with vitamin B-6 supplementation (Horrigan and Harris, 1968).

In the erythrocyte, PLP is a coenzyme for alanine and aspartic transaminases. Transaminase presence in the erythrocyte is well established and determination of their activity is used as indirect methods of assessing long-term vitamin B-6 status. In addition, the binding of PLP and PL to hemoglobin affects its affinity for oxygen. The binding of PL to the α-chain increases the affinity of oxygen to bind (Benesch et al, 1977), while PLP binding to the β-chain decreases the binding affinity (Maeda et al, 1976). The effect this may have on exercise performance is unknown.

Lipid Metabolism

The exact role of PLP in lipid metabolism remains elusive but is thought to impact triglyceride synthesis, fatty acid metabolism, carnitine metabolism, and, possibly, cholesterol synthesis. Early studies found that a vitamin B-6 deficiency in rats resulted in a decrease in body fats (McHenry and Gauvin, 1938). But not all subsequent studies showed a similar effect. One review of the literature reported numerous conflicting results with studies showing that a vitamin

B-6 deficiency increased fat synthesis, decreased fat synthesis, or had no effect on fat synthesis (Leklem, 1991). The disparity in results may in part due to differences in feeding patterns and the vitamin B-6 content of the diet. Vitamin B-6 also appears to be involved with the conversion of linoleic acid to arachidonic acid. Cunnane et al (1984) found that vitamin B-6 deficient rats had increased phospholipid levels of linoleic acid and decreased levels of arachidonic acid. Tsuge et al (2000) confirmed these findings in rats and found specifically that vitamin B-6 deficiency impaired the metabolism of (n-3) polyunsaturated fatty acids from alpha-linolenic acid to eicosapentaenoic acid and docosahexaenoic acid. In contrast, in one of the few human studies, Mueller and Iacono (1963) used desoxypyridoxine to induce a vitamin B-6 deficiency, but found only minor changes in plasma and erythrocyte fatty acid levels.

Carnitine is the carrier molecule that transfers fatty acyl units into the mitochondria for β-oxidation. Because PLP is required for the synthesis of carnitine from lysine, PLP has an indirect effect on fatty acid metabolism. Cho and Leklem (1990) found a decrease in total and free carnitine in the plasma, skeletal muscle, heart muscle, and urine of rats fed a vitamin B-6 deficient diet for six weeks. Subsequent repletion with vitamin B-6 increased carnitine concentrations, leading the researchers to conclude that vitamin B-6 is required for the synthesis of carnitine. To date, no human studies have looked into the effect of B-6 deficiency on carnitine.

The link between vitamin B-6 deficiency and cholesterol metabolism still remains weak. Fincnam et al, (1987) fed monkeys an atherogenic "Western" diet and a "prudent" antiatherogenic diet and found a significant positive correlation between plasma PLP and HDL cholesterol, and a negative correlation with total cholesterol and LDL. In a human study, Serfontein and Ubbink (1988) studied the effect of a 10-mg PN supplement and observed a 0.8 mmol/L reduction in serum cholesterol. This small of a change indicates the effect of vitamin B-6 on cholesterol is minimal and is most likely of little physiological significance.

It is unlikely that PLP is *directly* involved in lipid metabolism because the chemistry of PLP's action requires the substrate to contain nitrogen, which lipids do not. With the exception of carnitine, the exact biochemical pathway of the effect is not known. One promising explanation of how a vitamin B-6 deficiency may affect lipid metabolism involves PLP's role in methionine metabolism. The theory is that during a vitamin B-6 deficiency, S-adenosylmethionine (SAM) builds up because it cannot be converted to homocysteine, since homocysteine metabolism to cystathionine is a PLP dependent process. The build up in SAM exerts negative feedback on the conversion of phosphatidyl ethanolamine to phosphatidyl choline and may explain the changes seen in fatty acid metabolism (Leklem, 1991).

Nervous System

The role of vitamin B-6 in the nervous system is well documented. Several neurotransmitters, such as serotonin, taurine, dopamine, norepinephrine, histamine, and γ-aminobutyric acid, are synthesized by PLP-dependent enzymes (Dakshinamurti, 1982). As stated previously, infants who were fed a vitamin B-6 deficient formula developed convulsions and abnormal ECG tracings (Coursin, 1954). In adults, in 1969, abnormal ECG tracings were found in subjects consuming a high protein vitamin B-6 deficient diet (Canham et al, 1969). More recently Kretsch et al (1991) confined eight healthy young women to a metabolic ward and fed thema diet providing less than 0.05 mg vitamin B-6 per day. After only 12 days, two of the women exhibited abnormal EEG tracings. The changes were readily reversed by repletion of vitamin B-6 at 0.5 mg per day.

The role of vitamin B-6 in brain development has been studied in rats. Work by Aycock and Kirksey (1976) showed that dietary vitamin B-6 restriction of the dam resulted in low brain weight and a decrease in alanine aminotransferase and glutamic acid decarboxylase activity in the progeny. Supporting these findings, Thomas and Kirksey (1976) found in the cerebellum of pups from deficient dams, fatty acids (C18:2, C20:4, and C22:6) and ω–6 fatty

acids were lower, while Morre and Kirksey (1978) found decreased myelination. Other related findings cited in a review article (Leklem, 1991) include alteration in cerebellum and cerebrum fatty acid levels; a decrease in cerebral sphingolipids; and shorter Purkinje cell dendrites.

Hormone Modulation

A relatively new role for PLP is modulation of steroid action. During in vitro studies, Litwack et al (1985) found that PLP forms a Schiff base with a lysine residue on the steroid receptor and thereby inhibits the binding of the steroid complex to DNA. Compton and Cidlowski (1986) added to these findings by showing that in vitro PLP binds at two distinct locations: at the steroid binding site and at the DNA binding domain on the receptor. The end result was an altered binding of the steroid-receptor-DNA complex, resulting in decreased action of the steroid. The physiological effect of this action has been shown in rats. Bunce and Vessal (1987) studied the effect of a vitamin B-6 deficiency on estrogen uptake and retention by the uterus. They found that the number of receptors was not affected by the deficiency yet uptake was significantly increased, suggesting an increased sensitivity to the hormone.

Allgood and Cidlowski (1992) demonstrated that in vitro, vitamin B-6 modulates the transcriptional activation of androgen, progesterone, and estrogen receptors in a similar manner, suggesting the vitamin may modulate the expression of a diverse array of hormonally responsive genes. Further work suggests that the specific influence is on a functional or cooperative interaction between the hormone receptors and the transcription-factor, nuclear factor one (Allgood et al, 1993) and that the vitamin B-6 status of the cells modulates their capacity to respond to steroid hormones (Tully et al, 1994). As of yet there is no direct evidence suggesting physiological significance for these interactions. However, this area of study is in its infancy and more research is required to determine the possible impact on endocrine-mediated diseases.

Recommended Daily Intake

Several factors, including protein, age, bioavailability, and exercise affect the requirement for vitamin B-6 (Leklem, 1996; Reynolds, 1995). Of these, protein is the most studied and most understood. Because of vitamin B-6's role in protein metabolism, as protein intake increases so does the requirement for the vitamin. In both men and women, an inverse correlation exists between protein intake and plasma PLP concentration and protein intake and urinary 4-PA excretion (Leklem, 1996). Kretsch et al (1995) confined eight healthy women to a metabolic unit and studied the relationship between protein and vitamin B-6 status. They found that an intake of 0.015 mg vitamin B-6/g protein normalized most vitamin B-6 indices, and 0.02 mg/g protein normalized all the indices except for urinary vitamin B-6. These results confirm that the vitamin B-6 requirement is inextricably related to protein intake.

The 1989 Recommended Dietary Allowance (RDA) for vitamin B-6 was 2.0 mg for males and 1.6 mg for females and was based on a dietary vitamin B-6 ratio of 0.016 mg/g protein. The recommendation was based on extensive research that indicated it was the minimum level at which indices of vitamin B-6 status were maintained at an acceptable level (RDA, 1989). However, in 1998, the National Academy of Sciences, citing new research indicating vitamin B-6 requirement was not dependent on protein intake, lowered the RDA for vitamin B-6 to 1.7 mg for males and 1.4 mg for females. Calculating the vitamin B-6 to protein ratio using the average protein intake of adults (73–83 g/day, NHANES II) generates a 0.017–0.23 ratio, which, according to the work of Kretsch et al (1995) will be inadequate for 50% of the population. Alternatively, if the vitamin B-6 RDA is compared to the RDA for protein (58 g for males, 46 g for females) instead of actual intake, the ratio is greater than 0.028 which is above levels thought to be adequate.

Symptoms of vitamin B-6 deficiency, which are rare under normal circumstances, include dermatitis with cheilosis and glossitis, excretion of large amounts of xanthurenic acid (from abnormal tryptophan metabolism), dizziness, nausea, and nervous system disturbances.

However, drugs and poisons can induce deficiency states by reacting with the aldehyde, group thus blocking formation of a Schiff base (Mathews, 1996). An example of this is the treatment of tuberculosis with the drug isoniazid. In vitro, the drug blocks the growth of the mycrobacterium by binding PLP, and in humans prolonged treatment with this drug may cause a vitamin B-6 deficiency due to decreased absorption.

Food processing can also result in a deficiency state. In 1951, babies fed a specific canned formula began developing irritability, muscular twitching, and convulsions while babies on a similar powder formula were fine. A researcher recognized the similarity of the convulsive seizures to those he had seen in young rats deprived of vitamin B-6 and correctly diagnosed the babies with vitamin B-6 deficiency (Coursin, 1954). Further investigation revealed that the process of heat sterilization had destroyed most of the vitamin B-6 in the canned formula and the babies were indeed suffering from a vitamin B-6 deficiency.

The acute toxicity of vitamin B-6 is low. However, when taken in 7–10 g quantities for months, it can cause ataxia and a severe sensory neuropathy, although sensory neuropathies from long term ingestion of only 200 mg have been reported (Schaumburg et al, 1983). However, termination of supplementation results in reversal of the symptoms and complete recovery.

Food Sources

Vitamin B-6 is widely distributed in animal and plant tissues, where mainly the phosphorylated forms predominate. The richest unfortified sources of vitamin B-6 in the U.S. diet are meats (chicken, fish, kidney, liver, pork) which provide 0.4 mg per 100 g serving (Kant and Block, 1990). Other good sources include: unmilled rice, whole-wheat products, peanuts, walnuts, and fortified grain products. Data from the NHANES II project (Kant and Block, 1990) reveal that in the U.S., foods from animal and plant sources contribute 48% and 52% to

vitamin B-6 intake, respectively, and that beef, alcoholic beverages, potatoes, ready-to-eat cereals, and milk are important sources.

For most foods in the USDA data bank, the vitamin B-6 content was determined by a microbiological assay originally developed by Atkin in 1943 (Leklem, 1991). The major drawback of this assay is that it measures the total amount of the vitamin and does not discriminate between vitamers or the less bioavailable pyridoxine-5'-β-D-glucoside (PNG). PNG may make up 5–80% of the total vitamin B-6 content of fruits and vegetables (Gregory and Ink, 1987), but has only 58% of the bioavailability of PN (Gregory et al, 1991), therefore available vitamin B-6 may be substantially less than that determined by microbiological assay. Additionally, compared to PN, PL is not retained by the body as well, and a greater percentage is excreted in the urine as 4-PA (Wozenski et al, 1980). Therefore, a more discriminating method of analysis is desirable to assess the "quality" of vitamin B-6 in foods. More recently HPLC analyses of foodstuffs has been utilized to provide values on the individual forms (Reynolds, 1995), but this process is both expensive and time consuming and so is not yet utilized to any great extent. Also, even after the vitamin B-6 content is determined, food processing and storage can cause considerable vitamin B-6 loss in foods. Freezing fruits and vegetables may decrease vitamin B-6 by 15–70% and 50–90% may be lost in milling (RDA, 1979). Moreover, thermal processing (Gregory and Kirk, 1977) and low-moisture storage (Gregory and Kirk, 1978) may result in PL and PLP amino acid derivatives that have low or anti-vitamin B-6 activity.

Bioavailability

Bioavailability refers to the portion of an ingested nutrient that is absorbed and metabolically utilized. The bioavailability of dietary vitamin B-6 is generally greater than 75% (Leklem, 1996). There are eight chemical forms of vitamin B-6 known to exist in food: PN, PM, PL, PMP, PNP, PLP, PNG, and 4-PA (Reynolds, 1995). Red meats contain primarily PLP and PMP, milk contains PL, and plant foods contain PN and PNG. PNG is not present in

animal products but, as previously mentioned, makes up a large part of plant vitamin B-6 and is estimated to contribute 10–15% of the vitamin B-6 in a mixed diet (Gregory et al, 1991).

Some factors that can affect the bioavailability of a nutrient include food preparation techniques, storage, form of the nutrient, and food-nutrient interactions. Methods used to evaluate the bioavailability of vitamin B-6 include balance studies, in vivo response to repletion after a deficiency has been created, and vitamer concentration in the blood following feeding a specified food. Regarding bioavailability, it is important to remember that microbiological methods for determining the vitamin B-6 content of a food yields information on *total* vitamin B-6 activity. This includes the activity of PNG, which is cleaved to yield PN during preparation of the food sample Reynolds, 1990). Studies on bioavailability are important because they provide information on how much of, or if, the consumed vitamin B-6 is available to the tissue.

Leklem et al (1980), were among the first to directly determine the bioavailability of vitamin B-6. After feeding nine men either whole wheat bread, white bread enriched with 0.8 mg pyridoxine, or white bread plus a solution of containing 0.8 mg of pyridoxine for a week, urinary vitamin B-6, urinary 4-PA, and fecal vitamin B-6 were measured. They found that urinary 4-PA was reduced when wheat bread was fed compared to the other two treatments and concluded that the vitamin B-6 from this bread was 5–10% less available. Another study in Leklem's lab found that 15 g of cooked wheat bran slightly reduced vitamin B-6 bioavailability (Lindberg et al, 1983).

Using a comparative dose response technique, Nelson et al studied the bioavailability of vitamin B-6 in orange juice and found it was only 50% as well absorbed as crystalline pyridoxine (Nelson et al, 1976). Similarly, Kabir et al (1983) used a balance approach to compare the vitamin B-6 bioavailability from tuna, whole wheat bread, and peanut butter and found that compared to tuna, the vitamin B-6 in bread and peanut butter was 75 and 63% as available, respectively. In this study, assessment of urinary vitamin B-6 and 4-PA revealed an

inverse correlation between vitamin B-6 bioavailability and levels glycosylated vitamin B-6 in these foods. Further research in Leklem's lab on the effect of glycosylated vitamin B-6 on bioavailability revealed decreased bioavailability for the following foods: walnuts (78%), bananas (79%), tomato juice (25%), spinach (22%), orange juice (9%), and carrots (0%) (Bills and Leklem, 1987).

How PNG may affect vitamin B-6 status in vivo was studied by Hansen et al (1996a) who found that consuming a high PNG (27% of 9 μmol/day) diet for 18 days lowered total plasma vitamin B-6 and erythrocyte PLP compared to a low PNG (9% of 8.6 μmol/day). From this, they concluded that PNG was less bioavailable and was equal to a loss of 15–18% of the total vitamin B-6 intake. Further, work by Nakano et al (1997) indicates that along with being less bioavailable, PNG may partially inhibit the utilization of co-ingested pyridoxine.

In summary, food composition tables must be viewed with some degree of skepticism. It is inaccurate to assume that the amount of vitamin B-6 listed is the amount that is bioavailable to the tissues. Unfortunately the magnitude of the inaccuracy is not known and varies widely, especially among plant foods.

Status Assessment

Determination of vitamin B-6 status is important in determining the function and metabolism of vitamin B-6 and how a deficiency or excess may impact human physiology. As previously stated, severe deficiencies are rare, however, sub-clinical deficiencies due to inadequate intake, disease, or medication may occur.

The three ways to determine vitamin B-6 status are by evaluating direct indices, indirect indices, and dietary intake. Table 1.7 lists several of the methods as well as minimum values indicating adequate status. No one single index is adequate to make definitive statements about vitamin B-6 status. Utilizing a combination of indices is the most reliable approach. Leklem (1990) suggests that a minimum of three indices be used and should include a direct indicator,

such as plasma PLP, a short term indicator, such as urinary 4-PA, and an indirect measure, such as erythrocyte transaminase. Subject information regarding any history of estrogen therapy, alcoholism, uremia, liver disease, oral contraceptives, and the drugs isoniazid, cycloserine, penicillamine, and hydrocortisone is also important because they all can interfere with vitamin B-6 metabolism (Gibson, 1990).

Direct measures are those that measure a B-6 vitamer or 4-PA. Normally tissue samples are not available; therefore, direct measures are made on plasma, erythrocytes, and urine. Plasma pyridoxal-5'-phosphate concentration is considered to be one of the best indicators of vitamin B-6 status (Leklem, 1981), but its use as a status index is controversial (Reynolds, 1995). In humans, plasma PLP concentration is significantly correlated with dietary vitamin B-6 intake (Shultz and Leklem, 1981) and with tissue concentration (Lumeng et al, 1978). Plasma concentration of PLP normally ranges from 25 to 900 nmol/L (Leklem 1991). However, even if the same diet is consumed, between subjects concentration may vary by 25–35% (Leklem, 1985) making interpretation difficult. Table 1.8 lists several factors that influence plasma PLP. In spite of its limitations, plasma PLP is the best single measure of vitamin B-6 status.

Since plasma PLP crosses cell membranes as PL, plasma PL has been proposed as an indicator of vitamin B-6 status. Plasma PL makes up 8–30% of total plasma vitamin B-6 (Leklem, 1990, 1988). However, except during pregnancy, the concentrations of PL are fairly constant and do not tend to fluctuate, making it a poor indicator of status. The reason for this relative constancy is that, in contrast to PLP, PL can rapidly diffuse across cell membranes and be in equilibrium with the intracellular concentrations of PL (Reynolds, 1995).

Determination of urinary 4-PA concentration is a direct measure of short-term vitamin B-6 status and reflects intake/absorption for a 1–4 day period. As previously mentioned, 4-pyridoxic acid is the irreversible end product of PL (via aldehyde oxidase) and represents the

principal route of excretion for excess vitamin B-6 intake (Leklem, 1991). The amount of 4-PA excreted in the urine changes with intake. To assess 4-PA, a 24-hour urine collection is required. Under normal conditions, 40–60% of the daily vitamin B-6 intake will be excreted as 4-PA (Shultz and Leklem, 1981). However, like PLP, 4-PA concentration is potentially influenced by several factors (Table 1.8). Once in the urine, 4-PA is relatively stable. However, at room temperature or under acidic conditions, it can convert from the lactone form to the free acid form which could lead to an underestimation of concentration (Reynolds, 1995).

Table 1.7. Methods used to assess vitamin B-6 status and suggested minimal values[1].

Method	Minimum Level Indicating Adequate Status	Indicator of Status
Direct		
Plasma pyridoxal 5'phosphate	>30 nmol/L	Long term[2]
Plasma total vitamin B-6	>40 nmol/L	Long term
Urinary 4-pyridoxic acid	>3.0 μmol/day	Short term[3]
Urinary total vitamin B-6	>0.5 μmol/day	Short term
Indirect		
Erythrocyte alanine transaminase index	<1.25	Long term
Erythrocyte aspartic transaminase index	<1.80	Long term
2 g Tryptophan load; urinary xanthurenic acid	<65 μmol/day	Long term
3 g Methionine load; urinary cystathionine	<350 μmol/day	Long term
Dietary Intake		
Weekly average of vitamin B-6 intake	>1.25 mg/day	Long term
Vitamin B-6 to protein ratio (mg/g)	≥0.016	Long term

[1] Adapted from Leklem, 1990
[2] Greater than 15 days
[3] 1–4 days

Indirect measures are those that measure a metabolite produced from a PLP dependent pathway or measure the activity of a specific PLP dependent enzyme. If intracellular PLP is deficient, then the substrate will not be metabolized through the PLP dependent pathway. Two PLP dependent metabolic pathways for amino acids that are sensitive to a vitamin B-6

deficiency are those for tryptophan and methionine. An increased urinary excretion of xanthurenic acid following a 2-g tryptophan load has been one of the most widely used tests for assessing a vitamin B-6 deficiency (Brown, 1985). Some of the factors affecting results of the tryptophan load test include estrogen, hydrocortisone, liver disease, and some cancers (Gibson, 1990). Another common test, although less specific due to the multitude of conditions that can increase plasma homocysteine, is the methionine load test (Reynolds, 1995). This test challenges the methionine/homocysteine metabolic pathway and an increase in urinary excretion of cystathionine indicates a vitamin B-6 deficiency may exist.

Table 1.8. Factors Influencing Direct Measures

Factor	Effect on Plasma PLP	Effect on Urinary 4-PA
Diet		
↑ Vitamin B-6[2]	↑	↑
↑ Protein[2]	↓	↓
↑ Glucose[3]	↓, acute	No studies
↓ Bioavailability[4]	↓	↓
Physiologic		
↑ Exercise < 3 hr	↑, acute	↑ ↓(?)[1]
↑ Exercise > 6 hr	↓, acute	↑ ↓(?)[1]
Gender[5]	Males higher	Males higher
↑ Age[6]	↓	↑
Pregnancy[7]	↓	↓(?)[1]
↑ Alkaline Phosphatase activity[8]	↓	No change
Chronic Smoking[9]	↓	↓

[1] Effect controversial, data equivocal.
[2] Shultz and Leklem, 1981
[3] Leklem, 1985
[4] Hansen et al, 1996a
[5] Absolute excretion when intake was 2.2 mg vitamin B-6 for both males and females. Leklem, 1991.
[6] Lee and Leklem, 1985
[7] Kirksey and Udipi, 1985
[8] Whyte et al 1985
[9] Ruhumba-Sindihebura, 1999

Erythrocyte alanine and aspartate transaminase activity is PLP dependent and both are used as long-term indicators of vitamin B-6 status. Determining the activity index is a two step process. First, activity is measured in the absence of added PLP; then, activity is measured in the presence of excess PLP. The resulting index indicates the magnitude of increase when exogenous PLP is added (Reynolds, 1995). The higher the index, the lower the apparent vitamin B-6 status. Unfortunately, the correlation between plasma PLP and transaminase activity is poor and its usefulness as a status indicator has been questioned (Leklem, 1991). The lack of correlation may be partially due to the long life of the erythrocyte and PLPs binding to

hemoglobin. Additionally, oral contraceptives and anemia may affect transaminase activity (Reynolds, 1995).

Dietary assessment should include determination of vitamin B-6 *and* protein intake from a detailed multi-day diet record. Diet intake assessment can help explain data obtained from direct and indirect measures but is not sufficient in itself to assess vitamin B-6 status. In addition to problems associated with obtaining an accurate diet record, there are the aforementioned issues with the lack of specific vitamer data in the database.

Vitamin B-6 and Exercise

The primary link between vitamin B-6 and exercise is its role in fuel metabolism. However, beyond identification of the primary enzyme pathways involved, there is limited knowledge regarding vitamin B-6 metabolism during exercise, and what indirect effects it may have on physical performance. There are several theories, but because of the limitations of human research (tissue biopsies are not often practical or available), the puzzle still has numerous pieces missing. For example, it is known that plasma PLP is increased during exercise (Leklem and Shultz, 1983; Crozier et al, 1994, Leklem 1985) but where it came from and what happens to it is speculative and subject to debate. Does the increase indicate an increased requirement? Does poor status equate to poor performance? What are the effects of supplements? Research suggests that supplements can alter fuel substrates in a manner that increases glucose utilization (Virk et al, 1999). Might this help some athletes but hinder others? Additionally, it appears that vitamin B-6 may affect exercise induced plasma growth hormone concentration (Delitala et al, 1976). Other vitamin B-6 functions that may impact performance include synthesis of serotonin from tryptophan (central fatigue theory), carnitine from lysine (fatty acid metabolism), and hemoglobin (anemia). The remainder of this section will review the available research related to all these issues.

To summarize the role of vitamin B-6 in fuel metabolism, PLP is a coenzyme required for glycogenolysis, gluconeogenesis, and transamination prior to amino acid oxidation. Related to glycogen breakdown, PLP is bound to glycogen phosphorylase and is required for the conversion of glycogen to glucose-1-phosphate in both the muscle and liver (Krebs and Fischer, 1964). The role of PLP in gluconeogenesis is related to the glucose-alanine cycle, which is a multi-step process involving the muscle, gut, and liver (Figure 1.5). In this cycle, and for amino acid oxidation, PLP is a coenzyme for transferase, transaminase, and decarboxylase reactions (Sauberlich, 1968).

Except for one study by Leonard and Leklem (2000) that found decreased plasma PLP concentration following exercise greater than six hours, research shows that plasma PLP concentrations increase during exercise and stay elevated throughout the exercise bout (Leklem, 1985; Manore et al, 1987; Crozier et al, 1994). This is true for both short-term ($\leq$30 min) intense exercise [$\geq$80% maximal oxygen uptake (VO_{2max})] and long-term (50–180 min) moderate (60–75% VO_{2max}) exercise. Most of the increase (79%) occurs within five minutes and the total increase averages 10–35% (Crozier et al, 1994). Interestingly, the increase in plasma PLP concentration is similar to that seen after a 1–1.5 mg PN-HCl supplement is consumed (Wozenski et al, 1980). Reports on the effect of intensity on plasma PLP concentration are equivocal: Crozier et al (1994) also found no effect, and Leklem, in unpublished work, did (Leklem, 1985).

In the first study to demonstrate an increase in plasma PLP concentration during exercise, Leklem and Shultz (1983) investigated the effect of a 4500-m run on vitamin B-6 metabolism in seven male adolescent athletes. On three separate occasions during an 8-month period, blood was drawn before running 1500 meters and again 1–2 minutes after completion. The increase in PLP concentration ranged from 0.8 to 1.8 nmol/L equating to an increase of 18–33%. The change in total plasma vitamin B-6 was similar, indicating that most of the

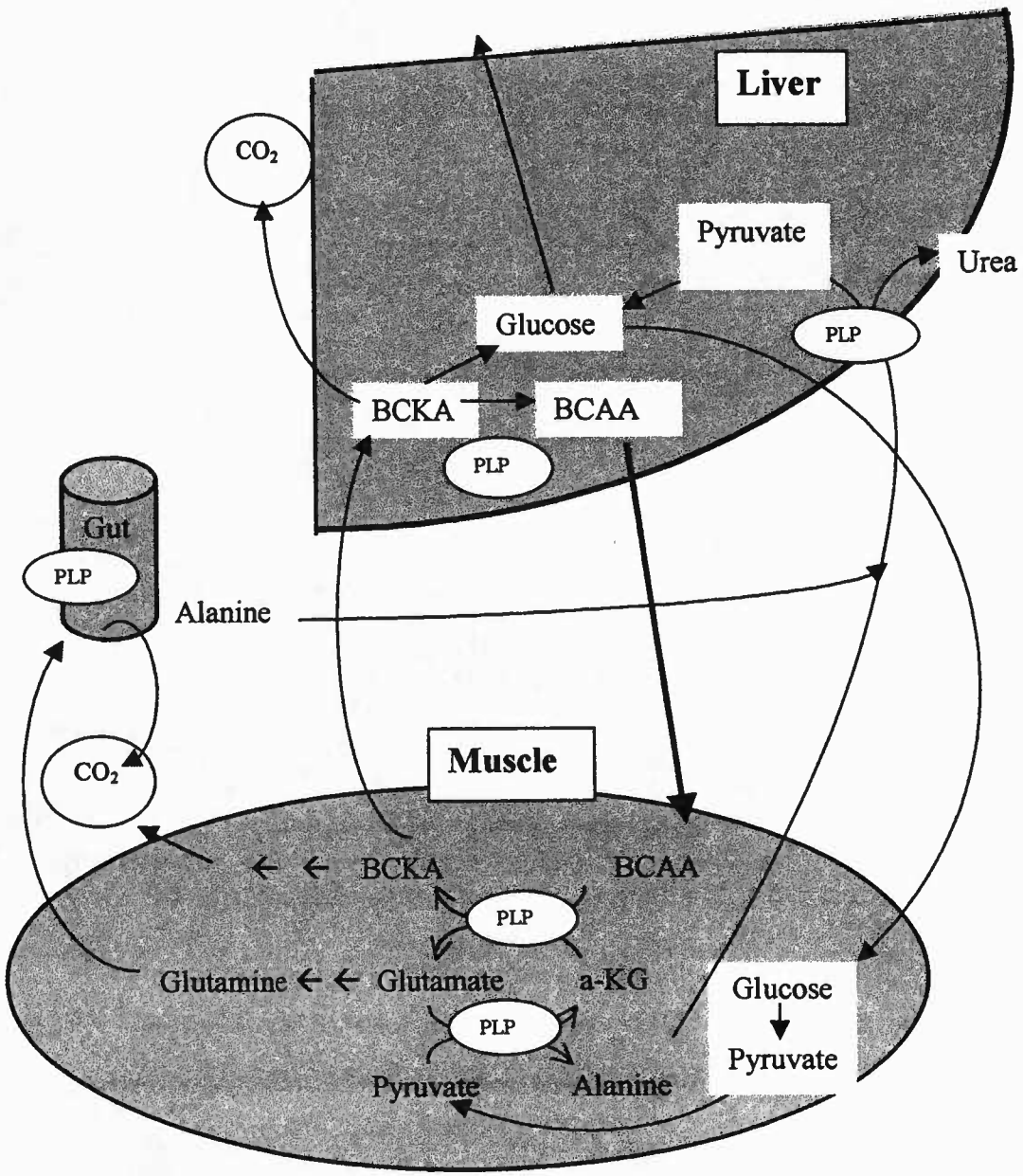

Figure 1.5. The role of the liver, muscle, gut and PLP in the glucose-alanine cycle and BCAA metabolism. Abbreviations: BCKA = branched chin keto acid; BCAA = branched chain amino acid; PLP = pyridoxal 5'-phosphate (Adapted from Harper et al, 1984).

variance was due to the increase in PLP concentration. The endogenous source of the PLP could not be determined from the study, but the Leklem and Shultz speculated it had four possible sources. The PLP could come from the lysis of red blood cells, an increase in plasma albumin, release or leakage from non-muscle tissue, or release or leakage from muscle stores.

Citing the facts that muscle stores of PLP decrease during periods of caloric deprivation (Black et al, 1978) and exercise induces such a state, the researchers concluded muscle was the most likely source of the plasma PLP.

In an attempt to correlate plasma PLP with blood glucose availability, Leklem and Hollenbeck (1990) studied the effect of acute carbohydrate ingestion on plasma PLP and total vitamin B-6 concentrations. Five males and four females were given 1 g D-glucose/kg body weight, and blood samples were collected over a 5-hour period. At 2 hours, there was a significant decrease in plasma PLP and total vitamin B-6 concentration. By 5 hours the mean change from fasting was 8–10 nmol/L for PLP and 8–12 nmol/L for total vitamin B-6 (an 18–21% decrease). An unexpected finding from this study was that ingestion of 300 mL of plain water resulted in a mean plasma PLP concentration decrease of 3.4 nmol/L, but still only explained part of the observed decrease. From this study, Leklem confirmed a relationship between glucose consumption and plasma PLP levels and speculated that the ingestion of glucose during exercise might lessen the need for PLP by the liver for gluconeogenesis.

Hofmann et al (1991) followed up Leklem's research and studied the effect of carbohydrate ingestion during exercise on vitamin B-6 metabolism. Six trained males completed four different conditions: a run with water ingestion, a run with glucose ingestion, rest with water ingestion, and rest with glucose ingestion. During the two running conditions, subjects ran for 120 minutes at 60–65% VO_{2max} while ingesting 200 ml of either glucose (7%) or 200 ml of water every 30 minutes. During the resting (control) conditions subjects remained standing and consumed 200 ml of glucose or water every 30 minutes. During each condition blood samples were drawn every 30 minutes for 180 minutes. During the two running conditions, in the first 60 minutes, plasma PLP increased significantly (19–26%) then plateaued until exercise ceased, and finally returned to baseline by 1-hour post exercise. Compared to the glucose condition, plasma PLP concentration was higher when water was consumed but the

difference was not significant. Additionally, plasma PL concentrations did not significantly change during any of the four conditions.

As a result of the aforementioned study, Hoffman proposed an alternative to Leklem's theory suggesting that the source of PLP was the liver and that it was released due to increased demand by the working muscle. The researchers had hypothesized that providing glucose during the run would attenuate the rise in PLP because of slower depletion of glycogen stores and a lower demand for glycogen phosphorylase activation. The lack of a significant difference between the glucose and water conditions during exercise could have been because there really was no difference or could have been the result of inadequate power ($n = 6$) to detect the difference. Another study finding, that supported the liver-to-muscle theory, was that free fatty acid concentration was lower during exercise with glucose compared to water. Lower plasma free fatty acids indicate that the exogenous glucose was being utilized, which would lessen the demand for fatty acid oxidation (Coyle, 1997), glycogen store utilization, and glycogen phosphorylase activation. The end result would be an acute decrease in PLP requirement and a concomitant lowering of plasma concentration when exogenous glucose was available.

In a study of longer duration exercise, Rokitzki et al (1994a) determined the acute changes in vitamin B-6 indicies in 13 men before and after a marathon. Subjects were allowed only water during the race and blood was drawn pre-, post-, and two hours post-race. Two random urine samples were also collected, one before and one after (within two hours) the event. Average run time was around three hours (exact time not reported), during which subjects experienced a 114% increase in serum vitamin B-6 [the vitamer(s) measured were not specified, so it is unclear if this increase is in PLP or all vitamers combined] that remained elevated (57%) above pre-race level) through two hours post-race. Using pre-race to post-race changes in the urinary 4-PA to creatinine ratio, Rokitzki estimated that 1-mg of vitamin B-6 was lost as a result of running a marathon race. However, this estimation was not based on 24-hour urine

collections, but was extrapolated from the 4-PA:creatinine ratio and must be viewed accordingly. Renal function is affected by exercise (Irving et al, 1990) but it is not known if, under varying plasma concentrations, 4-PA and creatinine are excreted in the same relative proportions. In fact, due to tubular secretion, depending on plasma 4-PA concentration, renal clearance of pyridoxic acid clearance may be twice that of creatinine (Zempleni and Kubler, 1995). After exercise, as normal renal function is restored, the rate of 4-PA clearance could be far greater than creatinine clearance and lead to a short-term elevation in the 4-PA:creatinine ratio and the erroneous assumption that total 4-PA excretion for the day exceeded normal levels.

Leonard and Leklem (2000), who evaluated vitamin B-6 metabolism in eight males and three females during a 50-km ultramarathon, have completed the study with the longest duration and distance. Event duration averaged over six hours, which is twice as long as any other study, and included 1829 meters of elevation change. Subjects were asked to forgo vitamin supplements containing vitamin B-6 for 48 hours prior to the race and a 500-calorie low vitamin B-6 (0.47 mg) breakfast was provided three hours prior to the race. During the race, water was available ad lib but food consumption was limited to specific foods containing less than 0.1-mg vitamin B-6 per 100 g. Plasma PLP, PL, and 4-PA concentration was determined pre-, post-, and one hour post-race. Compared to pre-race, post-race mean plasma PLP concentration *decreased* by 12.9 nmol/L (31%) and continued to decline through 1-hour post-race. Plasma PL concentration did not change significantly but pre-race to post-race plasma 4-PA concentration increased by 21%. Changes in plasma concentrations of glucose, lactate, albumin, and alkaline phosphatase activity were determined but were not correlated with changes in any plasma B-6 vitamer. The study suggests that the duration of exercise may affect vitamin B-6 metabolism in a manner not previously considered, but it is difficult to determine how because diet was not strictly controlled, blood was not sampled during the race, and urine was not collected.

The dramatic decline in PLP was unexpected and led Leonard and Leklem to expand on the theories of Leklem (1983) and Hoffman et al (1991). They theorized the change in PLP was linked to changes in fuel metabolism. Crozier et al (1994) noted that PLP increased significantly within 5 minutes of exercise onset, which would be approximately when fuel metabolism shifted from anaerobic to aerobic glycolytic. This change may stimulate release of PLP from storage pools (liver and/or muscle and/or interstitial space). PLP would then be available to the muscle and liver to increase glycogen phosphorylase activity, and would remain high until glycogen stores were depleted and fuel metabolism shifted predominantly to lipolytic and gluconeogenic pathways. Leonard and Leklem postulated that the depletion of the glycogen stores coupled with an increase in hepatic PLP requirement (gluconeogenesis) would cause a decline in plasma PLP levels during the later stages of prolonged (> 6 hours) endurance exercise.

A question yet to be answered is whether endurance exercise increases the requirement for vitamin B-6. The aforementioned study by Rokitzki (1994a) claims it does. However, if it does, an increase in 4-PA excretion would be expected and some endurance athletes might have marginal vitamin B-6 status. Manore et al (1987) fed five trained and 10 untrained women four diets (19% and 64% carbohydrate with and without 10 mg PN), then had them exercise at 80% VO_{2max} for 20 minutes. Blood was drawn pre- and post- exercise and 24 hour urines were collected. Plasma PLP increased an average of 13% and analysis of the urine revealed that for all diets, 4-PA increased significantly from the pre-exercise day to the day of exercise. There was no significant difference between the trained and untrained subjects, and for both the amount of increase was 0.5 μmol/24 hours, which is equivalent to less than 0.1 mg PN or 7% of the RDA for vitamin B-6. Dunton (1994) reported a similar but non-significant finding (0.4 μmol/day increase) in seven non-supplemented, trained men who exercised to exhaustion at 64–75% VO_{2max} on a cycle ergometer.

In contrast, Dreon and Butterfield (1986) studied four trained runners and six inactive controls all of whom consumed a controlled diet for eight weeks. Due to the greater caloric requirement of runners, between groups there was a slight difference in vitamin B-6 intake (range of intake 4.2–4.6 mg per day), but the vitamin B-6 to protein ratio was consistent (0.30) between groups. The runners ran 16 km (10 miles) per day for 29 days and then 8 km (5 miles) per day for another 29 days. The mean basal urinary excretion of 4-PA was significantly higher in the sedentary controls (11.2 μmol/day) compared to the 8 km/day period (7.6 μmol/day) and marginally higher than the 16/day period (8.9 μmol/day). Expressed as a percentage of pyridoxine intake, 4-PA excretion was less (30–40%) among the trained runners than the inactive controls (49%). To determine functional status, on the last day of each 29-day exercise period, urinary metabolites were evaluated in response to a methionine load. Results show that the active group increased 4-PA excretion and decreased cystathionine excretion while the sedentary group did the opposite (decreased 4-PA excretion and increased cystathionine excretion). Both changes were the opposite of what the investigators anticipated. The researchers expected the methionine load would increase the physiological demand for PLP and, thus, decrease the amount of 4-PA excreted and increase cystathionine excretion if vitamin B-6 status was marginal. These unexpected findings led the researchers to theorize that physically active individuals may have an increased need for vitamin B-6 that is unrelated to loss. They theorized that physical activity may act as a stimulus to expand a labile pool of pyridoxine, that is capable of redistribution under circumstances of increased need.

Another route of vitamin B-6 loss, that has not received much attention, is sweat. A significant loss of 4-PA through sweat could explain the results of Dreon and Butterfield (1986) who observed that sedentary individuals had a higher urinary 4-PA excretion than those who exercise. Sweat has been described as a filtrate of plasma because it contains many of the items present in the water phase of the blood (Costill, 1984). The solute concentration of sweat ranges

from 33–100% of the plasma concentration (Costill, 1984). Because whole body sweat is difficult to collect, most studies on sweat composition extrapolate whole body loss from regional samples which can deviate significantly from whole body sweat. Furthermore, the composition of sweat changes as exercise duration increases. In the only such study to date, Johnson et al (1945) completed 8-hour whole body sweat collections and 24-hour urinary collections on four male subjects resting at 100° F with 70% relative humidity. In both cases, he determined the concentration of PL, PN, and 4-PA. During the 8-hour period, sweat loss was negligible for PN, 0.031 mg (0.19 μmol) for PL, and 0.198 mg (1.16 μmol) for 4-PA. Of further interest was the fact that the concentration ratio of the 4-PA with the vitamers PN and PL was the same in the sweat as it was in the urine, indicating that during resting conditions, the B-6 vitamers were lost in sweat and urine in the same proportions.

One of the problems encountered when evaluating the vitamin B-6 status of athletes is the lack of reference values for athletes. Most studies use the reference values derived from inactive individuals, which may or may not be appropriate. The Rokitzki et al study (1994a) evaluated the vitamin B-6 status of 13 endurance athletes by examining plasma vitamin B-6, urinary 4-PA, erythrocyte transaminase, and a 7-day diet record. They determined that, even with exercise related vitamin B-6 losses estimated to be 1 mg/day, the vitamin B-6 status of the athletes was not different from normal reference values of vitamin B-6 status for untrained individuals. Utilizing the same methods, in a separate study on 57 strength and speedpower athletes, Rokitzki et al (1994b) found that 90% of the athletes did not meet the reference value (88 nmol/L) for whole blood vitamin B-6 concentration, 18% had 4-PA excretion below 2.73 μmol /g creatinine, and more than 30% did not meet the German recommended dietary allowance (1.8 mg/day male or 1.6 mg/day female) of vitamin B-6. An important aspect of this study is that the reference value of 88 nmol/L is very high compared to suggested minimal value of less than 30 nmol/L (Table 1.7). This is most likely the result of the specific microbiological

assay used to determine the reference value, which typically overestimates vitamin B-6 concentration. Three other studies reported that 35 to 60% of the athletes tested had poor vitamin B-6 status based on erythrocyte aspartic transaminase activity (EAST) indices (Fogelholm et al, 1993; Guilland et al, 1989; Telford et al, 1992). However, as previously mentioned, vitamin B-6 status cannot be accurately assessed by any one measure and since two of the three studies utilized only EAST to evaluate status, the results should be interpreted with caution. Some researchers believe that if athletes do have increased demands and/or increased losses of vitamin B-6, they also have a compensatory mechanism such that vitamin B-6 status is not compromised (Rokitzki et al, 1994a; Drenon and Butterfield, 1985).

Most studies on the dietary intake of endurance athletes show a mean vitamin B-6 intake that exceeds the RDA of 1.7 mg for men and 1.5 mg for women (Bazzare et al, 1993; Jensen et al 1992). However, there are studies that report athletes have low or marginal vitamin B-6 intakes. Guilland et al (1989) used weighed food records to assess the intake of cross country runners and found that in spite of consuming over 3,000 kcal/day, vitamin B-6 intake was only 1.5 mg. Rokitzki et al evaluated 7-day diet records of 13 endurance athletes (1994a) and 57 strength and speedpower athletes (1994b). They reported the respective daily calorie and vitamin B-6 intake for endurance athletes was 2,900 and 2.1 mg, for body builders was 4861 and 5.4 mg, for soccer players was 3588 and 2.1 mg, and for female handball players was 2324 and 1.4 mg. These results show that, with the exception of body builders, a high calorie diet was associated with a vitamin B-6 intake that ranged from 93–123% of the RDA. If, as suggested by Rokitzki, endurance exercise does increase vitamin B-6 loss, this population of athletes could be at risk by having suboptimal body reserves.

The general assumption is that individuals with poor vitamin B-6 status will have reduced exercise performance. This has been demonstrated by Suboticanec et al (1990) who measured the vitamin B-6 status of 124 boys aged 12–14 years and found 24% had poor status

based on erythrocyte aspartic acid transaminase stimulation index (EAST). Thirty-seven of the boys were then provided 2 mg PN for two months and retested on a cycle ergometer. Supplementation significantly improved vitamin B-6 status and maximal work capacity. The researchers reported a significant negative correlation ($p = 0.036$) between maximal work capacity and EAST values. van der Beek et al (1994) used a 2 by 2 by 2 factorial experiment to assess the effects of thiamin, riboflavin, and vitamin B-6 restriction of physical performance in 24 men. Subjects were provided 26 to 55% of the Dutch RDA for thiamin and/or riboflavin and/or vitamin B-6 (0.39–0.83 mg/day). After 11 weeks on the deficient diet, there was no vitamin-specific differences in physical performance. However, as a group, there was a significant 11.6 % decrease in aerobic power, a 7% decrease in lactic acid threshold, a 9.3% decrease in peak power generated, and a 6.0% decrease in mean power achieved. Because there was no quantitatively different effect observed for any one B vitamin, the researchers concluded that restriction of each had a significant but non-additive effect on physical performance.

Besides being directly involved in energy pathways, vitamin B-6 may also affect performance by affecting fuel substrate utilization. Studies in animals have shown that acute doses of vitamin B-6 can increase blood glucose by increasing catecholamine release from the adrenal medulla (Lau-Cam, 1991). A 1998 study by Virk et al examined the effect of vitamin B-6 supplementation on fuel metabolism during exercise. Eleven trained men were exercised to exhaustion at 71% VO_{2max} before and after 9 days of supplementation with 20 mg PN. Blood was drawn pre, during (60 min.), post, and 60 minutes post exercise and analyzed for free fatty acids, glucose, and catecholamines. The results of the study show that while blood glucose, lactate, and endurance were not affected, supplementation caused a significant decrease (8–25%) in plasma free fatty acids at *all* time points. Catecholamine concentration was not affected by supplementation. The authors concluded that vitamin B-6 supplementation

decreased total fat oxidation at rest and during exercise and/or increased intramuscular triglyceride utilization.

Supplemental vitamin B-6 has also been shown to produce an elevation of plasma growth hormone (GH). The proposed mechanism is that PLP is involved in the conversion of L-dopa to dopamine which, in turn, is the precursor for the growth hormone releasing hormone (Delitala et al, 1976). Delitala et al (1976) injected eight healthy volunteers with a single 300-mg IV dose of pyridoxine and by 120 minutes found a statistically significant rise in plasma GH. However, studies on oral ingested supplements have not found a similar effect. Dunton (1994) evaluated the effect of a 20-mg PN supplement and exercise on plasma growth hormone concentration but found no significant change.

Proteins and Amino Acids

Animal and plant proteins are made up of about 20 different amino acids and the ratio of amino acids varies depending on their source. For humans, there is a specific "pattern" of amino acids that the body requires (McLarney et al, 1996). In adults, there are nine amino acids (essential) that cannot be synthesized by the human body and must be consumed in adequate amounts as part of the diet. The remaining 11 amino acids (nonessential) the body can synthesize de novo and by rearranging existing amino acids, both of which require PLP. No matter the reason (i.e. growth, excess protein intake, exercise) the greater the quantity of amino acids to be metabolized, the greater the requirement for PLP.

Protein is unique among the energy nutrients because it is the only one that contains nitrogen. By weight, nitrogen makes up about 16% of the protein. Protein is required for the synthesis of body tissue and other important nitrogen containing compounds, such as peptide hormones, enzymes, and some neurotransmitters. Protein that is consumed in excess of the physiological requirements is degraded. The nitrogen portion of the amino acid is excreted as urea and the remaining keto acid is either oxidized for energy or is converted to fat and stored.

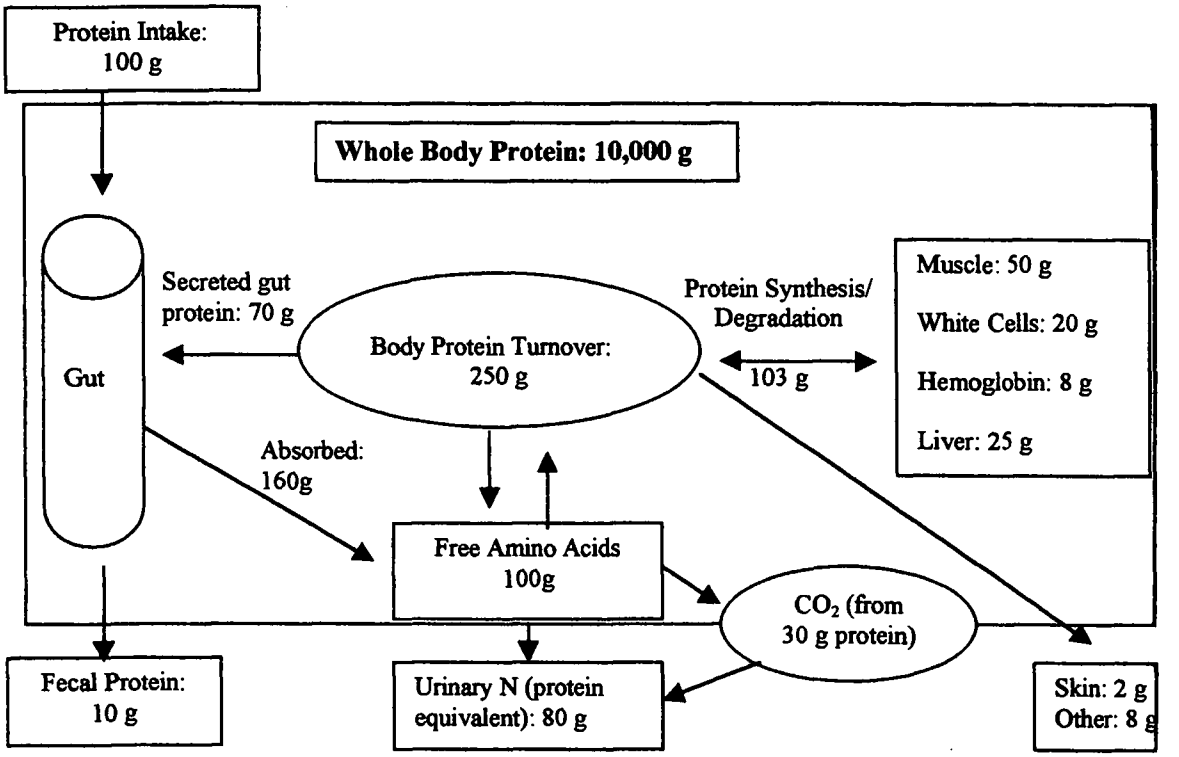

Figure 1.6. Estimated daily whole body turnover of protein in a 70-kg man. (Modified from Munro, H.N., Raven Press, New York, 1982)

In the body, there is a continual process of protein degradation and synthesis. Several times more protein is turned over daily than is ordinarily consumed, indicating that reutilization of amino acids is a major feature of the economy of protein metabolism. However, this recapture is not complete and some amino acids are lost through oxidative catabolism, urinary excretion (urea, creatinine, uric acid), sweat, feces, and sloughed skin, hair and nails (Figure 1.6). Therefore, a continuous daily supply of dietary amino acids is required.

Digestibility and Absorption

Differences in protein digestibility result from intrinsic differences in the nature of food protein, the presence of other dietary factors that modify digestion, and chemical reactions which may affect the release of amino acids by enzymatic processes. In general, proteins are well

absorbed and little dietary protein is lost in the feces. Eggs (97%), meat (94%), milk and cheese (95%), and refined wheat (96%) are the most digestibility, while rice (88%), oatmeal (86%), whole wheat (86%), and beans (78%) are less bioavailable. Overall, the protein in an American mixed diet is estimated to be 96% digestible (RDA, 1989).

Dietary protein is denatured in the stomach, broken down into peptides by gastric pepsin, digested into smaller peptides and amino acids by pancreatic and endothelial enzymes, and then absorbed through the mucosal cells of the small intestine (Vander et al, 1990). Amino acids are transported into the mucosal cells by energy-dependent carriers that have some specificity for neutral, basic, and acidic classes of amino acids (Mathews, 1999). In contrast, small peptides cannot be absorbed as such and are hydrolyzed by peptide hydrolases in the brush border and cytosol of the mucosal cells. Therefore, only free amino acids enter the portal vein for transport to the liver.

Role of the Liver

The absorbed amino acids are transported to the liver via the portal circulation. The liver is the main or only site of catabolism for six of the essential amino acids and the remaining three (the branched chain amino acids) are degraded primarily by the skeletal muscle and the kidney (Harper et al, 1984; Mathews, 1999). Because the liver gets "first pass" at the newly absorbed amino acids, the amount and pattern of amino acids in general circulation may be much different than that in the portal circulation. The hepatic process of "monitoring" amino acid levels regulates the amounts of individual essential amino acids available to the rest of the body. Breakdown of essential amino acids is minimal until the liver senses that requirements have been exceeded, and only then is a catabolic enzyme induced. In this manner, assuming an otherwise adequate nutritional status, when essential amino acids are limited they are preserved for the anabolic pathways requiring them. Conversely, with nonessential amino acids, there is

no threshold for induction and enzyme activity changes in direct relation to diet intake (Mathews, 1999).

There are two processes by which most amino acids are degraded: oxidative deamination and transamination. Oxidative deamination involves removing the amino group and replacing it with an oxygen atom derived from water:

$$\text{Amino acid A} + H_2O \rightarrow \text{keto acid A} + NH_4$$

The ammonia produced in this process is subsequently conjugated to form the non-toxic product urea and excreted in the urine. During transamination, which is a PLP dependent process, the amino acid still loses its amino group, but in this instance, it is transferred to a keto acid to form a different amino acid rather than being released as ammonia:

$$\text{Amino acid A} + \text{keto acid B} \rightarrow \text{Amino acid B} + \text{keto acid A}$$

In both cases, the end product of degradation always yields a keto acid that can be metabolized to produce another amino acid, produce adenosine triphosphate (ATP), create intermediates for the glucose synthetic pathway, or create acetyl coenzyme A (CoA) for synthesis of a fatty acid.

Regulation of Blood Amino Acid Levels

The process of amino acid monitoring by the liver does not immediately nor completely eliminate excess plasma amino acids. Therefore, after a meal, plasma levels of both essential amino acids and nonessential amino acids are generally elevated to some degree. Insulin mediates a large part of amino acid metabolism. Insulin stimulates active transport of amino acids into cells, increases the activity of enzymes associated with protein synthesis, and inhibits enzymes associated with protein catabolism (Vander et al, 1990; Brodsky, 1999). An increase in plasma glucose and/or amino acids will stimulate the pancreatic release of insulin and increase tissue uptake of amino acids. Therefore, shortly after a meal containing carbohydrate and/or protein, the concentration of most amino acids decreases. In the muscle the uptake is maximal

for branched-chain amino acids and can cause plasma levels to fall as much as 40% (Munro and Crim, 1988). Skeletal muscle is the largest tissue in the body, therefore, it is understandable that muscle would be a major destination and depot for amino acids.

Glucagon secretion, like insulin secretion is also stimulated by a rise in plasma amino acids. In as much as glucagon opposes the actions of insulin this appears to be a metabolic paradox. The explanation for this is, if amino acids stimulated insulin release only, the result of an all protein meal would be a precipitous drop in blood glucose as cells took up not only amino acids, but glucose as well. The increase in glucagon secretion caused by amino acids permits the plasma-glucose-raising effects of this hormone to counteract the plasma-glucose-lowering actions of insulin. Thus, a high-protein, carbohydrate-free meal can be absorbed with little change in plasma glucose concentration despite a marked increase in insulin secretion (Vander et al, 1990).

The steroid hormone cortisol is an insulin antagonist that assists in maintaining blood glucose by stimulating amino acid release from the muscle, by stimulating hepatic gluconeogenesis from amino acids, and by mobilizing free fatty acids (FFA) from adipose tissue (Brodsky, 1999). Cortisol is commonly referred to as a "stress" hormone and its plasma concentration increases in response to physical stress. Thus, both exercise intensity and duration affect plasma cortisol concentration. Research shows that skeletal BCAA transaminase activity is increased in cortisol-treated rats (Harper et al, 1984) and decreased in rats treated with growth hormone. It is documented that in humans heavy training increases serum cortisol (Brooks, 1996). Weight et al (1991) determined plasma cortisol changes as a result of running a marathon and found that post-race levels were two times higher than pre-race values. However, 24 hours after the race ,concentration dropped below pre-race levels and remained so for the next six days. It is unclear what long-term effect cortisol fluctuations have on protein metabolism, muscular growth, repair, and performance.

When glycogen stores are depleted, alanine is a major substrate for hepatic gluconeogenesis (Lemon and Mullin, 1980). Through hormone mediated pathways, severe caloric deficit can result in skeletal muscle loss of 50 g protein per day primarily through the release of alanine and glutamine (Munro and Crim, 1988). Because alanine is not a major muscle amino acid, it must be formed by transamination, a PLP dependant process, between pyruvate and another amino acid. Once produced, the alanine enters circulation and can be taken up by the liver, where the carbon skeleton is utilized for gluconeogenesis and the amino group is excreted as urea.

Another carrier of nitrogen from the skeletal muscle is glutamine. Glutamine is formed when α-ketogluterate accepts two ammonia moieties formed by the purine neucleotide cycle or amino acid deamination. The glutamine enters circulation and is taken up by the intestine where half undergoes transamination to alanine and is released back into the blood stream for circulation to the liver. After conversion to glucose, some of the alanine carbon comes back to the muscle as glucose. However, the majority of the glucose is taken up by the brain, nerves, and kidney. This cycle of exchange, depicted in Figue 5, is called the glucose-alanine cycle and allows transport of nitrogen and carbon to the liver (Munro and Crim, 1988). During periods of starvation, the muscle can provide substrate for production of 100 g of glucose per day (Brooks et al,1996b).

Branched-chain amino acids play a central and vital role in supplying the liver with alanine. The branched-chain amino acids (leucine, isoleucine, and valine) are essential amino acids and are unique because they are catabolized by the skeletal muscle, not by the liver. In the muscle, catabolism of a BCAA generates a carbon skeleton that can be utilized as a substrate for ATP generation, and a nitrogen which is used to synthesize alanine and glutamine (Harper et al, 1984) (See Figure 1.5). However, because BCAAs are essential amino acids under normal conditions, they are catabolized minimally for energy. Their primary function in

gluconeogenesis is to provide nitrogen for alanine and glutamine formation, and the remaining keto-acid is most often circulated to the liver for conversion back to its original form. However, if the keto acid is retained by the muscle and used for ATP, each has a different metabolic fate. The keto acid from valine is glucogenic because it yields succinyl-CoA, the keto acid from leucine is ketogenic because it yields acetyl-CoA, and the keto acid from isoleucine is both glucogenic and ketogenic because it yields propionyl-CoA and acetyl-CoA (Harper et al, 1984).

The transaminase that catalyzes the first step in BCAA degradation is PLP dependent. Its concentration is high in the skeletal muscle and low in the liver, and responds rapidly to changes in tissue BCAA concentration (Harper et al, 1984). Most of the resultant branched chain keto acids (BCKA) are transported out of the tissue, into circulation, and are taken up by the liver, which has high BCKA dehydrogenase activity (Harper et al, 1984). In the liver the BCKA is either reaminated or used for gluconeogenesis. See Figure 1.5 for more detail.

The purpose of the complex process of BCAA metabolism is to supply the muscle with a nitrogen source for synthesis of alanine and glutamine, yet allow conservation of the nutritionally indispensable BCAA carbon skeleton. BCAAs comprise about 35% of the essential amino acids in muscle proteins and make up almost 50% of the essential amino acids provided by the diet (Harper et al, 1984). In a fed state , 79% of the BCKAs are reaminated to their original BCAA and in a fasted state, 91% are reaminated (Harper et al, 1984). This suggests that cycling of BCAA carbon skeletons via transamination-reamination is critical to hepatic gluconeogenesis and is much more extensive than is decarboxylation and oxidation that results in the irreversible loss of the amino acid.

Recommended Daily Intake

The current RDA for protein is 0.8 g/kg of body weight. This figure is rounded up from 0.75 g/kg which was determined by a four step process (RDA, 1989) as listed below:

1. Data from both long term and short-term high-quality protein nitrogen balance studies were used to estimate the average protein requirements of sedentary male and female adults (Young et al, 1975, Calloway, 1975). The average was the level at which 50% of the U.S. population would meet their requirement and 50% would not. This requirement was determined to be 0.6 g/kg/day.

2. To determine if an additional protein allowance was warranted due to differences in protein digestibility, data from nitrogen balance studies on mixed diets were considered (Young et al, 1975, Atinmo, et al, 1988). The requirements, predicted from these studies, were 0.54 to 0.99 g/kg/day and were not found to be significantly different from the reference diet, and no additional allowance was made.

3. To meet the needs of 97.5% of the population, an amount of protein equal to two standard deviations from the mean was added. The standard deviation (SD) was 12.5% and resulted in an adjusted requirement of 0.75 g/kg/day.

4. Because protein requirements change throughout the lifecycle, the impact of age, pregnancy, and lactation on protein requirements was assessed and, when warranted, adjustments made. For instance, the protein content of breast milk is 0.011 g/mL which equates to an increase in maternal requirement of 15 g protein/day (750 mL produced with 70% efficiency plus 2 SDs).

Data from the NHANES III conducted from 1988–1991 indicates that for adults, 15% of total food energy intake (estimated at 2,095 calories) is derived from protein. This equates to approximately 80 g/day, indicating that protein deficiency is not likely under normal circumstances.

Nitrogen Balance

Nitrogen balance is the current standard method used for establishing total protein (nitrogen) requirements and allowances. The method is based on the assumption that nearly all of the total body nitrogen is incorporated into protein. To estimate protein requirements, subjects are fed adequate calories and levels of protein that are below and near predicted adequate intake. Nitrogen balance is then assessed by determining the difference between nitrogen intake and the amount excreted in urine, feces, sweat, and other minor losses. The requirement is estimated by interpolating or extrapolating the balance to the zero point for adults or to a defined level of positive balance for children (RDA, 1989). However, nitrogen balance studies are difficult to conduct and, in order to have reliable intake and loss data, require considerable cooperation from the subjects.

Under normal conditions, approximately 85% of the daily nitrogen loss is excreted in the urine and the remainder is lost through skin, feces, hair, and nails (Tome and Bos, 2000). Of the total nitrogen in urine, urea constitutes 80–90% and non-urea nitrogen components make up the remainder (creatinine 6.4%, ammonia 7.4%, uric acid 2–3%, and others 1–2%) (Allison and Bird, 1977). When protein intakes are low, urinary nitrogen excretion falls and the urea nitrogen accounts for a decreasing percentage of total urinary nitrogen (61–70%) (Allison and Bird, 1977).

Tarnopolsky et al (1988) determined the nitrogen balance point for six sedentary free-living males following a 13-day period of normal protein intake (1.05 g/kg) and a 13 day period of increased protein (1.9 g/kg) intake. Nitrogen balance was determined during the last three days of each period. To determine the total nitrogen excretion, three sequential 24-hour urine collection, 72-hour fecal collections, and 24-hour sweat secretion samples were obtained. The regression line calculated from the nitrogen balance data indicated 0.73 g/kg/day of protein would result in nitrogen balance. This agrees well with the work of Atinmo et al (1988), who used similar techniques and studied 15 sedentary medical students at four levels of protein intake

(0.3, 0.45, 0.6, and 0.75 g/kg). Again, using regression analysis, they estimated the mean protein requirement was 0.69 g/kg/day.

The quality of dietary protein as well as total caloric intake is also an important consideration when determining protein requirements. Young et al (1975) compared the effectiveness of beef protein to that of wheat protein in maintaining nitrogen balance in 16 young men. Results from regression analysis show that to achieve nitrogen balance, 0.6 g/kg of beef protein or 1.1 g/kg of wheat protein is required. The author concluded that variations in dietary protein quality should be considered when determining dietary adequacy. Calloway (1975) evaluated the relative importance of energy and protein intake on crude nitrogen balance (dietary N minus fecal and urinary N) and found the predicted minimum protein requirement was 0.56 g/kg/day when 100% of the energy requirement was met. However, if energy intake was only 85% of the requirement, nitrogen balance was minus 0.61 g/day and if it was 115%, nitrogen balance increased 0.59 g/day. From this, the author concluded that at marginally adequate protein intake, energy intake appears to have a much greater effect on nitrogen balance than does protein intake. Rao et al (1975) reached a similar conclusion when he found that zero nitrogen balance was achieved with 2, 249 calories providing 40 g protein, or 2,066 calories providing 60 g protein.

Because nitrogen balance studies are labor and time intensive, urea nitrogen alone is often used to estimate nitrogen balance. This technique appears adequate (but not exact) if two correction factors are made: (a) include a 2-g loss for the dermal and fecal losses and (b) include a 2-g loss for non-urea nitrogen components of the urine (MacKenzie et al, 1985). These simple adjustments are not appropriate in all circumstances. For instance, during severe energy restriction or starvation the production of renal ammonia is increased to such an extent that the non-urea nitrogen correction factor of 2 g is inadequate (Winterer et al, 1980).

Exercise Physiology

Exercise physiology deals with the functioning of the body during exercise. In many ways, the exercising human can be compared to an automobile, which takes one form of energy and converts it into another. Like an automobile, a human can increase speed or intensity by increasing the rate at which energy is converted. However, unlike an automobile, the human body can adapt to physical stress and improve its function. Another similarity is that, no matter how finely tuned either is for performance, without proper fuel performance is limited.

Understanding how the body acquires, converts, stores, and utilizes energy, is important to understanding human function during exercise. Physical performance depends largely on the energy available to the muscle fibers. Under normal conditions, ample fuel is stored in the chemical bonds of endogenous glycogen, fat, and protein, but the muscle cannot use these stores until the energy in the bonds has been transferred to ATP. This conversion process is fairly inefficient and ultimately only 50% of the original energy potential of food is transferred to ATP, and the remainder is lost as heat (Brooks, 1996a).

ATP, the Common Energy Currency

Adenosine triphosphate is the energy currency of the cell. However, the body stores only small amounts of ATP (about 80 to 100 g), enough to sustain maximum physical effort for only a few seconds. A unique feature of ATP is that it can act both as an energy donor and receiver, and can be quickly regenerated. Thus, even though ATP is stored in very limited quantities, its ability to be recycled provides the cell with a seemingly endless supply of energy.

Chemically, ATP contains a nitrogenous base, a five-carbon sugar, and three phosphates. 7.3 kcal/mol of energy is liberated from ATP by an ATPase enzyme during the following reaction (Brooks et al, 1996a):

$$ATP + H_2O \rightarrow ADP + \text{Inorganic phosphate } (P_i)$$

ATP serves numerous cellular functions and hydrolysis does not always result in energy release. Often, energy potential is transferred when ATP donates a phosphate group to form a low-energy phosphate compound, such as glucose 6-phosphate or glycerol 3-phosphate. However, during exercise the majority of ATP is hydrolyzed to release the energy to power muscle contraction.

The immediate precursor to ATP is always adenosine diphosphate (ADP). ADP is converted to ATP by phosphate transfer from a compound of higher energy, such as phosphoenolypyruvate, 1,3-diphosphoglycerate, or, especially in the muscle, creatine phosphate (CP). In the following equation, ATP is regenerated enzymatically by creatine kinase:

$$\text{ADP} + \text{Creatine phosphate} \rightarrow \text{ATP} + \text{Creatine}$$

Creatine is then rapidly rephosphorylated by another high energy phosphate compound so it may again participate in ATP regeneration. Thus, the cycle continues and the working muscle is provided with a continuous supply of ATP. When the demand for energy and ATP increases, the body immediately begins to breakdown energy stores. The pathway used to transfer energy to ATP depends on the availability of stored fuels, duration of activity, type of activity, intensity of activity, conditions of the cell, and training level of the individual.

Energy Systems

There are three systems that work together to transfer stored energy to ATP: the phosphagen system, the anaerobic glycolytic system, and the aerobic system. Briefly, the phosphagen system is the first system used and provides "quick" energy. The anaerobic system is the second system and produces ATP from glucose but does not require oxygen, and the final system, the aerobic system, requires oxygen but can use any fuel to produce ATP. Which system prevails depends on the type of activity in which the individual is engaged. Athletic activities can be categorized as one of three basic types: power, speed, and endurance events.

Table 1.9 summarizes the characteristics of the three primary energy systems as well as the type of activity they support.

Table 1.9. Characteristics of the Primary Energy Systems Utilized for Different Types of Activities[1]

	Power (i.e. Weight lifting)	Speed (i.e. Sprint)	Endurance (i.e. > 1500 m run)
System	Phosphagen	Anaerobic Glycolytic	Aerobic Glycolytic
Location	Cytosol	Cytosol	Cytosol and mitochondria
Rate of Induction	Immediate	Rapid	Slower
Duration	0 to 3 seconds	4 to 50 seconds	> 2 minutes
Fuel Storage Site	Cytosol	Cytosol	Cytosol, blood, liver, adipose tissue
Form of Stored Energy	ATP, creatine phosphate	Muscle glycogen and glucose	Muscle and liver: glycogen, glucose, triglycerides, and amino acids. Adipose tissue: lipids.
Oxygen Required?	No	No	Yes

[1] Adapted from Brooks, 1996a.

The phosphagen system provides immediate energy derived in three interconnected but distinct ways. First, as previously mentioned, ATP exists in the cell and is instantly available to do work. However, it is in very small quantities and is utilized in less than a few seconds (Brooks, 1996a). The second cellular source is creatine phosphate (CP), which exists in amounts 5–6 times greater than ATP. As the initial store of ATP is utilized, creatine kinase is actively regenerating ATP from ADP, utilizing the high energy phosphate bond in CP. The third immediate source of energy is catalyzed by adenylate kinase (myokinase) and generates one ATP from two ADPs (Brooks, 1996a).

The anaerobic glycolytic system allows for the continued production of ATP for up to three minutes when adequate oxygen for aerobic metabolism is not available. This system

converts endogenous glycogen and/or exogenous glucose to ATP without using oxygen. Glycolysis is summarized as follows:

$$\text{Glucose} \rightarrow 2\ \text{ATP} + 2\ \text{Lactic acid}$$

A limiting factor in anaerobic glycolysis is not the availability of glucose, but is a decrease in enzyme function due to lowered pH caused by an increase in cellular lactic acid and hydrogen ion concentration (Hawley and Hopkins, 1995).

The aerobic, or oxygen requiring system, is the main energy producing system at rest and for activity lasting longer than a minute (Hawley and Hopkins, 1995). An increase in the rate of oxidative metabolism is stimulated by a decrease in intracellular levels of ATP and CP and an increase in ADP and P_i (Brooks, 1996a). The system is made up of two sub-components: aerobic glycolysis and aerobic lipolysis, but is capable of using all three major energy sources (carbohydrate, lipids, and amino acids) to produce ATP. At intensities greater than 60–70% VO_{2max}, the system initially relies on glucose as the primary substrate (Hawley and Hopkins, 1995) and produces far more ATP from glucose than does nonoxidative glycolysis. Complete metabolism of glucose yields the following:

$$\text{Glucose} + O_2 \rightarrow 36\ \text{ATP} + CO_2 + H_2O$$

As glycogen stores diminish the system increasingly relies on fatty acids. Fats yield much more ATP than other fuels, but can be converted to energy only through oxidative metabolism. For palmitate, a commonly occurring 16-carbon fatty acid, the energy yield is as follows:

$$\text{Palmitate} + O_2 \rightarrow 129\ \text{ATP} + CO_2 + H_2O$$

Although carbohydrates and fats are the preferred fuel for producing ATP, amino acids can be catabolized through oxidative processes. Oxidation of alanine yields the following:

$$\text{Alanine} + \alpha\text{-Ketoglutarate} \rightarrow \text{Pyruvate} + \text{Glutamate}$$

$$\text{Pyruvate} + O_2 \rightarrow 15\ \text{ATP} + CO_2 + H_2O$$

In reality, all systems are contributing to some degree at any given time. It is primarily the exercise intensity, exercise duration, and individual training status that determines which system dominates. Brooks (1994) described the balance of carbohydrate and lipid utilization during exercise as the "crossover" point. He suggested that the pattern of substrate utilization in an individual depends on the interaction between exercise intensity induced responses (which increase carbohydrate utilization) and endurance training induced responses (which promote lipid oxidation). The crossover point is where the energy from lipids predominates over energy from derived from carbohydrate. Figure 1.7 illustrates the concept of the crossover point as well as the relative contributions of each system as a function of time when exercise intensity is 60–70% of VO_{2max}.

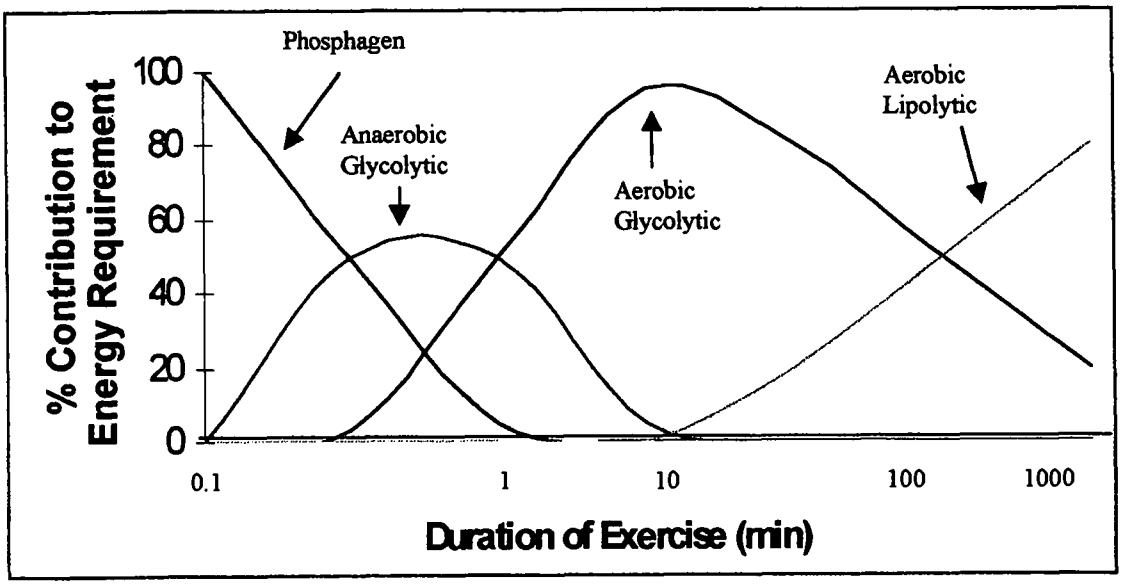

Figure 1.7. The crossover point and the relative contributions of the energy systems over time when exercise intensity is at 60–70% of VO_{2max}.

Because of chemical differences in the composition of carbohydrates, lipids, and proteins, complete oxidation of each to the end products of CO_2 and H_2O requires different amounts of oxygen (McArdle, 1999). The term respiratory exchange ratio (RER) is used to describe the ratio of CO_2 to O_2 and is determined by the following equation:

$$RQ = (CO_2 \text{ produced}) / O_2 \text{ uptake}$$

Determination of RER requires analysis of the difference between inspired and expired gases. The RER for glucose is 1.0, for lipid is 0.7, and for amino acids is 0.81. RER provides a convenient way to estimate which fuel, or mixture of fuels, is being utilized at any given time.

Regulation of Fuel Metabolism

In the resting state, the parasympathetic nervous system and the hormones insulin and glucagon exert primary control over fuel utilization. Under such conditions, metabolic requirements are met by a combination of carbohydrate (42%), fat (41%), and protein (17%) (Knoebel, 1971). The initiation of exercise causes stimulation of the sympathetic nervous system (SNS) via the brain's "central command" and metaboreceptors and ergoreceptors located in the exercising muscles. The stimulation results in the release of epinephrine and norepinephrine, which act on target tissues to stimulate glycogenolysis, inhibit insulin release, and stimulate glucagon release. The increase in glucagon is essential for the exercise-induced increase in gluconeogenesis and the fall in insulin enhances mobilization of fatty acids from the adipose tissue (Wasserman et al, 1995). The end result of exercise induced SNS and a hormonal change is maintenance of blood glucose despite a dramatic increase in demand and tissue uptake.

In addition to SNS and hormone modulation, oxygen availability strongly influences substrate utilization. Oxidation of fatty acids produces 8.2 ATPs per carbon atom while glucose yields only 6.2 ATPs (Connolly-Schooner, 2000). However, the ATP yield per oxygen molecule is 5.7 and 6.3 per carbon for fatty acids and glucose respectively. Therefore, when

oxygen is plentiful, fatty acids are the most economic resource, but when oxygen supply is limited, glucose is the preferred fuel.

During exercise, the aerobic system for ATP generation can utilize all three energy sources. Normally, glucose and fats contribute around 95% and protein contributes only 5%. The level of SNS stimulation and, hence, the balance of substrates utilized is affected by training status and exercise intensity and duration. These factors, as well as the specific contributions of carbohydrate, fat, and protein will be discussed in the following sections.

Training Status

Training can affect fuel utilization in numerous ways. Physiologically, the purpose of training is to stress or overload the body so that adaptation results. Endurance training results in muscular biochemical adaptations which include an increase in myoglobin, an increase in the number (120%) and size (14–40%) of mitochondria and an increase in enzymes involved in the Krebs cycle and electron transport system (Brooks, 1996a). Other adaptations to endurance training include an increase in VO_{2max} (greater ability to deliver oxygen), an increase in the lactic acid threshold, a decrease in exercise induced epinephrine and norepinephrine, and increased insulin sensitivity.

The body's ability to store and utilize fuel also adapts. Glycogen stores can increase by 65% (Hickner et al, 1997) and intracellular triglycerides by 100% (Hoppeler et al, 1985). Glucose cellular transport and utilization is increased due to greater glucose transporters (GLUT4) concentration, increased sensitivity to insulin, and greater hexokinase activity (Houmard, et al 1991). Similarly, lipid utilization is enhanced by an increase in the size and number of mitochondria, an increase in fatty acid oxidation enzymes, and a decrease in blood concentration of catecholamines and lactate (Brooks and Mercier, 1994).

One of the hallmarks of effective endurance training is a decrease in RER during submaximal exercise. This decrease indicates that lipid oxidation is providing a greater

percentage of the required energy which may spare glycogen stores and increase the time before exhaustion. Coggan et al(1990) found that a 12-week vigorous training program decreased the utilization of both muscle glycogen and plasma glucose during moderate intensity exercise. This decrease was accompanied by a decrease in RER indicating lipid oxidation was increased. More recently, Coggan et al (2000) exercised six endurance -trained and six untrained men at 75–80% VO_{2max} and found the rates of appearance and disappearance of glycerol and free fatty acids as well as RER. was higher in the trained group. However, the difference in plasma free fatty acid flux could not account for the total difference in fat oxidation, leading to the conclusion that utilization of intramuscular triglyceride stores was also greater in endurance-trained subjects.

Intensity and Duration

The effect of exercise intensity on aerobic glycolysis is fairly clear-cut: the higher the intensity, the greater dependence on carbohydrate relative to lipid. It takes only 15–30 minutes of exercise at 90–130% VO_{2max} to deplete glycogen stores compared to 2-three hours at 60–80% VO_{2max} (Coyle, 1991). In endurance trained athletes, the point at which carbohydrates and lipids contribute equally to energy requirements is at 60 to 70% of VO_{2max} (Hawley and Hopkins, 1995, Ranallo and Rhodes, 1998). At exercise intensity of greater than 70%, the primary source of ATP is glucose. However, as duration increases (3 to 5 hours) and carbohydrate stores diminish, lipid oxidation gradually increases to a point where it again equals that of carbohydrate oxidation (Hawley and Hopkins, 1995). The shift from carbohydrate to lipid is regulated by changes in metabolite concentration within the active cells. During exercise of long duration, increased lipid oxidation is stimulated by a fall in plasma glucose and insulin and a concomitant a rise in glucagon, catecholamines, growth hormone, and hepatic gluconeogenesis (Hawley and Hopkins, 1995).

The effects of exercise intensity and duration on amino acid metabolism are not at all clear cut. Although carbohydrate and fat are the major fuel sources for exercise, a number of studies have suggested that exercise can have a dramatic effect on amino acid metabolism (Wasserman et al, 1988; Millward et al, 1982). During endurance events, there is an increased demand for alternate glycolysis and Krebs cycle intermediates. In the 1970s, it was observed that exercising muscle released large quantities of alanine and glutamine in direct proportion to exercise intensity, and that plasma urea increased dramatically after 70 minutes (Felig and Wahren, 1971). Since then, several studies have shown that endurance exercise is associated with increases in BCAA oxidation and whole body nitrogen excretion, and that the rate of amino acid oxidation increases as intensity increases (White and Brooks, 1981; Lemon et al, 1982).

Carbohydrate Metabolism

Carbohydrate plays a significant role in energy production during endurance exercise. The body stores carbohydrate in the muscle and liver as glycogen. Dietary carbohydrate is the primary precursor to glycogen and amounts consumed in excess of immediate metabolic need are stored as glycogen (Hickner et al, 1997). Alternately, if carbohydrate consumption is in excess of total caloric requirements, it is converted into fatty acids and stored in adipose tissue. The conversion to fatty acids is irreversible: once glucose is converted to a fatty acid it cannot be converted back to glucose.

In trained individuals, muscles normally contain 300–400 g of glycogen and the liver 90–100 g. Thus, total glycogen stores average 500 g, which means the body has access to 2000 calories of stored carbohydrate (Felig, 1975). Liver glycogen stores maintain blood glucose at rest and during exercise. Initially, the majority of hepatic glucose output comes from glycogenolysis; however, when liver glycogen declines, the production of glucose via gluconeogenesis increases (Connolly-Schoonen, 2000). Maintenance of plasma glucose levels above 70 mg/100 mL is critical because the brain, CNS, and erythrocytes are dependent on

glucose for energy. Muscle, which can utilize alternate energy sources, normally accounts for less that 20% of blood glucose utilization. However, during exercise, plasma glucose uptake by the muscle can increase 30-fold and provides 15–30% of the carbohydrate oxidized in the exercising muscle (Holloszy and Kohrt, 1996). Under resting conditions hepatic glycogen stores are sufficient to maintain plasma glucose for more than 15 hours. However, a one-hour high intensity workout can decrease liver glycogen by 55% and a two hour strenuous workout may totally deplete stores (Kelly, 1995). It would seem logical, since plasma glucose is so important, that in times of need, muscle glycogen could be mobilized to help bolster levels. However, because skeletal muscle lacks the enzyme (glucose-6-phosphatase) required to convert glucose-6-phosphate to free glucose, the glucose is trapped in the muscle and cannot directly participate in the maintenance of plasma glucose levels.

There is a strong relationship between the pre-exercise muscle glycogen content and exercise duration. In one of the classic diet and endurance studies, Bergstrom et al (1971) compared the exercise time to exhaustion at 75% of VO_{2max} after three days of three different diets varying in carbohydrate content. Table 1.10 summarizes their findings.

Table 1.10. Effect of dietary carbohydrate content on muscle glycogen and exercise time to exhaustion

Carbohydrate Content of Diet	Muscle Glycogen Content (mmol/kg)	Exercise Time to Exhaustion (minutes)
< 5%	38	60
50%	106	115
> 82%	204	170

Thus, the correlation between diet, glycogen stores and endurance was clearly illustrated and further studies were initiated to illuminate the details of glycogen metabolism.

In trained athletes, exhaustive exercise can cause muscle glycogen to decline by over 100 mmol/kg and resynthesis does not normally exceed 5% per hour (5 mmol/kg/hour)

(Hickner et al, 1997). Thus, following exhaustive exercise, it will take at least 20 hours to replenish muscle glycogen stores (Coyle, 1991). To maximize glycogen resynthesis, the rate, type, and timing of carbohydrate ingestion are important factors. Blom et al (1987) studied differing amounts of carbohydrate intake and found that feeding 25 g carbohydrate every two hours increased glycogen stores by 2% per hour, whereas feeding 100 g increased resynthesis rates to 5–6% per hour. The recommendation to consume carbohydrate immediately after exercise resulted from a study by Ivey et al (1988) who determined that the rate of resynthesis is 7–8% per hour in the two hours immediately following exercise compared to the normal 5–6% per hour. This finding may be of little physiological significance however. Parkin et al (1997) studied the effect of timing and found that delaying carbohydrate intake by two hours did not effect the overall rate of glycogen resynthesis measured at 8 and 24 hours post-exercise.

The two major determinants of glycogen stores are training status and diet. Hickner et al (1997) determined the glycogen content of untrained subjects was 99 ± 16 mmol/kg of wet muscle compared to 164 ± 21 in trained cyclists. After depleting stores to below 20 mmol/kg by having subjects cycling at 75% VO_{2max} for two hours, they fed 10 g/kg of carbohydrate and assessed the rate of muscle glycogen accumulation and found a twofold greater rate in trained compared to untrained subjects. In the trained cyclists, the increased rate was positively correlated with the cellular concentration of glycogen synthase and glucose transporter protein (GLUT-4), further illustrating the specific adaptations that result from endurance training.

Adequate carbohydrate intake is critical to maximize glycogen stores and when suboptimal amounts are consumed, training and/or competitive performance may be impaired. Fallofield and Williams (1993) evaluated the effect of carbohydrate intake on recovery from prolonged exercise. Their subjects ran until fatigued and then consumed either 5.8 or 8.8 g carbohydrate/kg, rested, and then ran again. Those who consumed 8.8 g/kg were able to match their running time from the first trial and those who consumed 5.8 g/kg could not (averaging 15

minutes less). The standing recommendation is that athletes who train more than three hours per day should consume 7–10 g/kg/day of carbohydrate (Coleman, 2000).

Lactic Acid

Lactic acid is the end product of anaerobic glycolysis and was once viewed as a metabolic waste product. Radioactive tracer studies now show that, at rest and during moderate exercise, lactic acid is a dynamic metabolite and even when adequate oxygen is available, it is produced, removed, and taken up at equal rates (Connett et al, 1984). Thus, lack of change in blood concentration does not mean that lactic acid is not being produced. Lactic acid that is formed in metabolically active tissue can be circulated to less metabolically active tissue and utilized. For example, lactic acid produced by the exercising muscle is taken up by the liver where it is reconverted to glucose, oxidized for energy, or incorporated into amino acids and proteins. Both active and inactive muscle can also take up lactic acid and incorporate it into the Krebs Cycle for ATP production (Brooks, 1996a). During high intensity exercise production rate exceeds uptake and lactic acid accumulates in the blood. This pool provides a readily metabolizable reservoir of energy substrate that may be used to meet cellular energy needs or to restore blood glucose once exercise stops. It has also been suggested that lactic acid can be used to synthesize glycogen. The Glucose Paradox proposed by McGarry and associates (Newgard et al, 1983) describes an indirect route of liver glycogen production. In this scenario, dietary glucose is transported not to the liver, but to the muscle, where lactic acid is formed. In turn, the lactic acid is released into circulation where it is taken up by the liver and metabolized to glycogen.

Lipid Metabolism

The average athlete has lipid intramuscular and adipose lipid stores in excess of 8,000 g, representing over 72,000 calories of stored energy. Compared to carbohydrate stores, fat stores

represent a virtually unlimited energy supply. The primary storage form of fat is triglyceride, which is hydrolyzed by lipase to glycerol and free fatty acids. The amount hydrolyzed is determined by lipase activity, which is impacted by plasma levels of epinephrine, norepinephrine, glucagon, insulin, and lactate. FFA originating from the adipose tissue are released into circulation for subsequent uptake and utilization by the muscle and other tissues. Intracellularly derived FFA are primarily transported into the mitochondria for oxidation, however, they too can be released into the plasma.

Because adipose lipid stores are more abundant than intracellular lipid stores it would seem logical that they would provide the majority of FFA for oxidation. However, intracellular and plasma FFA are believed to contribute equally to the total fat oxidized during sustained exercise (Carlson et al, 1971). Martin et al (1993) determined the source of lipids oxidized during two hours of exercise at 50–75% VO_{2max} and found that less than half of the lipid oxidized came from the plasma and the remainder came from intramuscular triglyceride stores. However, during prolonged exercise the contribution of blood borne FFA increases as a result of their progressive increase in the plasma, whereas the contribution of muscle triglycerides decreases as they are gradually depleted (Romijn et al, 1993).

Generally, oxidation of plasma FFA can provide most of the energy needed during mild (< 30% VO_{2max}) exercise, with little or no net utilization of intramuscular substrates and with little oxidation of plasma glucose (Romijn et al, 1993). During moderately intense exercise (50–70% of VO_{2max}) lasting 60–120 minutes, fat oxidation provides 40–60% of the energy requirements. However, when exercise intensity exceeds 60–70% VO_{2max} the demand for ATP begins to outstrip the ability of the aerobic lipolytic system to produce it, and there is an increased reliance on glycolytic and gluconeogenic pathways (Deuster et al, 1989).

Diet has both a long-term and short-term effect on fatty acid oxidation. A common assumption among athletes is that consumption of carbohydrate during prolonged exercise will

spare muscle glycogen and increase time to exhaustion. While there is evidence that this practice will prevent hypoglycemia and can delay the development of fatigue (Coggan et al, 1991), it appears to be unrelated to glycogen stores. Coyle et al (1986) determined that carbohydrate ingestion during exercise resulted in an increase in carbohydrate oxidation mirrored by a proportional decrease in fat oxidation. After further investigation Coyle et al (1997) asserted that fatty acid oxidation was *regulated* by carbohydrate metabolism during exercise. In their study on six endurance trained cyclists, they found that lipid oxidation could be directly affected by manipulation of glycolytic flux, indicating that carbohydrate availability directly regulated fat oxidation during exercise. The researchers attributed the reduction in fat oxidation to the specific inhibition of long-chain fatty acid oxidation as well as a reduced mobilization of intramuscular and adipose triglycerides. In a similar investigation, Glisezinski et al (1998) found that sucrose ingestion decreased lipid oxidation by blunting the exercise induced epinephrine response and increasing plasma insulin.

Long-term adaptations to diet also affect lipid oxidation. It is well established that consuming a low-carbohydrate, high-fat diet for 1–5 days impairs endurance (Bergstrom et al, 1971; Karlsson and Saltin, 1971). However, there is evidence that long-term adaptation to a high-fat diet will induce cellular adaptations that in turn could improve performance during long-duration sub-maximal exercise (Helge et al, 1998). Lambert et al (1994) studied the effect of diet on moderate intensity exercise and found that feeding a 76% fat diet for two weeks neither impaired, nor improved, power output and time to exhaustion compared to two weeks of a 74% carbohydrate diet. The specific mechanisms are unknown, but Kiens (1997) found a strong positive correlation between B-hydroxyacyl CoA dehydrogenase activity, fatty acid uptake, and fatty acid oxidation. However, he also found that consumption of a high fat diet for seven weeks was associated with a reduction in time to exhaustion, suggesting that there is a

plateau in fat utilization adaptations which may be followed by a decline in other performance related variables.

For events performed at greater than 70% VO_{2max} and lasting less than four hours, the primary source of ATP is likely to be aerobic glycolysis, and a high carbohydrate diet would be advantageous. However, if an event is lower in intensity and lasts longer than four hours, aerobic lypolysis will most likely provide the majority of ATP and there may be some advantage to a chronic high fat diet (Lambert et al, 1994). However, given the long-term risks of a high-fat, diet this is generally ill advised.

Amino Acid Metabolism

The mechanisms involved in regulating protein metabolism during exercise are not well known. However, it is now well established that the rates of amino acid oxidation and the conversion of amino acids to glucose are increased during endurance exercise and these changes may be physiologically important. The quantity of amino acids metabolized during exercise is often greater than what is available in the body's free amino acid pool, and their source appears to be tissue protein (Felig and Wahren, 1971). Additionally, there is substantial evidence that during exercise protein synthesis is depressed (Dohm et al 1985), freeing up more amino acids for energy metabolism.

The contribution of amino acids to energy expenditure has been estimated to be as low as 5% and as high as 15% (Paul, 1989). Quantitatively, this means an endurance athlete training two to three hours per day would catabolize 20–70 g of amino acids per day. The majority of these amino acids would be used to provide a carbon skeleton for ATP production and/or a gluconeogenic substrate for hepatic glucose production. Because the liver has primary catabolic function for all amino acids except BCAAs, direct peripheral oxidation is limited to carbon skeletons provided by BCAAs. In the liver, alanine, lactate and pyruvate are the primary substrates for gluconeogenesis. The lactate and pyruvate are products of glycolysis and alanine

is produced via a cooperative interorgan effort previously described as the glucose-alanine cycle (Refer to Figure 1.5). The utilization of amino acids for ATP and glucose increases as exercise intensity increases, as glycogen stores decrease, and as blood glucose begins to fall.

Felig and Wahren (1971), who studied amino acid metabolism in exercising men, provided evidence of the role of amino acids in exercise. Using 18 subjects, they inserted catheters into a brachial artery, a femoral vein, a antecubital vein, and a hepatic vein, and then evaluated arterial concentration and net exchange across the leg and splanchnic bed for 19 amino acids. In a resting, fasted state they noted the peripheral release and splanchnic uptake of alanine exceeded that of all other amino acids and accounted for 35–40% of total net amino acid exchange. Relative to net splanchnic uptake, hepatic release of alanine and other amino acids was small. During exercise, leg alanine output increased in proportion to the workload and splanchnic alanine uptake increased 15–20%, a rate that exceeded all other amino acids. Additionally, at heavier workloads, splanchnic release of isoleucine, leucine, methionine, tyrosine, and phenylalanine increased by 8–35%. This data, coupled with the fact that alanine comprises only 5–7% of the amino acid residues in muscle protein, led the authors to conclude the following: (a) alanine must be peripherally synthesized to be released at the observed rates, (b) during exercise the muscle synthesizes alanine from pyruvate and amino groups derived via transamination of other amino acids, (c) a glucose-alanine cycle exists whereby alanine, synthesized in muscle, is taken up by the liver and its carbon skeleton is reconverted to glucose, and (d) alanine serves as a carrier to transport amino groups from peripheral muscle to the liver.

Additional data on alanine metabolism was provided by Wasserman et al (1988) who assessed the importance of intrahepatic mechanisms to gluconeogenesis in dogs. Using labeled alanine, they found, during 150 min of treadmill exercise and 90 minutes of recovery, the conversion rate of alanine to glucose increased by 248% at 150 minutes of exercise with a further increase during recovery. However, it should be noted that dogs are carnivorous animals

adapted for lipid and amino acid metabolism and may metabolize alanine very differently than humans. Additional studies on humans show that alanine alone may account for half of the total glucose production from amino acids (Brooks, 1987). Sumida and Donovan (1995) demonstrated that, after four hours of continuous light exercise, the liver's output of alanine-derived glucose accounted for 45% of the liver's total glucose release and contributed 10–15% of the total exercise energy requirement. A further observation is that the role of alanine is not limited to just the exercise session; after exercise, gluconeogenesis from alanine is markedly elevated and its overall contribution to glucose production is significant (Wasserman et al 1988; Brooks, 1987).

With respect to the source of the amino group for alanine formation, because BCAAs are preferentially catabolized in the muscle rather than the liver they are potential nitrogen group donors. Of the BCAAs, leucine is the one most often studied because of its role in the regulation of tissue protein metabolism (Tischler et al, 1982), and because it is purely ketogenic and oxidation is easy to trace. The work of Wolfe et al (1984) was important in proving the link between leucine catabolism and alanine formation. In their study they used dual (^{15}N and ^{13}C) labeling techniques to show the transfer of leucine nitrogen to alanine. Furthermore, they determined that 10% of the alanine formed during exercise was from transamination via leucine. However, an unexpected outcome was that, despite the increased formation of alanine, there was no increase in the rate of total or labeled urea production. The authors attributed this finding to a decreased catabolic rate for other amino acids and/or the fact that other amino acids supply the nitrogen for alanine formation.

Nutritionally, leucine metabolism is of interest because it is an essential amino acid and a deficiency of it could be a limiting factor in total body protein synthesis. White and Brooks (1981) used rats to study the effect of exercise intensity on leucine metabolism and oxidation. Using pulse injection of ^{14}C labeled leucine tracers, they found that, as exercise intensity

increased, so did exhalation of labeled CO_2, indicating that progressively greater amounts of leucine were being oxidized for ATP. Similar findings were reported by Lemon et al (1982) who determined that labeled leucine oxidation was greater at 80% VO_{2max} than at 40% VO_{2max} and oxidation remained elevated until five hours post-exercise. In humans, the role of leucine metabolism during exercise was investigated by Evans et al (1983) who demonstrated that during two hours of exercise at 55% VO_{2max}, 86% (680 mg) of the daily requirement for leucine was oxidized. Fortunately, estimates of the leucine intake of physically active individuals is 57–100 mg/kg/day which is well above the daily requirement of 14–39 mg/kg/day (Carroll, 2000). However, in ultraendurance events lasting longer than six hours it is conceivable that leucine oxidation could exceed intake.

Exercise intensity impacts amino acid metabolism but there appears to be a minimum threshold for increased catabolism. Lemon et al (1984) had subjects run on a treadmill for one-hour at three different intensities and evaluated sweat and 72 hour urinary urea excretion. Compared to exercise at 41% VO_{2max}, sweat urea nitrogen levels were higher when subjects exercised at 55% and 67% VO_{2max}. Also, urinary urea nitrogen excretion was greater three days after exercise for the 55% and 67% VO_{2max} runs, and not the 41% VO_{2max} run. These results led the authors to conclude that the amino acid utilization threshold was between 41 and 55% VO_{2max} and that amino acids are utilized during the recovery period as well as during exercise.

Nitrogen Balance During Exercise

The fact that exercise alters amino acid metabolism is irrefutable, however its impact on overall protein requirements is less clear. Nitrogen balance studies provide a method of determining the impact of exercise on overall protein requirements. As previously mentioned, even in sedentary individuals determining nitrogen balance is difficult, thus, the introduction of exercise as an additional variable further complicates interpretation. However, a few intrepid groups have conducted well designed nitrogen balance studies on exercising subjects.

Tarnopolsky et al (1988) calculated regression lines from nitrogen balance data obtained when subjects consumed isocaloric, calorie-sufficient diets containing 1.7 g/kg/day then 2.65 g/kg/day of protein. The authors found that endurance athletes who trained at least 12 hours per week required 1.37 g/kg/day of protein to maintain nitrogen balance compared to 0.73 g/kg/day for sedentary controls. Friedman and Lemon (1989) had similar findings when they studied endurance runners during their regular training program and found that nitrogen balance was negative when 0.86 g/kg/day of protein was consumed and positive when 1.5 g/kg/day was consumed. The consensus of most research on the protein requirements of endurance athletes is that the current USRDA does not meet their needs. However, due to the increased caloric requirements of athletes, if a calorie sufficient diet containing at least 10% of the calories as protein is consumed, nitrogen balance will be maintained (Tarnopolsky et al, 1988). Based on a protein requirement of 1.37 g/kg/day and a vitamin B-6 to protein ratio of 0.2, a 70-kg male would require 96 g of protein and 1.9 mg vitamin B-6, which are 176% and 146% of the respective RDAs of 56 g protein and 1.3 mg vitamin B-6.

During exercise, an indirect method thought to quantify amino acid degradation is assessment of urea nitrogen production. Urea is formed in the liver via the urea cycle and is the major route of nitrogen removal from the body. After formation, urea is released into the blood and is removed by the kidney and sweat glands. Haralambie and Berg (1976) provided evidence linking the exercise-induced increase in serum urea nitrogen to increased amino acid catabolism. Their study included six groups of male athletes participating in various forms of physical activity ranging from 70 to 765 minutes. Analysis revealed a significant correlation between the decrease in serum amino acid nitrogen and an increase in serum urea nitrogen. Furthermore, the magnitude of the changes correlated with the duration of exercise. Under some conditions, altered kidney function during exercise may invalidate urinary urea nitrogen as a comprehensive status indicator. Rennie et al (1981a) noted increased plasma urea and

decreased urinary urea production during cycling at 50% VO_{2max}. Renal clearance resumed normal rates five hours after exercise, at which time urea excretion showed marked increases, which lasted throughout the 20-hour recovery period. Although urinary loss represents the primary route to eliminate urea from the body, a significant amount may also be excreted via sweat.

A confounding factor in most nitrogen balance exercise studies is accurate assessment of sweat urea nitrogen losses. Individual sweat rate varies and overall volume is the most important factor in determining the amount of urea nitrogen lost in this manner. Sweat collection is difficult and the whole-body wash-down method (WBW) is generally considered the most accurate (Brooks, 1987). However, recent research by Colombani et al (1997) comparing the sum of urea and ammonia nitrogen assessed by the WBW and a regional collection method showed no significant difference. The magnitude of difference in published works points to the method of collection and/or analysis as a source of error. Using regional collection techniques, Calles-Escandon et al (1984) calculated that 30% of the total urea excretion during 90 minutes of cycling at 45% VO_{2max} was in the sweat. This estimation is extraordinarily high compared to other studies. Friedman and Lemon (1989) examined the effect of chronic endurance exercise on protein metabolism in five well-trained endurance runners. Following 75 minutes of treadmill running at 72% VO_{2max}, they determined total sweat urea by whole body washdown and found it amounted to only 3.5% (260 mg/hr) of the total 24 hour urea excretion. Tarnopolsky et al (1988) had remarkably similar findings after they collected "representative" sweat samples from six elite endurance athletes during 70-80 minutes of running at 75% VO_{2max} and estimated sweat contained 1.6% (240 mg/hr) of the total urea.

There are no estimates of sweat urea nitrogen for exercise sessions lasting more than 80 minutes. During exercise of long duration, it is reasonable to assume that as amino acid catabolism increases so does the urea nitrogen concentration of sweat. The potential impact of

this effect is illustrated in the work of Lemon and Mullin (1980). In their study, they exercised glycogen loaded and glycogen depleted subjects on a cycle ergometer at 60% VO_{2max} and noted a 66-fold increase in sweat urea in the loaded subjects and a 154-fold increase in the depleted subjects.

The aforementioned study also illustrates the impact of diet on amino acid oxidation. Chronic and acute consumption of carbohydrate appears to have an amino acid sparing effect. Training (Layman et al, 1994) and exercise (Kasperek et al 1985) increase both liver and muscle BCKA dehydrogenase activity. However, the availability of glycogen and glucose will partially modulate the impact of this adaptation. Diets chronically low in carbohydrate decrease glycogen stores and thus increase reliance on amino acid oxidation for energy. In support of the 1980 Lemon and Mullin study, Davies et al (1982) confirmed the concept of amino acid sparing by carbohydrates when he found that glycogen depleted subjects utilized a greater amount of protein for energy than did those glycogen replete. Specifically, they found the three-fold increase in exercise induced leucine oxidation was decreased when glucose was infused during the exercise. The decrease in leucine oxidation indicates a decreased hepatic requirement for alanine as well as a decreased need for ATP substrate by the muscle. Thus, protein requirements are likely to increase if exercise is undertaken when carbohydrate stores are low.

Fluid Volume Shifts

It is not uncommon for athletes to experience sweat rates over a liter per hour, and when it is hot losses may easily exceed 2 liters per hour (Brouns, 1997). The rate of sweat secretion increases progressively with exercise intensity and is influenced by environmental conditions such as temperature, humidity, solar and ground radiation, as well as training status and type of clothing. Dehydration is known to have metabolic and physiological consequences, such as reduced cardiac output, impaired heat exchange, reduced oxygen supply to muscle cells, increased catecholamine levels and enhanced glycogen breakdown rates (Brouns, 1997). The

general consequence of ongoing dehydration is that health may be threatened and performance capacity will be reduced.

Water loss during exercise-induced sweating leads to dehydration of both intracellular and extracellular fluid compartments of the body. However, shifts in plasma volume are not simply a result of sweating; they are a complex interaction of several factors. At the onset of exercise, there is a marked flux of water out of plasma into the active tissue and plasma volume decreases around 13% (Costill, 1984). This fluid shift is a result of hyperosmolality created by metabolic processes in the active tissue. Following this initial rapid flux out of the plasma, there is a slow shift toward replacing plasma volume. The loss of hypotonic sweat and an increase in intravascular protein content causes an increase in plasma osmolality that pulls water from extravascular spaces to the vascular compartment. Additionally, metabolic water produced from the active tissue may add to plasma volume. Exercise intensity and duration as well as fluid intake and environmental factors all affect plasma volume and help determine the magnitude and direction of fluid shifts.

Costill et al (1976) induced two levels of dehydration by having subjects cycle in the heat and then determined from which compartments the water was lost. When 2% of the body weight was lost, 10% came from the plasma, 60% from of interstitial fluid, and 30% from intracellular fluid. With a 6% loss of body weight, still only 11% came from the plasma fluid, 39% from interstitial fluid, and 50% from intracellular fluid. These results indicate that plasma volume is maintained at the expense of other fluid compartments. When losses are minimal, the extracellular compartments account for the majority of the loss. However, when body water losses increase, a greater percentage of the water deficit comes from the intracellular compartment.

Dietary Recommendations for Ultraendurance Sports

The predominant energy system for ultraendurance athletes is aerobic, with brief, intermittent involvement of anaerobic energy systems. During the event, exercise intensity ranges between 50 and 90% VO_{2max} and duration typically ranges from 4 to 24 hours, although multi-day events are not uncommon. The athlete's total energy expenditure depends on the intensity, duration, and type of activity, but may range between 5,000 to 10,000 kcal/day (Kreider, 1991). Tarnopolsky et al (1988) estimated the energy requirements of male endurance runners training over 125 km/week was 4,570 calories.

The top nutrition concerns for ultraendurance athletes are to match caloric intake with expenditure, consume adequate carbohydrates, match fluid intake with fluid loss, and to drink and eat according to a schedule that promotes hydration, maintains blood glucose, and avoids gastrointestinal upset. Specific macronutrient recommendations during training are as follows (Coleman, 2000):

7 to 10 g carbohydrate per day

$\geq$ 1.2 to 1.4 g/kg of protein per day

$\geq$ 1 g/kg of fat per day

During competition, macronutrient needs increase and carbohydrate should be increased to 12–22 g/kg, protein to 1.5–3.0 g/kg, and fat to 1–3 g/kg. The carbohydrate should be consumed at the rate of 1–4 g/kg 1–4 hours prior to exercise, 30–60 g every hour during exercise, 1.5 g/kg immediately after exercise, and 1.5 g/kg every two hours post-exercise.

Of equal importance is adequate fluid consumption. Athletes should be well hydrated during training and competition and drink 1000–1300 mL for each kg lost. A beverage containing 500–700 mg of sodium/L is recommended because it will enhance palatability, promote fluid retention, and prevent hyponatremia (Coleman, 2000).

THE EFFECT OF A 50-KM ULTRAMARATHON ON VITAMIN B-6 METABOLISM

Abstract

Purpose: To examine the effect of a 50-km ultramarathon on vitamin B-6 metabolism. **Methods**: Nine men and five women (44 ± 11 years) completed two five-day trials separated by three weeks. During both trials subjects were fed a controlled diet providing men 2.0 mg vitamin B-6, 114 g protein, and > 3000 kcal, and women 1.5 mg vitamin B-6, 88 g protein, and > 2500 kcal. On day four of Trial 1 (T1) an ultramarathon (ultra) was run, and on day four of Trial 2 (T2) the subjects were inactive. Twenty four-hour urine collections were completed for each day of each trial and blood was drawn pre- race (pre), mid-race (mid), post-race (post), and 60 minutes post race (P-60). On the inactive day blood was drawn at the same intervals. During the ultramarathon all food/beverage selections were recorded and on the inactive day subjects consumed the same foods at the same intervals. Plasma was analyzed for pyridoxal 5'-phosphate (PLP), pyridoxal (PL), 4-pyridoxic acid (4-PA), urea nitrogen (PUN), creatinine, albumin, glucose, and lactate concentration as well as alkaline phosphatase activity. Urine was analyzed for 4-PA and creatinine. **Results**: During T1, compared to pre levels, plasma PLP concentration increased 17% by mid, decreased 5% by post, for a total decrease of 19% by P-60 [pre to P-60 ($p < 0.02$), mid to post ($p < 0.01$), mid to P-60 ($p < 0.00$), and post to P-60 ($p < 0.05$)]. During T2 plasma PLP concentration decreased 13% ($p < 0.05$) pre to P-60. There was no correlation between the change in PLP concentration and the change in the plasma concentration of glucose, albumin, lactate, or alkaline phosphatase activity. During T1 plasma 4-PA concentration increased 135% ($p < 0.02$) and was significantly higher than T2 at mid, post, and P-60 ($p < 0.03$). Compared to the day before the ultra, there was no change in urinary 4-PA excretion the day of or after the event. However, the percent of dietary vitamin B-6 that was

excreted as 4-PA the day of the ultra was higher ($p > 0.05$) than that excreted the day before and the day after. **Conclusion**: Extreme exercise of greater than six hours initially increases the plasma concentration of PLP but ultimately results in a significant decrease in plasma PLP, an increase in plasma 4-PA, and a increase in percent of dietary vitamin B-6 (as 4-PA) excreted in the urine.

Introduction

Vitamin B-6 (pyridoxal 5'-phosphate) has numerous critical roles in fuel metabolism. during exercise. During exercise there is an increase in glucose utilization by the muscle (Kelly, 1995), a need that is initially met by increasing glycogen phosphorylase activity (Brooks et al, 1996a), for which pyridoxal 5'-phosphate (PLP) is a required coenzyme (Taylor et al., 1972). However, as glycogen stores are depleted there is an increased reliance on amino acid derived glucose to maintain blood glucose levels (Brooks, 1987). In this gluconeogenic role, PLP is required by both the muscle and liver as a cofactor for transaminase and decarboxylase enzymes involved in the glucose-alanine cycle (Harper et al., 1984). Additionally, PLP is required in the muscle when amino acids are metabolized for energy.

Vitamin B-6 metabolism is significantly altered by exercise intensities above 50% VO_{2max} (Rokitzki et al., 1994a, Leklem and Shultz 1983; Hoffman et al., 1991; Dreon and Butterfield, 1986). Leklem and Shultz (1983) were the first to document an increase (18–33%) in plasma PLP during exercise, a finding that has subsequently been confirmed in both human and animal studies (Hadj-Sadd et al, 1995; Manore et al, 1987). Further, Crozier et al. (1994) determined that the majority of the increase (79%) occurred during the first five minutes of exercise and that exercise intensity (60 vs 85% VO_{2max}) does not affect the magnitude of response. However, Leklem (1984) found that intensity does affect the response. The only study to demonstrate a *decrease* in plasma PLP was also the only study that had exercise duration greater than three hours. In this study Leonard and Leklem (2000) found that following

a 50-km ultramarathon plasma PLP was 31% below pre race levels and one hour post race the concentration was 44% below pre race values. This study suggests that exercise duration may affect vitamin B-6 metabolism in a manner not previously considered.

There have been several theories proposed to explain the source and destination of mobilized PLP. Leklem and Shultz (1983) suggested the "muscle to liver", Dreon and Butterfield (1986) the "labile pool", and Hoffman (1991) the "liver to muscle" theory. The common theme of all the theories is that PLP is released from a tissue/pool with a low metabolic need and redistributed to a tissue with greater metabolic need. Hadj-Saad et al. (1995) demonstrated that in rats 2.5 hours of swimming resulted in a 50% increase in total muscle PLP and no change in total liver PLP suggesting involvement of, and redistribution from, a "pool" other than muscle or liver. However, thus far in humans there is insufficient evidence to unequivocally reject or accept any one theory. One of the lingering questions is, "does the increased mobilization of vitamin B-6 lead to an increased loss of the vitamin, and thus, an increased requirement?" Several studies have attempted to answer this question through evaluation of 4-pyridoxic acid, the irreversible end product of vitamin B-6 metabolism, excretion. The results are inconclusive. Some human and animal studies have shown an increase in urinary 4-PA excretion following exercise (Rokitzki et al., 1994a; Manore, 1987), others have found no significant difference (Leklem and Shultz, 1983; Dunton, 1994), and still others indicate that excretion may be less (Drenon and Butterfield, 1986; Efremor and Zaburkin 1972). However, comparison of these studies is complicated by differences in experimental design and type of exercise.

The intent of this study is to evaluate the effect of extreme exercise of long duration (>6 hours) on phases of vitamin B-6 metabolism. We hypothesized that during the first two to three hours of exercise plasma PLP concentration will increase and during the last three to four hours concentration will decrease and ultimately drop below pre race concentration.

Additionally, during exercise there will be a continual increase in plasma 4-PA concentration and following exercise urinary excretion of 4-PA will be increased above baseline levels. The diet was controlled to minimize the affect of dietary intake on plasma and urinary variables. Additionally, to elucidate the mechanism(s) leading to the increase and decline in PLP, plasma albumin, alkaline phosphatase, blood urea nitrogen, and lactate were measured.

Methods

Subjects

Fourteen healthy, 25–57 year-old male and female subjects were recruited from local area residents interested in completing an ultramarathon. Recruitment included mailing information to registered runners and posting flyers around Oregon State University (OSU). Subjects selected were healthy (no metabolic disorders), physically fit, non-smoking, endurance trained runners (7.5 ± 2.9 hours per week) who were not taking any medications that would affect vitamin B-6 metabolism. Five of the subjects (four males and one female) reported habitual consumption of a vitamin and mineral supplement in excess of the RDA 3–6 times per week, a practice they agreed to discontinue nine days prior to Trial 1. The OSU Human Subjects Committee approved the study, and written informed consent as well as a medical history was obtained from each subject. Physical characteristics of the subjects are given in Table 2.1.

Table 2.1. Mean (± SD) physical characteristics of subjects

	Age (years)	Weight (kg)	Height (cm)
Male (n = 9)	43.5 ± 11	77.4 ± 8.5	179 ± 7
Female (n = 5)	40.6 ± 10	57.5 ± 3.5	161 ± 4

Experimental Protocol

All subjects participated in two five-day trials. During each trial subjects were weighed daily, consumed a controlled diet, completed five blood draws, collected urine, and recorded activity (Figure 2.1). During Trial 1 (T1) on day four, subjects completed a 50-km ultramarathon, and during Trial 2 (T2) on day four subjects were inactive. Subjects agreed to keep a 24-hour activity log and stop all supplements (vitamin, mineral, and herbal) nine days prior to beginning each trial. To evaluate vitamin B-6 metabolism, plasma was analyzed for PLP, pyridoxal (PL), pyridoxamine (PN), 4-PA, glucose, lactate, urea nitrogen, alkaline phosphatase activity, and albumin. Whole blood was analyzed for hemoglobin (Hb) and hematocrit (hct), and urine was analyzed for creatinine and 4-PA.

Trial 1	←No Vitamin or Mineral or Herbal Supplements→ ←Record the Intensity and Duration of all Activities (24 hour log)→					
	Normal Diet	***Trial 1, Controlled Diet*** ***24 Hour Urine Collections; Daily Weights***				
	9 Day Pre-Trial Period	Day 1 Blood Draw: Fasting	Day 2	Day 3	Day 4 Ultramarathon Blood Draws: Pre, Mid, Post, P60	Day 5
	//Trials Separated by 22 Days//					
Trial 2	←No Vitamin or Mineral or Herbal Supplements→ ←Replicate Daily Activity Recorded for Trial 1→					
	Normal Diet	***Trial 2, Controlled Diet*** ***24 Hour Urine Collections; Daily Weights***				
	9 Day Pre-Trial Period	Day 1 Blood Draw: *Fasting*	Day 2	Day 3	Day 4 **No Exercise** Blood Draws: Four at Trial 1, day 4 intervals	Day 5

Figure 2.1. Timeline and major activities during Trial 1 and Trial 2. Twenty-two days elapsed between day five of Trial 1, and day one of Trial 2 (inclusive of the Trail 2 nine-day pre-trial period).

Trial 1

The ultramarathon was completed on day four of T1. Subjects were instructed to consume their breakfast at 5 a.m. and report for a pre-race blood draw and weigh-in at 7:30 a.m.. Breakfast consisted of a bagel (100 g), jelly (30 g), and orange juice (200 g) and provided approximately 500 kcal, 0.4 mg vitamin B-6, and 10 g protein. The 50-km ultramarathon trail run began at 8 am and had a total elevation change of 3,902 m. During the race six aid stations provided subjects with urine bottles as well as unlimited quantities of specific food and beverages (see diet section for more detail). At each aid station an attendant recorded the time the subject arrived and the quantity of all food and beverages consumed. The mid-point and post race blood draws were accomplished within three minutes of their arrival and between the post-race blood draw and the P-60 draw subjects consumed 500 mL of water and nothing more. Post-race weight was recorded prior to the water consumption. Following their P-60 blood draw subjects were provided their normal lunch and reported to the nutrition lab at the standard time for dinner. Adjustments were made to dinner selections to compensate for the total amount of vitamin B-6 and protein consumed during the run.

Trial 2

Trial 2 was the mirror image of T1 with the exception of the ultramarathon: the subjects duplicated the intensity and duration of activity recorded during T1 and followed the same diet, urine, and blood draw procedures. The difference was that on day four of the controlled diet, instead of running an ultramarathon the participants were "inactive" for a time equal to the duration it took them to complete the race. During this "inactive" time subjects were allowed to continue non-vigorous daily activities including desk work and walking to and from the nutrition lab where they had blood drawn at intervals established according to their ultramarathon blood draw times. Between blood draws subjects consumed the same quantity of food and drink that they had during the ultramarathon.

Blood collections

Nineteen milliliters of blood was drawn from the anticubital vein into heparinized tubes five times per trial. The time of each blood draw was recorded and all draws were made 60 to 90 seconds after the subjects assumed a seated position. The first draw was a fasting sample (no food or drink after 8 p.m. the night before) taken three days prior to the race. The remaining four blood draws were completed 30–60 minutes prior to the ultramarathon (pre), during the race at mile 15 (mid), immediately after race completion (post), and 60 minutes following the race (P-60). Pre, post, and P-60 race day blood draws were completed in a building at the race site. The mid-point blood draw was completed in the open at a site approximately mid way through the course. In all cases blood was immediately placed on ice, covered, and delivered to the OSU Nutrition Lab (15-minute drive) at 30-minute intervals. The technicians at the lab recorded the time each sample arrived, determined the hematocrit and hemoglobin concentration, then centrifuged the blood and extracted plasma aliquots which were frozen at ˉ40°C. To duplicate the travel and delivery time that occurred in T1, during T2, blood was held on ice 30 to 40 minutes before processing.

Diet

During T1 and T2 all foods consumed by the subjects were prepared in the metabolic kitchen at OSU. Breakfast and lunch meals were consumed at home or work and the dinner meal was consumed on site. At the dinner meal each day the subjects completed a daily record verifying food and beverage consumption for that day. The diet, consisting of two alternating menus, was the same for both trials and provided 55–65% carbohydrate, 15–20% protein, and 15–30% fat. The range of intake was a result of allowing subjects to consume simple carbohydrates and fats *ad libitum* to adjust for each individual's variation in energy need while keeping the vitamin B-6 and protein constant. Using a computerized dietary analysis program (Food Processor II, ESHA Research, Salem, OR), the diet was designed to provide male

subjects, 130 g of protein, 2.3 mg of vitamin B-6, and >3,000 calories per day; and female subjects, 97 g of protein, 1.7 mg of vitamin B-6, and > 2500 calories per day. The diet also provided 100% of the RDA for vitamins and minerals (RDA, 1989). The morning of, and during, the ultramarathon only foods with negligible vitamin B-6 (<0.1mg/100g) and protein (<6g/100g) were allowed. Table 2.2 lists the vitamin B-6 and protein content of food and beverages available during the ultramarathon. Prior to dinner following the race, the vitamin B-6 and protein content of foods consumed during the ultramarathon was calculated and an equivalent amount was subtracted from the evening meal. This was done so that the total amount of vitamin B-6 and protein consumed during that day would not be significantly different from the other days. The vitamin B-6 and nitrogen content of the meals was verified in our laboratory using aliquots of food composites prepared on the last two days of each trial.

All food served during this study was purchased in bulk from the same lot when possible. Prior to T1, all meats for both trials were sliced, weighed, and frozen in daily use portions. Fresh produce and milk was purchased immediately prior to each trial. All foods were weighed to the nearest 0.2 g. To ensure complete consumption, subjects were instructed to rinse all food containers with water and then consume the rinse water.

Urine Collection

For each day of both trials, beginning in the morning, urine (24-hour) was collected. Samples were kept refrigerated between collections and were delivered to the nutrition lab daily where individual 24-hour samples were mixed, measured, and aliquots were frozen at -20° C. The completness of each 24-hour collection was assessed by measuring creatinine content.

Table 2.2. Vitamin B-6 and protein content of foods available during the ultramarathon.

Food Item	Serving Size	B-6 (mg)	Protein (g)	Calories
Hydra fuel ®[1]	6 oz	0	0	42
Cola	4 oz	0	0	56
Cliff Bar ®[2]	1 (68g)	.11	4	250
Cliff Shot ®[2]	64g	0	0	194
Gu ®[3]	32g	0	0	100
Oreo ®[4]	2 (22g)	.02	1.3	107
Vanilla Wafers	4 (16g)	.01	0.9	77
Pretzels	15g	.02	1.4	57
Apple Slice	¼ (30g)	.01	0.1	18
Orange Slice	¼ (35g)	.02	0.3	16

[1] Hydra Fuel is a trademark of TwinLab, Ronkonoma, NY.
[2] Clif Bar and Clif Shot are trademarks of Clif Bar Inc., Berkeley CA. Each subject was limited to one Clif Bar during the event.
[3] Gu is a trademark of Sports Street Marketing, Berkeley, CA.
[4] Oreo is a trademark of Nabisco Foods, East Hanover, NJ.

Biochemical Analysis

All samples were analyzed in duplicate unless otherwise specified. Hematocrit and hemoglobin (in triplicate) was determined using the micro Hct and cyanmet Hb methods, respectively. These data were used to calculate plasma volume changes using a method described by Dill and Costill (1974). On the last two days of both T1 andT2, a food composite was made for each male and female menu and dietary vitamin B-6 was determined by a microbiological method (Miller and Edwards, 1981) using Saccharomyces uvarum (ATCC 9080). Dietary nitrogen was determined using the boric acid modification of the Kjeldahl method (Scales and Harrison, 1920).

Plasma vitamin B-6 vitamers were analyzed by high performance liquid chromatography (HPLC) using the method of Sampson and O'Conner (1989). The method utilizes reverse-phase ion pairing to separate PLP, PM, PL, PN, and 4-PA which are chromatographically measured at an excitation wavelength of 330 nm and fluorescent emission at 400 nm. The first mobile phase of the gradient system was 0.033 M phosphate/8mM octane

sulfonic acid, and the second mobile phase was 0.033 M phosphate/isopropanol (18%, v:v). Both phases were pH 2.3 with a flow rate of 1.0 mL/min. The post column reagent (1.0 g/L sodium bisulfite in 1 M KH2PO4, pH 7.5) was added to the column eluate at 0.2 mL/min. The Shimadzu HPLC system includes a SCL-10H controller, two LC-10AD pumps; a 250 ul injection loop; a RF-10A fluorometer, and a CR501 recorder/integrator. The post column pump was an Eldex model A-30-S and the column was a Rainin C18 ion-pair analytical column (3-μm particle size, 4.6 x 100 mm, Rainin Instrument Co, Emeryville, CA). Sample preparation was conducted under yellow fluorescent lighting to minimize photodegradation of B-6 vitamers. A set of standards (4) and a control sample was analyzed daily and the sample B-6 vitamer concentration was calculated from the 0.05 μmol standard peak height. The interassay coefficient of variation for the control sample (n = 15) was: PLP = 10%, 4-PA = 8.3%, PL = 15.1% and PN = 36%. Single samples from each subject were analyzed in one assay to minimize the effect of interassay variation.

Urinary 4-PA was analyzed by a modification of a HPLC procedure (Gregory and Kirk, 1979) using a Beckman HPLC with a 0.034M, 2.2 pH phosphate methanol mobile phase buffer (1.25% acetonitrile, 5% methanol). A reverse phase column (Econsil C18 10μ; 250 mm x 4.6 mm) was used to separate the 4-PA and flourescence was measured at 425 nm (320 nm excitation) and peak area was recorded by a Hewlett-Packard Integrator (3390A). All samples were run in duplicate in one assay along with a standard and a control. Control sample (n = 14) interassay CV was 3.6% and recovery of added 4-PA averaged 95.5 ± 5 %.

Glucose was determined using a modified procedure of Trinder (1969) utilizing an Alpkem Autoanalyzer II. CV for the control samples (n = 4) was < 1%. Alkaline phosphatase concentration was estimated by determining alkaline phosphatase activity using a colorimetric assay described by Roy (1970). Interassay CV for the control samples (n = 14) was 4%. Plasma albumin was determined using a method by Slater et al (1975) and CV for the control

samples (n = 5) was 5.7%. Plasma lactic acid was measured spectrophotometrically (Sigma Chemical Co. Procedure No. 726-UV/826-UV) using the method of Hohorst (1963). Interassy CV for the control samples (n = 4) was 5%. Blood and urinary urea nitrogen was analyzed using an automated method described by Di Giorgio (1974). Interassay CV for the control samples was 2.6% for PUN (n = 3). Urinary and plasma creatinine was determined using an automated method based on the modified procedure of Pino et al (1965).

Statistics

The SPSS statistical analysis software package (version 9.0) was used to analyze all data (SPSS Base 9.0 Applications Guide, Chicago IL). Significance for all analyses was set at $p \leq 0.05$. A GLM (general linear model) repeated measures was used to determine main effects of trial, day or time by trial, and the within trial effect of day or time. For multiple comparisons of day or time, the Bonferroni adjustment was used. Between trial comparisons of the same time points was made using the Student's paired t-test. Bivariate associations were assessed by Pearson's r.

Results

The response of males and females did not differ from one another for any of the dependent variables studied. Therefore, the data was analyzed collectively and, unless otherwise noted, means reported represent data from n = 14. Additionally, some of the changes in plasma volume were significant, therefore the data reported for mid, post and P-60 plasma measures has been corrected for plasma volume change. Table 2.3 lists the mean, SD, and range of % change in plasma volume for these measures.

Table 2.3. Mean percent change (± SD) and the range of observed changes for the change in plasma volume from pre to mid, post, and P-60 during T1 and T2.

	Percent change from pre:		
	Mid	Post	P-60
T1	-3.7 ± 6	0.3 ± 7	5.4 ± 8
(Range)	(-11.7 to 8.6)	(-9.5 to 12.3)	(-5.9 to 25.7)
T2	2.7 ± 6	4.8 ± 5[1]	3.4 ± 4[2]
(Range)	(-4.9 to 13)	(-2.9 to 10.3)	(-5.6 to 8.3)

[1] $p = 0.01$
[2] $p = 0.04$

Diet

All subjects completed all five days of each trial. Dietary compliance verified by the daily report was 98% with each of the three reported infractions involving less than 0.05 mg of vitamin B-6. Analysis of food composites revealed the average vitamin B-6 content of the diet was 1.97 mg (11.6 μmol) for males and 1.51 mg (8.9 μmol) for females, which was slightly below the respective estimates of 2.3 mg (13.6 μmol) and 1.7 mg (10.0 μmol). However, since analysis of dietary protein revealed it too was lower than expected (males 113.5 g; females 87.8 g) the ratio of vitamin B-6 to protein remained between 0.016 and 0.019. Based on the computerized diet analysis and reported *ad libitum* food intake, the average number of calories consumed by males was 3150 ± 116 per day (41 kcal per kg) and by females was 2570 ± 90 kcal per day (45kcal per kg). Of this total, *ad libitum* food intake accounted for 150 kcal per day for males and 70 kcal per day for females. In spite of the availability of such foods, during the five days the controlled diet was consumed, during T1 five males and one female lost weight (mean of 1.3 and 0.9 kg, respectively) and during T2 five males and two females lost weight (mean of 0.6 and 0.5 kg, respectively). However, the difference in weight loss between groups was not significantly different.

Ultramarathon

During the race the air temperature increased 12 ° C (from 11 to 23°C) and humidity decreased from 83% to 34%. The average time elapsed before the mid point blood draw was 166 ± 31 minutes and subjects completed the race in 396 ± 68 minutes with males finishing an average of 22 minutes (5.6%) before the females (388 ± 77 vs. 410 ± 52 min). After completion of the run, the average rate of perceived exertion (RPE) reported was 14 (Borg scale 6 to 20) which correlates to an intensity of approximately 75% VO_{2max} (Borg, 1982). The average weight loss during the race was 1.5 kg, equaling 2.1 ± 1.5% of their total body weight and equating to a fluid deficit of 1.5 L. Average fluid intake was 3.8 L, with males consuming 27.8% (1.2 L) more than females. During the race an average of 1285 calories, 0.25 mg (1.5 μmol) vitamin B-6, and 6.1 g of protein was consumed. Table 2.4 lists the average food and beverage consumption per subject, per aid station.

Table 2.4. The nutritional composition of the food and beverages consumed per subject per aid station.[1]

Aid Station #	B-6 (μmol)	Protein (g)	CHO (g)	Total Calories	Fluid (mL)
1	1.5	1.6	57	267	573
2	0.1	0.9	57	238	567
3	0.4	1.4	58	258	746
4	0.1	0.9	56	240	807
5	0.1	0.6	32	142	398
6	0.2	0.7	31	140	741
Total ± SD	1.5 ± 0.2	6.1 ± 0.9	291 ± 47	1285 ± 127	3832 ± 618

[1] Values listed represent average per subject per aid station

Plasma PLP, PL, PN and 4-PA

Figure 2.2 graphically depicts the mean pre, mid, post, and P-60 PLP concentration during T1 and T2. Fasting values are not included because they were taken before the subjects started consuming a controlled diet. There was no significant difference between the mean

fasting PLP concentrations of T1 (58.5 ± 18.4 nmol/L) and T2 (52.4 ± 27.5 nmol/L) indicating the pretrial the intake of vitamin B-6 was similar for T1 and T2. Comparing males to females, the mean fasting concentration of PLP nmol/L was 63.7 ± 14.3 vs. 49.0 ± 22.9 for T1, and 57.6 ± 32.2 vs. 43.1 ± 14.4 for T2 ($p > 0.05$). Subjects with a history of supplement usage had higher mean fasting PLP concentrations than non-users but the difference was not significant and was less during T2 compared to T1 (T1, 66 ± 17 vs 54 ± 19 and T2, 55 ± 38 vs 51 ± 22 nmol/L, respectively, for users and nonusers). Compared to pre levels, during T1 (ultramarathon) mean plasma PLP concentration increased 17% by mid, decreased 5% by post, for a total decrease of 19% by P-60 [significant decrease pre to P-60 ($p < 0.02$), mid to post ($p < 0.01$), mid to P-60 ($p < 0.00$), and post to P-60 ($p < 0.5$)]. T2 plasma PLP concentration showed a significant mean decrease of 13% between the pre and P-60 measures ($p < 0.05$). For pre to mid, mid to post, and post to P-60, there was no significant correlation between the change in plasma PLP concentration and the change in plasma concentration of glucose, albumin, lactate, or alkaline phosphatase activity.

There was no difference in mean fasting PL or PN concentrations (PL:15.4 ± 4.9 vs 17.2 ± 6.9 nmol/L and PN 6.9 ± 4.0 vs 9.8 ± 4.8 nmol/L, for T1 and T2 respectively). Figure 2.3 illustrates the mean PL concentrations during T1 and T2. There were no significant within or between trial differences in mean plasma PN concentration. During T1 mean PN concentration was 6.9 ± 3, 7.8 ± 6, 8.2 ± 6, 6.8 ± 6 nmol/L and during T2 7.0 ± 5, 6.2 ± 3, 6.0 ± 4.6, 6.8 ± 4 nmol/L respectively, for pre, mid, post, and P-60 measures.

There was no difference in the mean fasting plasma 4-PA concentration between T1 (35.2 ± 12.3 μmol/L) and T2 (32.7 ± 14.1 μmol/L). Compared to the pre value, during T1 the mean mid plasma 4-PA concentration was 116% ($p < 0.00$) higher and at post was 135% ($p < 0.2$) higher. However, by P-60 the mean plasma 4-PA concentration began to decline but was still 90% above

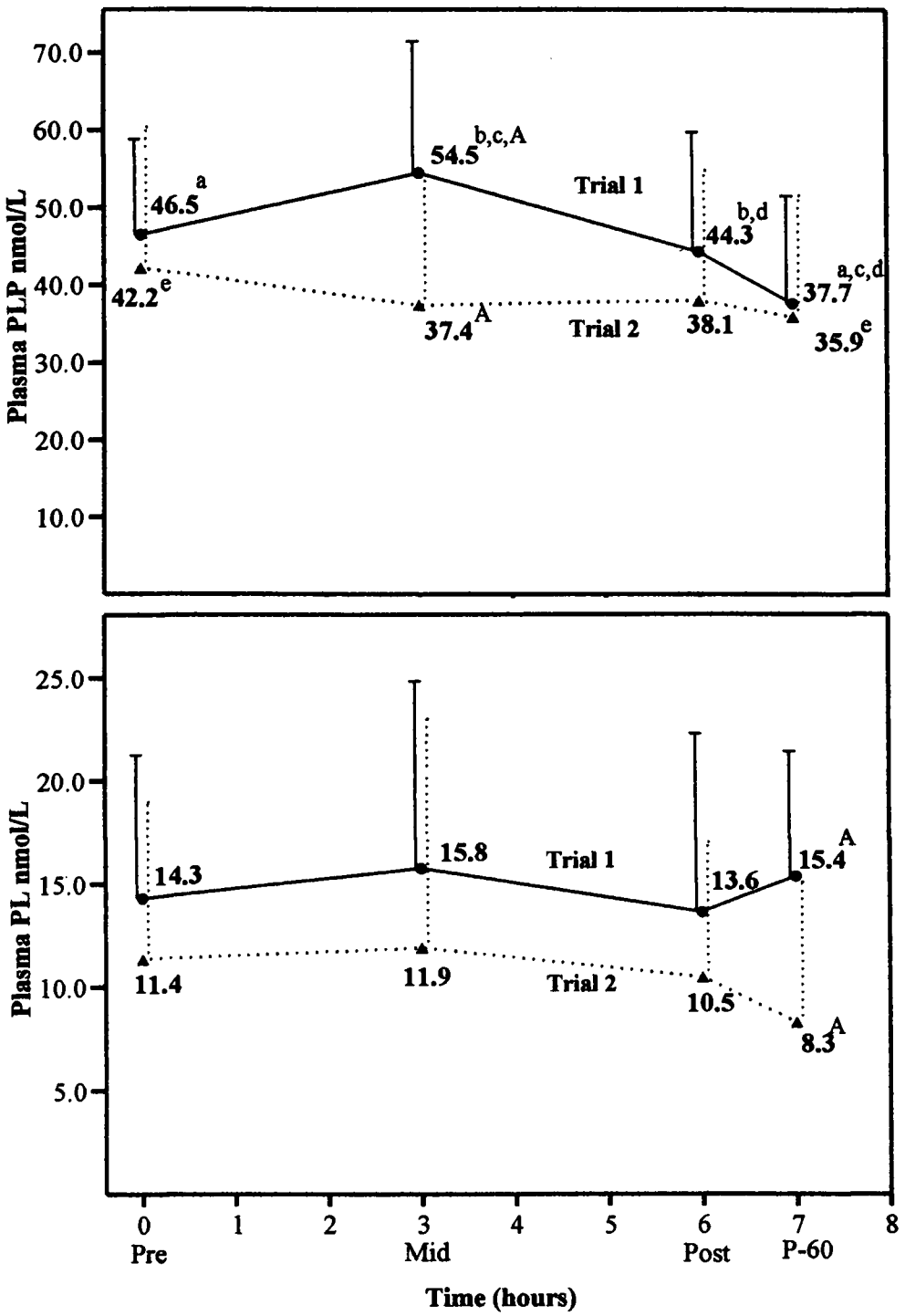

Figure 2.2. Mean plasma pyridoxal 5'-phosphate (PLP, top graph) concentration and mean plasma pyridoxal concentration (PL, bottom graph) at before (pre), during (mid), after (post), and 60 minutes after (P-60) an ultramarathon (Trial 1 = ——) and inactivity (Trial 2 = ····). Error bars indicate ± SD. Sampling time is rounded to the nearest hour. Like letters indicate a significant ($p < 0.05$) difference between two time points (uppercase for between trial, lowercase for within trial). For PLP, there was a significant univariate Time by Trial ($p = 0.00$) interaction that explains 40% of the observed variability. For PL, the Time by Trial interaction was not significant.

pre levels. During T2 there was no significant change in plasma 4-PA concentration (12% increase between pre and P-60). Figure 2.3 graphically depicts the change in mean plasma 4-PA concentration for T1 compared to T2.

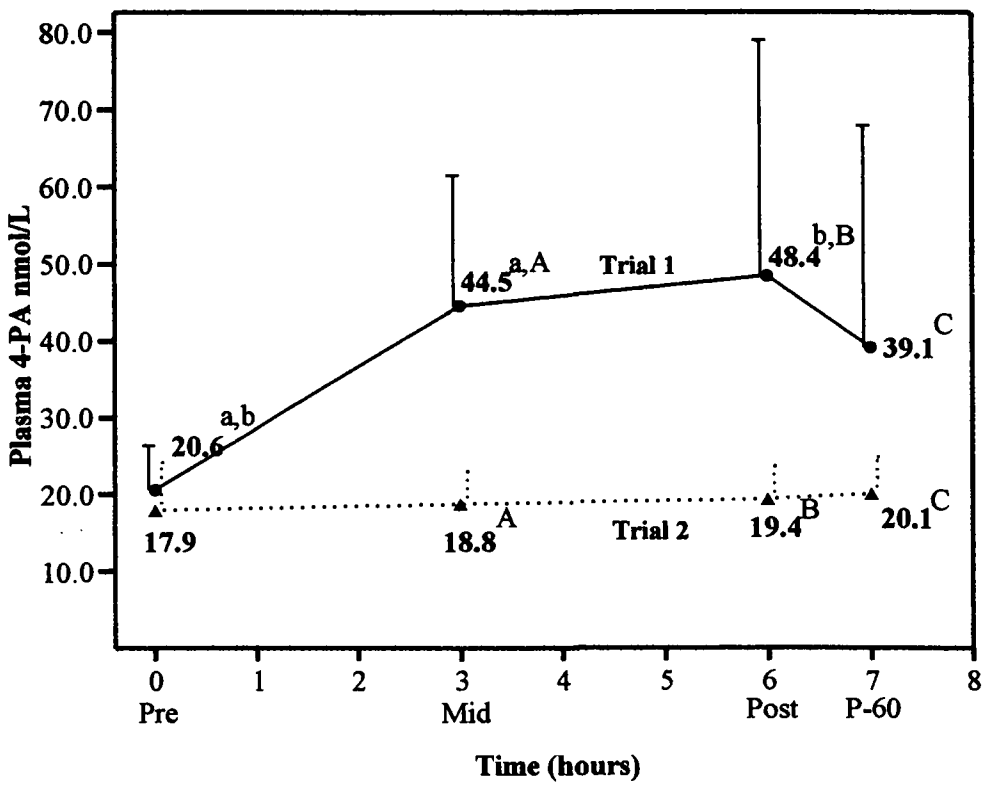

Figure 2.3. Mean plasma 4 pyridoxal (4-PA) concentration at before (pre), during (mid), after (post), and 60 minutes after (P-60) an ultramarathon (Trial 1 = ——) and inactivity (Trial 2 = ····). Error bars indicate ± SD. Sampling time is rounded to the nearest hour. Like letters indicate a significant ($p < 0.05$) difference between two time points (uppercase for between trial, lowercase for within trial). There was a significant univariate Time by Trial ($p = 0.006$) interaction that explains 36% of the observed variability.

Plasma Glucose, Lactate, Albumin, Alkaline Phosphatase and Creatinine

There were no differences between T1 andT2 in fasting mean plasma concentration of glucose (4.8 ± 0.4 vs 4.6 ± 0.3 mmol/L), lactate (1.2 ± 0.4 vs 1.2 ± 0.4 mmol/L), albumin (53 ± 4 vs 53 ± 4 g/L), creatinine (72.4 ± 17 vs 72.5 ± 16 μmol/L), or alkaline phosphatase activity (19.6 ± 5 vs 19.3 ± 5 U/L). The data for the pre, mid, post, and P-60 concentration of these variables is summarized in Table 2.5. During T1 there was no significant change in mean plasma glucose concentrations, although mid, post, and P-60 concentrations were slightly higher than pre concentration. During T2 the mean plasma glucose concentration significantly increased (27%) pre to post but returned to pre levels by P-60. During T1, from pre to post the mean plasma lactate concentration increased 55% ($p < 0.01$) and was significantly different from T2 at pre, mid, post and P-60 by 0.2, 0.8, 1.4, and 1.3 mmol/L, respectively. The mean plasma albumin concentration increased significantly ($p < 0.006$) between pre and post measures for both T1 and T2. For T1, this increase continued through P-60 at which point the concentration was significantly higher than at P-60 during T2. During T1, between pre and P-60 the mean plasma ALP activity increased significantly (13%, $p < 0.00$) but during T2 the increase was not significant (4%, $p > 0.05$). Between T1 and T2 the mean activity of ALP was significantly different ($p < 0.01$) at pre (0.8 U/L difference), mid (1.2 U/L difference), post (2.3 U/L difference) and P-60 (2.6 U/L difference) measures. During T1 there was a significant ($p < 0.01$) 43% increase in mean plasma creatinine from pre to P-60, and for T2 there was no significant change. The between trial differences in mean plasma creatinine concentrations were significant ($p < 0.00$) at mid (12.6 ± 7.4 μmol/L), post (25.6 ± 16.2 μmol/L), and P-60 (33.0 ± 12.0 μmol/L).

Table 2.5. Mean (± SD) plasma glucose, lactate, albumin, and alkaline phosphatase (ALP) concentrations before (pre), during (mid), after (post) and one hour after (P-60) an ultramarathon (T1) and rest (T2).

		Pre	Mid	Post	P-60
Glucose*	T1	4.6 ± 0.9	5.3 ± 1.1	5.0 ± 0.8^{1}	5.1 ± 1.1^{2}
(mmol/L)	T2	4.1 ± 0.8^{a}	5.0 ± 1.2	$5.2 \pm 0.9^{a,1}$	4.1 ± 0.7^{2}
Lactate*	T1	$2.1 \pm 0.5^{A,1}$	2.9 ± 0.8^{2}	$3.3 \pm 1.0^{A,3}$	2.9 ± 1.0^{4}
(mmol/L)	T2	1.9 ± 0.6^{1}	2.0 ± 0.9^{2}	1.9 ± 0.6^{3}	1.6 ± 0.5^{4}
Albumin*	T1	$52 \pm 4^{A,B,C}$	$54 \pm 5^{A,D,E}$	$58 \pm 5^{B,D}$	$59 \pm 6^{C,E,1}$
(g/L)	T2	$51 \pm 4^{a,b,c}$	$54 \pm 4^{a,d}$	$56 \pm 5^{b,d}$	$55 \pm 5^{c,1}$
ALP*	T1	$19.2 \pm 5^{A,B,C,1}$	$20.0 \pm 5^{A,D,E,2}$	$21.4 \pm 5^{B,D,3}$	$21.7 \pm 5^{C,E,4}$
(U/L)	T2	18.4 ± 4^{1}	18.8 ± 4^{2}	19.2 ± 5^{3}	19.1 ± 5^{4}
Creatinine	T1	$72 \pm 17^{A,B,C}$	$82 \pm 20^{A,D,E,1}$	$98 \pm 22^{B,D,2}$	$103 \pm 24^{C,E,3}$
(μmol/L)	T2	72 ± 16	69 ± 17^{1}	72 ± 20^{2}	70 ± 17^{3}

^{A,B,C,D,E,F,a,b,c,d} Like letters (upper case for T1, lower case for T2) represent a significant ($p < 0.05$) *within trial* difference between 2 time points.

^{1,2,3,4} Like numbers represent a significant ($p < 0.05$) *between trial* difference.

* Significant univariate Time by Trial interaction that explains 16% of the observed variability for glucose, 32% for lactate, 30% for albumin, and 57% for alkaline phosphatase activity.

Urinary Creatinine and Urinary 4-PA.

There was no significant between or within trial difference in mean daily urinary creatinine excretion (Table 2.6). The grand mean for T1 was 1.67 ± 0.48 g/day (range of means 1.61 to 1.80 g/day) and 1.72 ± 0.48 T2 (range of means 1.62 to 1.82 g/day) for T2. Mean creatinine excretion the day of and the day after the ultramarathon was 1.80 ± 0.54 and 1.65 ± 0.43 g/day, respectively, and was not significantly different compared to the same days during T2 (1.82 ± 0.50 and 1.73 ± 0.43 g/day).

For both T1 and T2, urinary 4-PA excretion was significantly correlated with fasting plasma PLP concentration (T1: $r = 0.549$, $p = 0.042$; T2: $r = 0.807$, $p = 0.000$) and fasting plasma 4-PA concentration (T1: $r = 0.905$, $p = 0.000$ and T2: $r = 0.821$, $p = 0.000$). On day one of T1, the mean urinary 4-PA excretion was 2 μmol/day higher ($p < 0.02$) than that for the same day during T2.

Table 2.6. Mean (± SD) urinary creatinine excretion and 4-PA to creatinine ratio, for days one through five, during Trial 1 and Trial 2.

		Day 1	Day 2	Day 3	Day 4	Day 5
Creatinine (g/24 hr)	T1	1.6 ± 0.5	1.6 ± 0.5	1.7 ± 0.4	1.8 ± 0.5	1.7 ± 0.4
	T2	1.7 ± 0.5	1.6 ± 0.5	1.8 ± 0.5	1.8 ± 0.5	1.7 ± 0.4
4-PA per g creatinine	T1	6.7 ± 2.9^{A}	$5.3 \pm 1.2^{B,1}$	4.9 ± 1.0	$4.4 \pm 0.7^{A,B,2}$	5.0 ± 0.9^{3}
	T2	$5.1 \pm 1.5^{a,b}$	$4.5 \pm 1.1^{a,c,1}$	4.4 ± 1.0^{d}	$4.0 \pm 0.9^{b,c,d,2}$	3.9 ± 0.8^{3}

^{A,B,a,b,c,d} Like letters (upper case for T1, lower case for T2) represent a significant ($p < 0.05$) *within trial* difference between 2 time points.
^{1,2,3} Like numbers represent a significant ($p < 0.05$) *between trial* difference.

This difference is not surprising because the subjects had been consuming their regular diets and 4-PA excretion reflects intake of the past 24–48 hours. As expected as subjects adjusted to the controlled diet, by day three there was a non-significant 0.6 μmol/day between trial difference in 4-PA exertion. Thus, because of the time to adapt to a specific intake of vitamin B-6, assessment of 4-PA excretion is limited to comparing days 3, 4 and 5 of each trial. Figure 2.4 graphically depicts daily mean urinary 4-PA excretion on these days during T1 and T2. During T2 the progressive significant decline in daily excretion of 4-PA cannot be explained by differences in excretion between supplement users and non supplement users or by dietary adaptation, which is normally complete in two to three days. However, expressing urinary excretion as μmol 4-PA per mg of creatinine, the mean amount excreted on days four (4.4 ± 0.7) and five (5.0 ± 0.9) of T1, were significantly ($p < 0.04$) greater than that for days four (4.0 ± 0.9) and five (3.9 ± 0.8) of T2. This trend is nearly identical to evaluation of urinary 4-PA alone, which indicates observed differences are not due to incomplete urine collections. In contrast, expressing 4-PA excretion as a percent of dietary vitamin B-6 intake revealed that excretion the day of the ultramarathon was greater ($p > 0.05$) than either the day before or they day after (Figure 2.4).

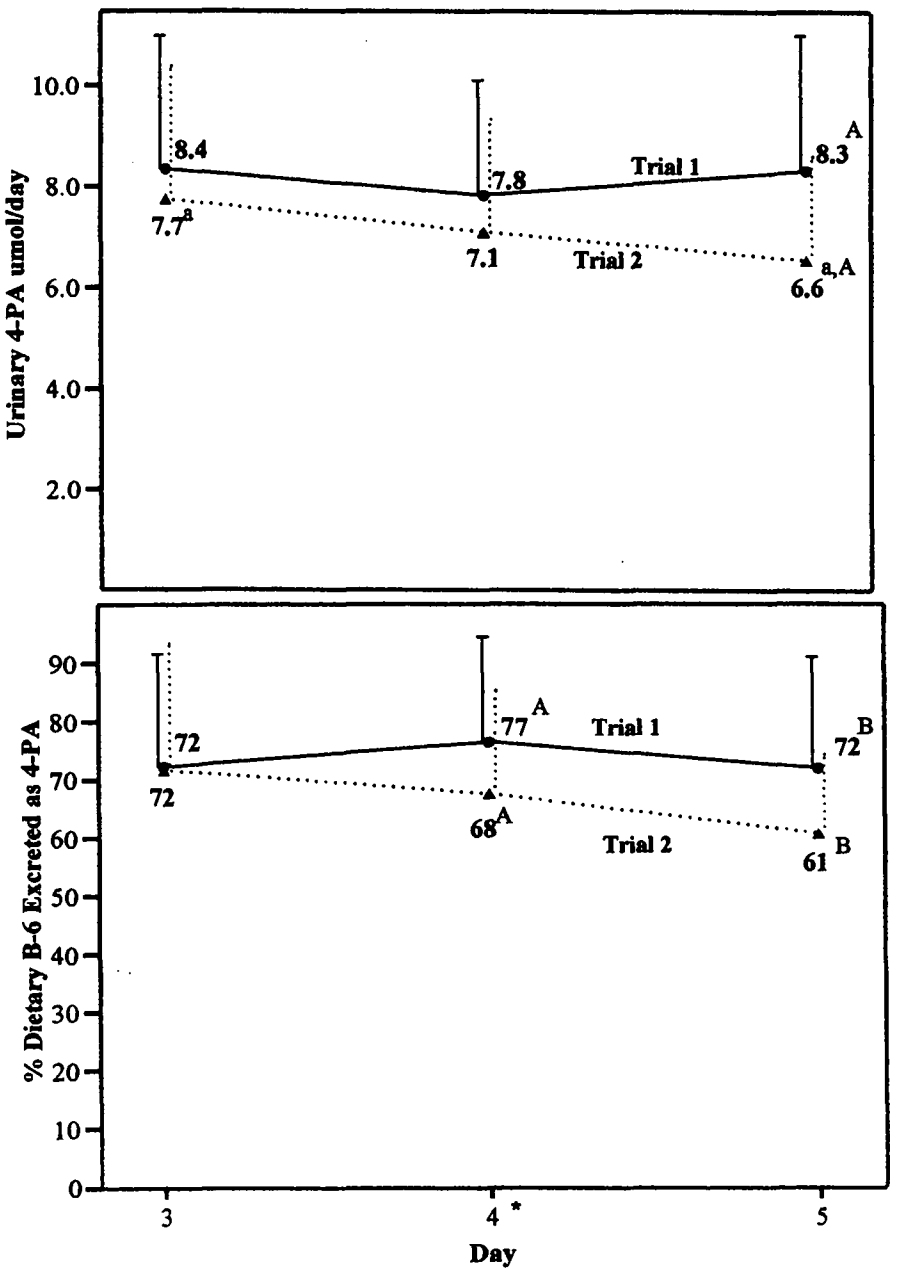

Figure 2.4. Mean urinary 4-pyridoxic acid (4-PA, top graph) excretion and the mean percent of total dietary vitamin B-6 intake excreted as urinary 4-PA (bottom graph) for Trial 1 (—) and Trial 2 (·····). Due to the time required to adapt to the controlled diet only data from days 3, 4 and 5 is reported. Error bars indicate ± SD. Like letters indicate a significant ($p < 0.05$) difference between two time points (upper case for between trial, lower case for within trial). During Trial 1 an ultramarathon was run on day 4, during Trial 2 subjects were inactive on day 4. For urinary 4-PA excretion, there was a significant univariate Day by Trial ($p = 0.021$) interaction that explains 26+% of the observed variability. For the percent of dietary vitamin B-6 excreted in the urine as 4-PA, there was a significant univariate Day by Trial ($p = 0.037$) interaction that explains 32% of the observed variability.

Plasma Urea Nitrogen

The mean fasting (6.2 ± 1.5 vs 6.4 ± 1.4 mmol/L) and mean pre concentrations of plasma urea nitrogen (PUN) were not different between T1 and T2. However, during T1 there was a significant ($p < 0.01$) increase in the mean PUN concentration from pre to mid (0.8 ± 0.2 mmol/L), pre to post (3.2 ± 0.5 mmol/L), and pre to P-60 (3.9 ± 0.4 mmol/L). The mean percent increase during T1 was 14.7% pre to mid, 36.9% mid to post, and 8.1% post to P-60. During T2 the mean PUN concentration decreased significantly ($p < 0.02$) from pre to post (-0.6 ± 0.2 mmol/L) and pre to P-60 (-0.9 ± 0.2 mmol/L) measures. Overall, the mean PUN concentration increased 70.5% pre to P-60 during T1, compared to a decrease of 15.9% pre to P-60 during T2.

Discussion

This study indicates that during extreme exercise there are at least two distinct phases of vitamin B-6 metabolism. During the first phase (2–3 hours) plasma PLP and 4-PA concentrations both increase. However, during the second phase plasma 4-PA concentration plateaus, while plasma PLP concentration decreases and eventually drops below pre exercise levels. Moreover, it appears that around the time that plasma PLP concentration begins to decline the rate of urea production increases suggesting that the decrease may be associated with a change in amino acid metabolism.

The only other study to show a decrease in plasma PLP following exercise greater than 6 hours was that of Leonard and Leklem (2000). This study was designed to validate and expand on their findings. The ultramarathon course was the same, the subject characteristics were similar (same ratio male to female, average age 43.5 vs. 43.7 years), the race day foods were similar, and the same analytical techniques were used. The difference is that in the present study subjects consumed a controlled diet, urine was collected, a mid race blood draw was obtained, and a control trial was completed. Our findings confirm the findings of Leonard and Leklem (2000), and the findings of shorter duration studies of the impact of exercise on plasma

PLP concentration. In the present study, by mid-point the subjects had exercised an average of 166 minutes (range 125 to 225) and had a mean increase in plasma PLP concentration of 18% (range -22 to 65) which is within the range (10–35% increase after 20–180 minutes) reported by others (Leklem and Shultz 1983; Hoffman et al., 1991; Manore et al., 1987). However, this study did not find the same magnitude of decrease that Leonard and Leklem did. In their study they reported a 31% post and 44% P-60 decrease in plasma PLP concentration, while we found a 4% and 19% decrease, respectively (5% and 24% respectively, if data is not corrected for plasma volume change). Part of the difference may be that during the Leonard and Leklem study their subjects consumed an average of 0.16 mg (0.9 μmol) vitamin B-6 during the race while ours consumed 0.25 mg (1.5 μmol) which added an additional 0.6 μmol of B-6 into circulation. The biggest difference between our study and theirs was the change in plasma 4-PA. The mean pre-race 4-PA concentrations were similar (20.6 vs 25.5 nmol/L) but we found a 135% pre to post-race increase while Leonard and Leklem (2000) reported only a 21% increase. There is no obvious explanation for this finding but it may provide a clue as to the fate of plasma PLP. One explanation for a decrease in plasma PLP is that it is converted to 4-PA and is excreted from the body. If this were the case, a smaller decrease in PLP concentration should be associated with a reciprocally smaller increase in plasma 4-PA concentration, while a greater decrease in plasma PLP would be associated with a greater increase in plasma 4-PA. However, this did not occur, and it seems likely that at least part of the decline in plasma PLP concentration is due to tissue redistribution rather than only conversion to 4-PA.

It is well established that PLP concentration increases during exercise but where it comes from and its fate continue to be debated. The recent work of Hadj-Saad et al (1995) supports the theory that it is redistributed to tissues with greater need. They found a sharp increase in plasma PLP and 4-PA concentration after one hour (55% and 283%, respectively) and 2.5 hours (130% and 390%, respectively) of exercise, suggesting that vitamin B-6

metabolism occurs in phases linked to duration. Additionally, they found that the muscle content (nmol/organ) of PLP and PMP *increased* by 40–50%, and the liver B-6 vitamer content decreased 5% ($p > 0.05$). Although glycogen stores were not measured, other rat studies have demonstrated a decrease in muscle glycogen during exercise (Furler et al, 1998). Combined, the studies indicate that during exercise muscle PLP content may increase while glycogen stores decrease and the source the PLP is other than the liver. To date there has been no similar study on humans, and it is not known if during exercise the muscle concentration of PLP increases, decreases, or does not change.

It is plausible that the muscle content of PLP might increase during exercise of both short and long duration. To break down glycogen, glycogen phosphorylase requires PLP to be bound to each of its two subunits (Krebs and Fisher, 1964). However, the binding affinity of PLP to the enzyme may lessen as exercise decreases cellular pH (Munoz et al., 1984). Thus, the uptake of additional PL, with subsequent conversion to PLP, may maximize glycogen phosphorylase activity by keeping it fully saturated with PLP. This theory would be consistent with Croziers finding that the majority of increase in PLP occurs in the first five minutes of exercise and the findings of Greenhaff et al (1988) who showed that three minutes of intense exercise decreases cellular pH from 7.4 to 7.2.

From the reported RPE of 14, and the significant increase in plasma lactate concentration, we estimated the average intensity of exercise may have been as high as 75% of VO_{2max} (Borg 1982, Brooks 1996c). At this intensity, after two to three hours of running glycogen stores would become depleted (Parkin et al., 1997), and there would be a greater dependence on amino acids as a gluconeogenic substrate and as a fuel for the muscle (Lemon and Mullin, 1980). In this study support for this assertion is found in the increase in plasma urea during the last half (36.9%) of the race compared to the first half (14.7%). Further evidence linking urea production to glucose availability is that during T2, when subjects were inactive and

consuming carbohydrate (mean 44 g/hr), there was a continual decline in mean PUN concentration. This amino acid sparing effect of exogenous glucose has been previously demonstrated by Millward et al. (1982). Additionally, the slight ($p > 0.05$) drop in plasma glucose between mid and post may indicate increased uptake by the muscles and a depletion of hepatic glycogen stores. These events would increase the PLP requirement of both the muscle and liver for transamination and decarboxylation reactions to support gluconeogenesis and amino acid oxidation to meet the energy requirements of exercise.

Several attempts have been made at identifying body pools of vitamin B-6. Initially, Johansson (1966) proposed a two-compartment model in which a large pool with slow turnover was in equilibrium with a small pool with rapid turnover. More recently Coburn (1990, 1996) used tracer studies and enzyme activities to model vitamin B-6 metabolism and found 75 pools to be insufficient. Human vitamin B-6 pools estimated through muscle biopsies (Coburn et al., 1988) indicate that the total body pool of vitamin B-6 is around 1000 μmol, of which 750–800 μmol is in the muscle, 50–100 μmol is in the liver, and 100–200 μmol is in other body tissues or pools. In the current study plasma PLP concentration averaged 45 nmol/L PLP and, assuming an average plasma volume of 3 L (Vander et al, 1990), totaled 135 nmol (0.135 μmol) or 0.0135% of total body vitamin B-6. To increase plasma PLP concentration by 18% would require mobilization of 0.081 μmol of PLP, which does not represent a major, or even noticeable, loss from either the muscle or liver. However, if the rate of turnover of this 0.081 μmol was only 10 minutes, then in 6 hours 2.9 μmol (0.7 mg) of PLP would be mobilized. This is highly speculative because the turnover rate of PLP during exercise has never been studied but does illustrate how a modest increase over time can become significant.

Once glycogen stores are depleted it is unclear what happens to the PLP bound to glycogen phosphorylase. The PLP could remain in the muscle to meet the increased requirement for amino acid metabolism or it could be released into the plasma and taken up by

the liver to support gluconeogenesis. The work by Hadj-Saad (1995) indicates it may remain in the muscle while the work of Black et al. (1978), who found that in rats B-6 vitamers were released from the muscle during periods of caloric deprivation, indicates it may be released. However, an important point is that PLP must be dephosphorylated to PL before release from a tissue, therefore, an increase in plasma PL concentration should precede an increase in plasma PLP concentration. In the present study, as in others (Hofman et al., 1991; Crozier et al., 1994), such an increase was not observed though it is possible that plasma PLP and PL concentration peaked and declined prior to our mid point measure. However, Crozier et al. (1994), who documented a significant 11% increase in plasma PLP concentration after five minutes of exercise, also saw no concurrent change in plasma PL concentration. The lack of change in plasma PL concentration could mean metabolism was unaffected by exercise or could mean hepatic uptake and conversion of PL to PLP was so efficient that no visible change occurred. However, during exercise hepatic circulation is decreased by as much as 50–60% (Felig and Wahren, 1971) making this explanation less likely. Consumption of a 1–2 mg PN hydrochloride supplement also causes an increase in plasma PLP concentration similar to that observed by mid point (Wozenski et al., 1980), an increase that is preceded by a significant increase in free B-6 vitamer concentration (Ubbink, 1987). This suggests that in the present study PLP is acquired from a reservoir, such as the liver or albumin, that does not require dephosphorylation and rephosphorylation prior to entering the blood stream. Lumeng et al. (1974) found the short term distribution of intravenously administered PLP was twice that accounted for in the plasma volume and speculated the plasma PLP was in equilibration with albumin bound PLP in an interstitial pool and/or bound to the walls of the vascular system. The liver represents a particularly rich reservoir of PLP and albumin because cytosolic PLP concentration is high and the interstitial protein content approaches that of plasma, (Landis and Pappenheimer, 1963). Thus, a rapid change in plasma PLP concentration without a change in

the precursors, PN or PL could logically be attributed to changes in plasma albumin concentration. However, in this study during T1 the change in PLP and albumin were not correlated and their ratio increased from 0.9 at pre, to 1 at mid, but then *dropped* to 0.8 at post and 0.6 by P-60. Exercise related variables that may explain this inconsistency include an increase in dephosphorylation by ALP (9 % increase in ALP mid to P-60), and a decreased affinity of PLP for albumin due to an increase in plasma 4-PA (Fonda et al., 1990) and a decrease in plasma pH (Wilson et al., 1983; Greenhaff et al., 1988). Additionally, free fatty acids, which are also transported bound to albumin (Brodersen et al., 1990), may increase over 600% following four hours of exercise (Maron et al., 1977) and may displace PLP from its binding sites on albumin. Another factor that has not been studied, but holds promise, is the hormonal milieu that occurs during exercise. Recently Mahuren et al. (1999) examined the interaction of adrenocorticotropic hormone (ACTH) and vitamin B-6 metabolism in pigs and found that in the adrenal glands of ACTH treated pigs pyridoxal 5'-phosphatse activity increased from 67 to 764 nmol/g/min) and increased the dephosphorylation of PMP. Thus, hormones may partially govern B-6 vitamer metabolism and, in humans, during exercise ACTH increases (Wittert et al., 1996). In summary, the increase in plasma PLP concentration (11%) observed by mid-point may be attributable to an initial influx of albumin (6%), and the mid to post decrease a result of duration related plasma variables (ALP, 4-PA, pH, fatty acids, hormones) that may stimulate tissue uptake and/or impinge on the ability of PLP to bind to albumin.

The cause of the initial albumin and PLP flux may be related to fluid shifts that are a characteristic of exercise. The initial net flux of fluid from the plasma into the active tissue caused by exercise is followed by a replacement of plasma volume with fluids from interstitial and intracellular stores. Sweat rate also affects fluid distribution. Costill et al (1976) determined the fluid for a 1.5 L sweat loss came from the plasma (10%), interstitial space (60%), and intracellular space (30%). Thus, because the amount of PLP needed to cause a 20% increase or

decrease is so small (0.081 μmol) it is easy to imagine the change could be caused by fluid shifts between the plasma and interstitial space.

One of the biggest inconsistencies in the literature is the effect of exercise on 4-PA formation and excretion. A significant increase in urinary 4-PA may have physiological implications that would translate into an increased vitamin B-6 requirement for athletes. Most human and rat studies show that exercise causes an increase in plasma 4-PA (Crozier et al., 1994; Hadj-Saad et al., 1995; Leonard and Leklem, 2000). However, it is unclear if the increase represents a significant increase in the conversion of PL to 4-PA. The total change in urinary 4-PA excretion reported ranges from 0.4 to 3.6 μmol/day and may or may not be found to be significant. Manore et al. (1987) reported that for trained women who cycled 20 min at 80%VO_{2max} a 0.5 μmol/day increase in excretion was significant, while Dunton (1994) determined that for trained men who cycled to exhaustion at 75%, a 0.4 μmol/day increase in excretion was not significant, and Drenon and Butterfield (1986) reported that for trained men who ran 5 or 10 miles per day a 2.3 to 3.6 μmol/day decrease in excretion was significant.

In the present study, interpretation of the urinary 4-PA data is complicated by a successive decrease in 4-PA excretion during T2 (Fig 2.5) and the uncertainty of whether or not the ultramarathon masked the same pattern during T1. One explanation for a gradual decline in urinary 4-PA excretion is that for the 5 individuals who regularly consumed supplements in excess of the RDA, the 9-day pre-trial washout period was insufficient to achieve a new steady state in plasma PLP concentration and urinary 4-PA excretion. During T1 supplement users had fasting plasma PLP concentrations 12 μmol/L higher and excreted 3.2 μmol/day more 4-PA than non-supplement users but by T2 the differences were 4 μmol/L and 2.0 μmol/day, respectively. This agrees well with research by Lui et al (1985) who found that following 28 days of supplementation (25 mg by I.V.) the greatest decline in plasma PLP and urinary 4-PA excretion occurred within 5 days but a notable decrease continued for greater than 20 days.

In this study, because 4-PA excretion the day of the ultramarathon was 0.4 to 0.5 μmol/day less ($p > 0.05$) than the day before or the day after, it appears that exercise may have decreased urinary 4-PA excretion. However, for reasons related to renal function, dietary intake, and possible 4-PA excretion via sweat, this conclusion is probably inaccurate. A common effect of extreme exercise is a decrease in renal blood flow (Suzuki et al., 1996) that may result in delayed clearance of substances such as creatinine, urea, and 4-PA. In the present study, following the ultramarathon (day 5), 4-PA excretion was 0.5 μmol/day higher ($p > 0.05$) than the day of the ultramarathon and 1.7 μmol/day higher than day 5 during T2. However, because we did not draw blood the day after the ultra we do not know how long it took to excrete excess plasma 4-PA. Another factor that may have affected urinary 4-PA excretion is dietary intake. Although the difference was statistically insignificant, the menu the day of the ultra provided 1.3 μmol less vitamin B-6 than the day before or after. Therefore, urinary 4-PA excretion on this day should be 0.8–0.9 μmol less based on average excretion of approximately 60–70% of dietary intake (Table 2.x). In relative terms, the day of the ultra more 4-PA was excreted in the urine (77% of intake) than the day prior (72%). The last factor confounding evaluation of 4-PA formation and excretion is that sweat losses of 4-PA were not accounted for. Historically, sweat losses are assumed to be negligible but as event duration increases the total amount of 4-PA lost through this route may become substantial. In the current study the average sweat loss, estimated from fluid intake (3.8 L), weight loss (1.5 kg) and estimated respiratory loss (600 mL) (Mitchell et al., 1972), totaled 4.7 L and represents more than a complete turnover in plasma volume. To date, only one study has determined 4-PA losses through sweat. Johnson et al. (1945) completed whole body sweat collections on 4 unsupplemented male subjects resting at 100°F with 70% humidity and found in 8-hours 0.198 mg (1.2 μmol) of 4-PA was lost. Thus, during exercise when plasma 4-PA concentration is doubled it is conceivable that in a 6 hour period over 2 μmol of 4-PA could be excreted through sweat. In the current study this would

make the total 4-PA excretion the day of the ultramarathon 1.4 μmol (0.23 mg) greater than the day before or the day after. This same rationale may partially explain the results of Dreon and Butterfield who found that subjects running 5–10 miles/day excreted 2.3–3.6 μmol/day less 4-PA than the sedentary controls.

Limitations of this study include lack of data on sweat 4-PA losses, lack of direct assessment of muscle and liver tissue, and the frequency of blood sampling. Future studies should include direct evaluation of sweat for 4-PA and total vitamin B-6 loss. Ideally, direct sampling of target tissues would be used to determine vitamin B-6 metabolism during exercise. However, this technique is often impractical therefore, the alternative use of labeled vitamin B-6 would facilitate determining the source and destination of PLP mobilized during exercise. Additionally, a study involving more frequent measures of plasma PLP and/or fluid shifts would provide additional insight into when and how plasma PLP concentration peaked.

In conclusion, during six hours of running, there are at least two phases of altered vitamin B-6 metabolism. Phase one is characterized by an increase in both plasma PLP and 4-PA concentrations, while phase two is characterized by a decrease in plasma PLP concentration and a plateau in plasma 4-PA concentration. During phase one, the source of increased PLP is unknown. However, because plasma PL, the PLP precursor, concentration does not change, it is likely the source of PLP is from interstitial spaces and mobilization is the result of exercise induced fluid shifts. Additionally, the overall increase in 4-PA indicates hepatic exposure to, and/or uptake of, PLP is elevated and may increase the vitamin B-6 requirement of endurance athletes.

THE EFFECT OF A 50-KM ULTRAMARATHON ON PLASMA UREA NITROGEN CONCENTRATION AND URINARY NITROGEN EXCRETION

Abstract

Purpose: To examine the effect of a 50-km ultramarathon on plasma urea nitrogen concentration and urinary nitrogen excretion. **Methods**: Nine men and five women (44 ± 11 years) completed two five-day trials separated by three weeks. During both trials the subjects consumed a controlled diet providing men 114 g protein and 3000 kcal, and women 88 g protein and 2500 kcal. During Trial 1 (T1) on day four an ultramarathon was run and during Trial 2 (T2) on day four the subjects were inactive. With the exception of day four, the activity during T1 was replicated during T2. Twenty-four hour urine collections were completed for each day of each trial and blood was drawn pre- race (pre), mid-race (mid), post-race (post) and 60 minutes post race (P-60). On the inactive day of T2 blood was drawn at the same intervals. During the ultramarathon all food/beverage selections were recorded and on the inactive day subjects consumed the same foods at the same intervals. Plasma was analyzed for urea nitrogen (PUN), creatinine (Cr), glucose, and lactate concentration; urine was analyzed for total urinary nitrogen (TUN), urea nitrogen (UUN), and creatinine; and the diet was analyzed for total nitrogen. **Results**: During T1, from pre to mid the mean PUN concentration increased 14.7%, and from mid to post it increased 36.9%. During T2, mean PUN concentration decreased 17% pre to P60. During T1 the mean rate of PUN concentration increase from pre to mid, mid to post, and post to P60 was 0.5, 1.75, and 2 mg/100 mL/hour, respectively. The ratio of the mean PUN to the mean plasma Cr concentration (μmol/L) did not change between pre (22.3:1) and mid (22.6:1) but increased to 26.2:1 by post and 26.5:1 by P-60. During both trials on days 3, 4, and 5 there was a significant gender effect for the absolute amount of TUN and UUN excreted (mean 16.4 ± 2.3 vs 12.2 ± 1.4, $p = 0.003$ and 13.4 ± 0.5 vs 10.0 ± 0.5, $p = 0.004$ for males and

females respectively). However, gender did not significantly affect the relative excretion of nitrogen. During T1 on days 3, 4, and 5, 88%, 100%, and 95% of the nitrogen intake was excreted in the urine compared to 86%, 83%, and 84% for the same days during T2. The day of the ultramarathon 24-hour TUN was 2 g higher than the day before and 2.8 g higher than the same day during T2. **Conclusion**: During moderate to intense exercise the rate of change in PUN increases as duration increases and when duration exceeds six hours there is a significant increase in urinary urea nitrogen and total urinary nitrogen excretion.

Introduction

During the 19th century protein was thought to be the major exercise fuel, then in the 20th century it was discovered that carbohydrates and fats were the primary fuels utilized and protein was assigned an insignificant role. Currently, the contribution of amino acids to energy expenditure during exercise is estimated to be between 5% and 15% (Paul, 1989; Dohm, 1983). The increased metabolism of amino acids during exercise is well documented and the quantity is often greater than that available in the body's free amino acid pool. (Felig and Wahren, 1971). For this to occur the amino acids pool must be augmented by tissue protein breakdown and/or a decrease in the rate of protein synthesis (Dohm et al 1985). The net effect of exercise on nitrogen balance is controversial. During exercise, protein synthesis can decrease 30–70% (Dohm et al, 1985, 1987) and partially offsets the increase in amino acid catabolism. However, to maintain positive nitrogen balance most research indicates endurance athletes require 1.2 to 1.4 g/kg of protein (1.5 times the current RDA) (Tarnopolsky et al, 1988; Lemon, 1991).

Urea, the end product of protein catabolism, is formed in the liver and released into circulation for excretion by the kidney. Consequently, urea production and excretion is used to assess amino acid degradation. Haralambie and Berg (1976) provided evidence linking the exercise-induced increase in serum urea nitrogen to increased amino acid catabolism. Their study of athletes participating in 70 to 765 minutes of activity revealed a significant correlation

between the decrease in serum amino acid nitrogen concentration and the increase in serum urea nitrogen concentration. Furthermore, the magnitude of the changes correlated with the duration of exercise. Since then numerous studies have reported an increase in urinary nitrogen excretion following exercise (Dohm et al 1982; Calles-Escandon et al,1984; Decombaz, et al 1979). To examine the possibility that urea production occurred during recovery not exercise, Millward et al (1982) found that during 3.75 hours of treadmill exercise, there was a 71 mg/kg (equal to 5 g for a 70 kg subject) increase in urea production but excretion was delayed due to altered kidney function. Altered renal function has been reported by others (Rennie, 1981a) and appears to be negatively correlated with exercise intensity and temperature, both of which increase the amount of cardiac output shunted away from the viscera and to peripheral tissues (Kanstrup, 1999). In contrast, in a frequently cited study, Carraro et al (1993) reported that during 3 hours of exercise at 40% VO_{2max} or one hour at 70% VO_{2max}, there was no change in the rate of new or total urea formation during exercise. An additional finding was that recycling of urea nitrogen increased while overall excretion decreased, leading to the conclusion that 50% of urea nitrogen was recycled back into the body, presumable into protein. Similarly, using labeled isotopes, Wolfe et al (1984) found that despite an increase in alanine metabolism during exercise, there was no increase in the rate of total or labeled urea production.

Comparing the results of different studies is confounded by factors that impact urea production. Research suggests that urea production is positively correlated with exercise intensity (Millward et al, 1982; Lemon et al, 1984), exercise duration (Haralambie and Berg, 1976), and fitness (Layman et al, 1994); negatively correlated with glycogen stores and carbohydrate intake (Lemon and Mullin, 1980); and is higher for males than for females (Tarnopolsky et al, 1990). A particular area of interest, given the recent popularity of ultraendurance events, is the impact of exercise duration on urea production. There have been few urea studies for events longer than a marathon, and those that have been completed assessed

the overall effect (pre to post changes) and did not collect serial blood samples during the event to determine if the rate of change is constant or variable. Irving et al (1990) found an increase in plasma urea and the rate of urea production following a 56km race. Decombaz, et al, (1979) observed similar findings following a 100 km run.

The purpose of this investigation was to determine if the rate that PUN concentration increases is constant throughout the ultramarathon or if production increases as duration increases. Additionally, the overall effect on total urinary nitrogen excretion and urea nitrogen concentration was assessed to determine possible gender differences. To accomplish these goals, in addition to pre and post blood samples, a mid race sample was collected and food consumption during the race was limited to foods without significant protein content. This research was completed as a component of another study investigating the impact of an ultramarathon on vitamin B-6 metabolism (Grediagin, 2000).

Methods

Subjects

Fourteen healthy, 25–57 year-old male and female subjects were recruited from local area residents interested in completing an ultramarathon. Recruitment included mailing information to registered runners and posting flyers around Oregon State University (OSU). Twelve of the selected participants had already successfully completed an ultramarathon and all subjects were healthy (no metabolic disorders), physically fit, non-smoking, experienced runners who trained an average of 7.5 ± 2.9 hours per week. The OSU Human Subjects Committee approved the study, and written informed consent as well as a medical history was obtained from each subject. Physical characteristics of the subjects are in presented Table 3.1.

Table 3.1. Mean (± SD) physical characteristics of subjects

	Age (years)	Weight (kg)	Height (cm)
Male (n = 9)	43.5 ± 11	77.4 ± 8.5	179 ± 7
Female (n = 5)	40.6 ± 10	57.5 ± 3.5	161 ± 4

Experimental Protocol

All subjects participated in two five-day trials: one requiring a 50-km ultramarathon on day four (T1), and one requiring inactivity on day four (T2). Subjects agreed to keep a 24 hour activity log and stop the use of all supplements (vitamin, mineral, and herbal) nine days prior to each trial (five subjects reported habitual supplement usage). During each five-day trial subjects were weighed daily, consumed a controlled diet, completed 5 blood draws, collected 24-hour urines, and recorded their activity (Figure 3.1). Plasma was analyzed for urea nitrogen (PUN), glucose (Glu), and lactate (Lac) concentration; blood was analyzed for hemoglobin (Hb) and hematocrit (hct); and urine was analyzed for creatinine, urea nitrogen (UUN), and total nitrogen (TUN) excretion.

Trial 1

Subjects were instructed to consume their breakfast at 5 a.m. and report for the pre-race (pre) blood draw and weigh-in at 7:30 a.m.. Breakfast consisted of a bagel, jelly, and orange juice and provided approximately 500 kcal and 10g protein. The 50-km ultramarathon trail run began at 8 am and included a total elevation change of 3,902 meters. During the race six aid stations provided subjects with urine bottles as well as unlimited quantities of specific foods and beverages (see diet section for more detail). At each aid station an attendant recorded the time the subject arrived and the quantity of all foods and beverages consumed. The mid-point (mid) and post race (post) blood draws were accomplished within three minutes of subject arrival. Between the post-race blood draw and the post 60 minute (P-60) draw subjects consumed 500 ml of water and nothing more. Post-race weight was recorded prior to the water consumption.

Following their P-60 blood draw subjects were provided their normal lunch and reported to the nutrition lab at the standard time for dinner. Adjustments were made to dinner selections to account for the total amount of protein consumed during the run.

Trial 1	←No Vitamin or Mineral or Herbal Supplements→ ←Record the Intensity and Duration of all Activities (24 hour log)→					
	Normal Diet	***Trial 1, Controlled Diet*** ***24 Hour Urine Collections; Daily Weights***				
	9 Day Pre-Trial Period	Day 1 Blood Draw: Fasting	Day 2	Day 3	Day 4 Ultramarathon Blood Draws: Pre, Mid, Post, P60	Day 5
	//Trials Separated by 22 Days//					
Trial 2	←No Vitamin or Mineral or Herbal Supplements→ ←Replicate Daily Activity Recorded for Trial 1→					
	Normal Diet	***Trial 2, Controlled Diet*** ***24 Hour Urine Collections; Daily Weights***				
	9 Day Pre-Trial Period	Day 1 Blood Draw: *Fasting*	Day 2	Day 3	Day 4 **No Exercise** Blood Draws: Four at Trial 1, day 4 intervals	Day 5

Figure 3.1. Timeline and major activities during Trial 1 and Trial 2. Twenty-two days elapsed between day five of Trial 1, and day one of Trial 2 (inclusive of the Trail 2 nine-day pre-trial period).

Trial 2

T2 was the mirror image of T1 with the exception of the ultramarathon: the subjects duplicated the intensity and duration of activity recorded during T1 and followed the same diet, urine, and blood draw procedures. The difference was that on day 4 of the controlled diet, instead of running an ultramarathon the participants were "inactive" for a time equal to the duration it took them to complete the race. During this "inactive" time subjects were allowed to

continue non-vigorous daily activities including desk work and walking to and from the nutrition lab where they had blood drawn at intervals established according to their ultramarathon blood draw times. Between blood draws, subjects consumed the same quantity of food and drink that they had during the ultramarathon.

Blood collections

Nineteen milliliters of blood was drawn from the anticubital vein into heparinized tubes five times per trial. The time of each blood draw was recorded and all draws were made 60 to 90 seconds after the subjects assumed a seated position. The first draw was a fasting sample (no food or drink after 8 p.m. the night before) taken 3 days prior to the race. The remaining four blood draws were completed 30–60 minutes prior to the ultramarathon (pre), during the race at mile 15 (mid), immediately after race completion (post), and 60 minutes following the race (P-60). Pre, post, and P-60 race day blood draws were completed in a structure at the race start, and during the race blood was drawn in the open at a site mid way through the course. In all cases blood was immediately placed on ice, covered, and delivered to the OSU Nutrition Lab (15-minute drive) at 30-minute intervals. The technicians at the lab recorded the time each sample arrived, determined the Hct and Hb concentration, then centrifuged the blood and extracted plasma aliquots which were frozen at - 40° C. To duplicate the travel and delivery time that occurred in T1, during T2, blood was held on ice 30 to 40 minutes before processing.

Diet

During T1 and T2 all foods consumed by the subjects were prepared in the metabolic kitchen at OSU. Breakfast and lunch meals were consumed at home or work and the dinner meal was consumed on site. At the dinner meal each day the subjects completed a daily record verifying food and beverage consumption for that day. The diet, consisting of two alternating menus, was the same for both trials. The percent of calories provided by carbohydrate was

55–65%, by protein 15–20%, and by fat 15–30%. The range of intake was a result of allowing subjects to consume simple carbohydrates and fats *ad libitum* to adjust for each individual's variation in energy need while keeping protein intake constant. Using a computerized dietary analysis program (Food Processor II, ESHA Research, Salem, OR), the diet was designed to provide males 130 grams of protein and more than 3,000 calories per day; and females 97 grams of protein, and more than 2500 calories per day, and both, 100% of the RDA for vitamins and minerals. The morning of, and during the ultramarathon only foods with negligible protein (<6g/100g) were allowed. Breakfast consisted of a bagel (100 g), orange juice (200 g), and jelly (30 g) and was consumed two to three hours before the start of the race and provided approximately 10 g of protein, and 500 calories. Table 3.2 lists the protein content of food and beverages available during the ultramarathon. Prior to dinner following the race, the protein content of foods consumed during the ultramarathon was calculated and an equivalent amount was subtracted from the evening meal. The nitrogen content of the meals was determined from aliquots of food composites prepared on the last two days of each trial.

Table 3.2. Protein and calorie content of foods available during the ultramarathon.

Food Item	Serving Size	Protein (g)	Calories
Hydra fuel ®[1]	6 oz	0	42
Cola	4 oz	0	56
Cliff Bar ®[2]	1 (68g)	4	250
Cliff Shot ®[2]	64g	0	194
Gu ®[3]	32g	0	100
Oreo ®[4]	2 (22g)	1.3	107
Vanilla wafers	4 (16g)	0.9	77
Pretzels	15g	1.4	57
Apple slice	¼ (30g)	0.1	18
Orange slice	¼ (35g)	0.3	16

[1] Hydra Fuel is a trademark of TwinLab, Ronkonoma, NY.
[2] Clif Bar and Clif Shot are trademarks of Clif Bar Inc., Berkeley CA. Each subject was limited to one Clif Bar during the event.
[3] Gu is a trademark of Sports Street Marketing, Berkeley, CA.
[4] Oreo is a trademark of Nabisco Foods, East Hanover, NJ.

All food served during this study was purchased in bulk from the same lot when possible. Prior to T1, all meats for both trials were sliced, weighed, and frozen in daily use portions. Fresh produce and milk was purchased immediately prior to each trial. All foods were weighed to the nearest 0.2 g. To ensure complete consumption, subjects were instructed to rinse all food containers with water and then consume the rinse water.

Urine Collection

For each day of both trials, beginning in the morning, urine (24-hour) was collected. Samples were kept refrigerated between collections and were delivered to the nutrition lab daily where individual 24 hour samples were mixed, measured, and aliquots frozen at -20° C. The accuracy of each 24-hour collection was assessed by measuring creatinine excretion.

Biochemical Analysis

All samples were done in duplicate unless otherwise specified. Plasma and urinary urea nitrogen was analyzed using an automated method described by Di Giorgio (1974). Interassay CV for the control samples was 2.6% for PUN (n=3) and 3.6% for UUN (n=4). Dietary nitrogen and urinary nitrogen was determined using the boric acid modification of the Kjeldahl method (Scales and Harrison, 1920). Urinary and plasma creatinine was determined by an automated method based on the procedure of Pino et al (1965). Glucose was determined using a modified procedure of Trinder (1969) utilizing an Alpkem Autoanalyzer II. CV for the control samples (n=4) was <1%. Plasma lactic acid was measured spectrophotometrically (Sigma Chemical Co. Procedure No. 726-UV/826-UV) using the method of Hohorst (1963). Interassy CV for the control samples (n=4) was 5%. Hematocrit and hemoglobin (in triplicate) was determined using the micro Hct and cyanmet Hb methods, respectively. This data was used to calculate plasma volume changes using the method of Dill and Costill (1974).

Statistics

The SPSS statistical analysis software package (version 9.0) was used to analyze all data (SPSS Base 9.0 Applications Guide, Chicago IL). Significance for all analyses was set at $p \leq 0.05$. A GLM (general linear model) repeated measures was used to determine main effects of trial, day or time by trial, and the within trial effect of day or time. For multiple comparisons of day or time, the Bonferroni adjustment was used. Between trial comparisons of the same time points was made using the Student's paired t-test.

Results

With the exception of UUN and TUN, there were no significant differences in the response of males and females for any of the dependent variables studied. Therefore, the data was analyzed collectively and, unless otherwise noted, means reported represent data from n = 14. Additionally, some of the changes in plasma volume were significant, therefore, the values reported for the mid, post and P-60 plasma measures have been corrected accordingly. Table 3.3 lists the mean, and range, for percent change in plasma volume at mid, post, and P-60.

Table 3.3. Mean percent change (± SD) and the range of observed changes for the change in plasma volume from pre to mid, post, and P-60 during T1 and T2.

	Percent change from pre:		
	Mid	Post	P-60
T1	-3.7 ± 6	0.3 ± 7	5.4 ± 8
(Range)	(-11.7 to 8.6)	(-9.5 to 12.3)	(-5.9 to 25.7)
T2	2.7 ± 6	4.8 ± 5[1]	3.4 ± 4[2]
(Range)	(-4.9 to 13)	(-2.9 to 10.3)	(-5.6 to 8.3)

[1] p = 0.01
[2] p = 0.04

Diet

All subjects completed all five days of each trial. Dietary compliance verified by the daily report was 98% with each of the 3 reported infractions involving less than 4 g protein. The

nitrogen content of the meals consumed during T1 and T2 is presented in Table 3.5. Using 6.25% as the average nitrogen content of protein, average dietary protein intake was estimated to be 113.5 g for males and 87.8 g for females. The average number of calories consumed by males was 3,150 ± 116 per day (41 kcal per kg) and by females was 2,570 ± 90 kcal per day(45kcal per kg). Included in this average was an *ad libitum* food intake of 150 kcal per day for males and 70 kcal per day for females. In spite of the availability of *ad libitum* foods, during the five days of controlled diet in T1, five males and one female lost weight (mean of 1.3 and 0.9 kg, respectively), and during the same timeframe in T2, five males and two females lost weight (mean of 0.6 and 0.5 kg, respectively).

Ultramarathon

During the race the air temperature increased 12° C (11–23° C) and humidity decreased from 83% to 34%. The average time elapsed before the mid-race blood draw was 166 ± 31 minutes. All subjects completed the race in an average of 396 ± 68 minutes, with males finishing an average of 22 minutes (5.6%) before the females (388 ± 77 vs. 410 ± 52). After completion of the run, the average rate of perceived exertion (RPE) reported was 14 (Borg scale 6 to 20) which correlates to an intensity of approximately 75% VO_{2max} (Borg, 1982). The average weight loss during the race was 1.5 kg, equaling 2.1 ± 1.5% of their total body weight and equating to a fluid deficit of 1.5 L. Average fluid intake was 3.8 L with males consuming 27.8% (1.2 L) more than females. During the race an average of 1,285 calories and 6.1 g of protein was consumed. Table 3.4 lists the average food and beverage consumption per subject, per aid station.

Table 3.4. The nutritional composition of the food and beverages consumed per aid station.[1]

Aid Station #	Protein (g)	CHO (g)	Total Calories	Fluid (mL)
1	1.6	57	267	573
2	0.9	57	238	567
3	1.4	58	258	746
4	0.9	56	240	807
5	0.6	32	142	398
6	0.7	31	140	741
Total ± SD	6.1 ± 3.5	291 ± 47	1285 ± 127	3832 ± 418

[1]Values listed represent average per subject

Plasma Urea Nitrogen and Plasma Creatinine

The mean fasting (6.2 ± 1.5 vs 6.4 ± 1.4 mmol/L) and pre concentrations of PUN were not different between T1 and T2. Figure 3.2 depicts the mean PUN concentration for T1 and T2. Overall, during T1 the mean PUN concentration increased 70.5% pre to P-60 ($p = 0.00$), and during T2, for the same period, there was a decrease of 15.9% ($p = 0.003$). During T1 the average rate of PUN concentration increase from pre to mid, mid to post, and post to P60 was 0.17, 0.62, and 0.71 mmol/L/hr respectively. For the same time periods the mean plasma creatinine concentration increased 3.6, 4.1, and 5.5 μmol/L/hr respectively for an overall increase of 43% ($p = 0.00$). During T1, the mean change in PUN concentration from pre to mid was correlated with the mean change in creatinine concentration ($r = .73$, $p = 0.003$) but from mid to post ($r = -0.11$, $p = 0.71$) and post to P-60 ($r = -0.253$, $p = 0.38$) it was not. See Table 3.6 for the mean plasma creatinine concentration and mean PUN to creatinine ratio at pre, mid, post, and P-60 for T1 and T2.

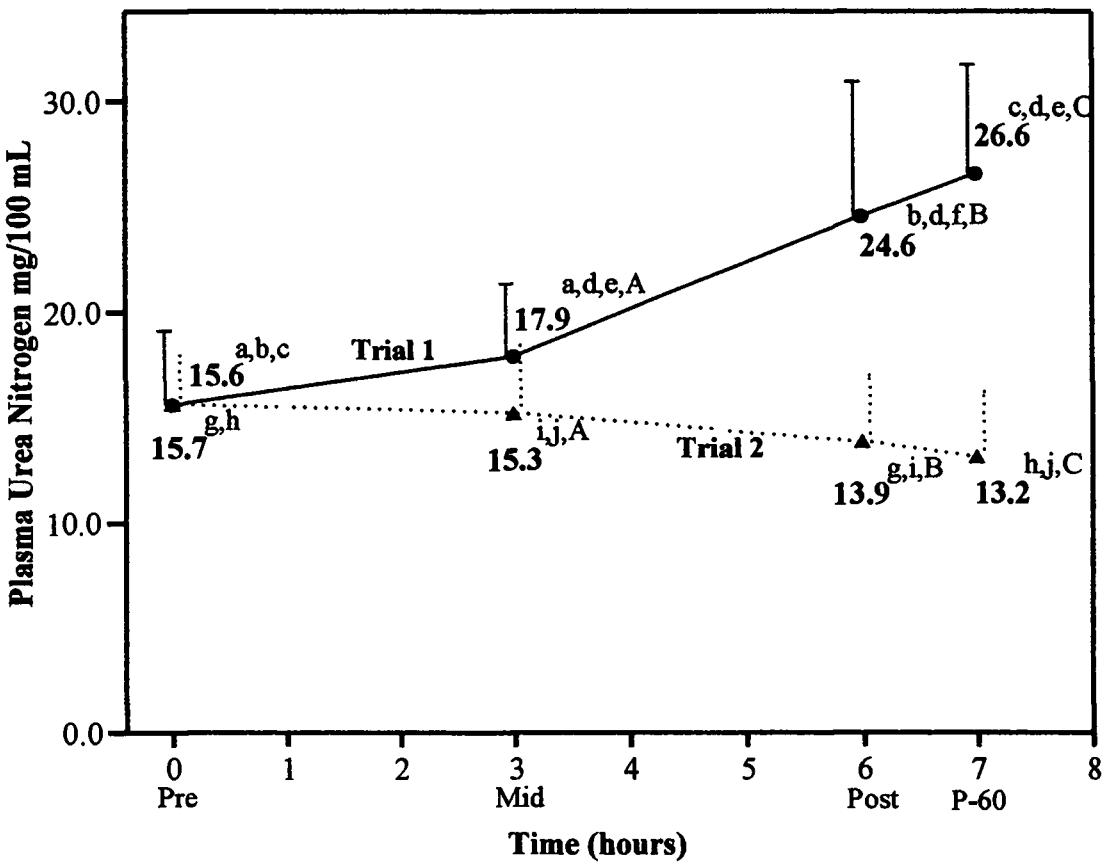

Figure 3.2. Plasma urea nitrogen concentration before (pre), during (mid), after (post), and 60 minutes after (P-60) an ultramarathon (Trial 1—) and rest (Trial 2 ⁻). Error bars show mean ± SD. Like letters (uppercase for between trial, lowercase for within trial) indicate a significant ($p < 0.01$) difference between two measures. There was a significant univariate Time by Trial interaction ($p = 0.00$) that explains 83% of the observed variability.

Urinary Creatinine, Total Urinary Nitrogen, and Urinary Urea Nitrogen.

There was no significant between or within group difference in the daily urinary creatinine excretion (Figure 3.3). The grand mean for T1 was 14.8 ± 4.2 mmol/day (range of means 14.2 to 15.9 mmol/day) and for T2 was 15.2 ± 4.2 (range of means 14.3 to 16.1 mmol/day). The mean creatinine excretion the day of, and the day after, the ultramarathon was 15.9 ± 4.8 and 14.6 ± 3.8 mmol/day, respectively, and was not significantly different compared to values for the same days during T2 (16.1 ± 4.42 and 15.3 ± 3.8 mmol/day, respectively).

Table 3.5. Mean (± SD) dietary nitrogen (N) intake, total urinary N excretion, percent dietary N excretion, urinary urea N excretion and percent total urinary N as urea for males and females during trial 1 (T1) and trial 2 (T2).

		Males[1] (n = 9)			Females (n = 5)		
		Day 3	Day 4	Day 5	Day 3	Day 4	Day 5
Dietary N g/per day	T1	18.3	18.0	18.3	13.8	14.4	13.8
	T2	18.5	18.1	18.5	13.6	14.4	13.6
Total urinary N g/24 hr (as a % of intake)	T1[2]	16.6±2.1 (91%)	17.9±3.6^{A} (100%)	17.9±1.9 (98%)	11.4±1.4^{a} (83%)	14.4±0.7^{B,a} (100%)	12.2±1.8 (88%)
	T2	16.0±3.5 (86%)	15.2±0.9^{A} (84%)	15.1±2.0 (81%)	11.4±0.7 (84%)	11.5±1.7^{B} (80%)	12.1±2.1 (89%)
Urinary Urea N g/24 hr (as a % of intake)	T1[3]	13.2±2^{b} (72%)^{c}	14.7±2.9^{C} (82%)	15.1±1.9^{D,b} (83%)^{c}	9.6±1.3 (70%)	11.6±0.7^{E} (81%)	10.7±3.1 (78%)
	T2	13.3±2.9 (72%)	12.1±1.0^{C} (67%)	11.9±1.1^{D} (64%)	9.2±0.5 (67%)	9.0±1.4^{E} (63%)	9.8±2.2 (72%)

[1] There was a significant gender effect for the absolute amounts of dietary nitrogen, total urinary nitrogen, and urinary urea nitrogen, with males consuming and excreting significantly more than females on each day of each trial. However, relative to intake (percent excreted) there is no gender effect for total urinary nitrogen or urinary urea nitrogen.

[2] There was a significant univariate Trial interaction for males ($p = 0.003$) and females ($p = 0.008$) that explains 69% and 85%, respectively, of the observed variability.

[3] There was a significant univariate Trial interaction for males ($p = 0.004$) and females ($p = 0.015$) that explains 67% and 81%, respectively, of the observed variability.

[A,B,C,D,E] Like letters indicate a significant ($p < 0.05$) *between trial* difference.

[a,b,c,d,e] Like letters indicate a significant ($p < 0.05$) *within trial* difference.

There were significant differences between males and females for the absolute amount of TUN and UUN excreted. Table 3.5 lists the amounts for each. However, there was no difference in relative excretion (i.e. the percent of dietary nitrogen excreted) between males and females so the data was combined. Figure 3.3 depicts the mean intake and excretion of nitrogen. For both TUN and UUN excretion, there were no within trial differences, but for both, more was excreted on days 4 and 5 of T1 compared to the excretion the same days during T2. Analysis of TUN excretion identifying weight loss as a between subjects factor revealed that individuals

who lost weight during T1 and T2 excreted a mean of 3 ± 0.5 g/day and 1.8 ± 0.6 more, respectively, than individuals who did not lose weight, but this difference was not significant.

Plasma Glucose, Lactate, and Creatinine

There were no differences between T1 andT2 in the mean fasting concentration for plasma glucose (4.8 ± 0.4 vs 4.6 ± 0.3mmol/L), lactate (1.2 ± 0.4 vs 1.2 ± 0.4mmol/L), or creatinine (72.4 ± 17 vs 72.5 ± 16μmol/L). The data for the pre, mid, post, and P-60 concentration of these variables is summarized in Table 3.6. During T1 there was no significant change in mean plasma glucose concentration although mid, post, and P-60 concentrations were slightly higher than pre concentration. During T2, mean plasma glucose concentration significantly increased (27%) pre to post but returned to pre levels by P-60. During T1 mean plasma lactate concentration increased 55% ($p < 0.01$) between the pre and post measures and was significantly different from T2 at pre 0.2mmol/L difference), mid (0.8mmol/L difference), post (1.4mmol/L difference), and P-60 (1.4mmol/L difference). For plasma creatinine, within T1 there was a significant ($p < 0.01$) 43% increase in mean concentration between the pre and P-60 measures and for T2 there was no change. Between trials there were significant ($p < 0.00$) differences in mean creatinine concentration at the mid (12.6 ± 7.4 μmol/L difference), post (25.6 ± 16.2 μmol/L difference), and P-60 (33.0 ± 12.0 μmol/L difference) measures.

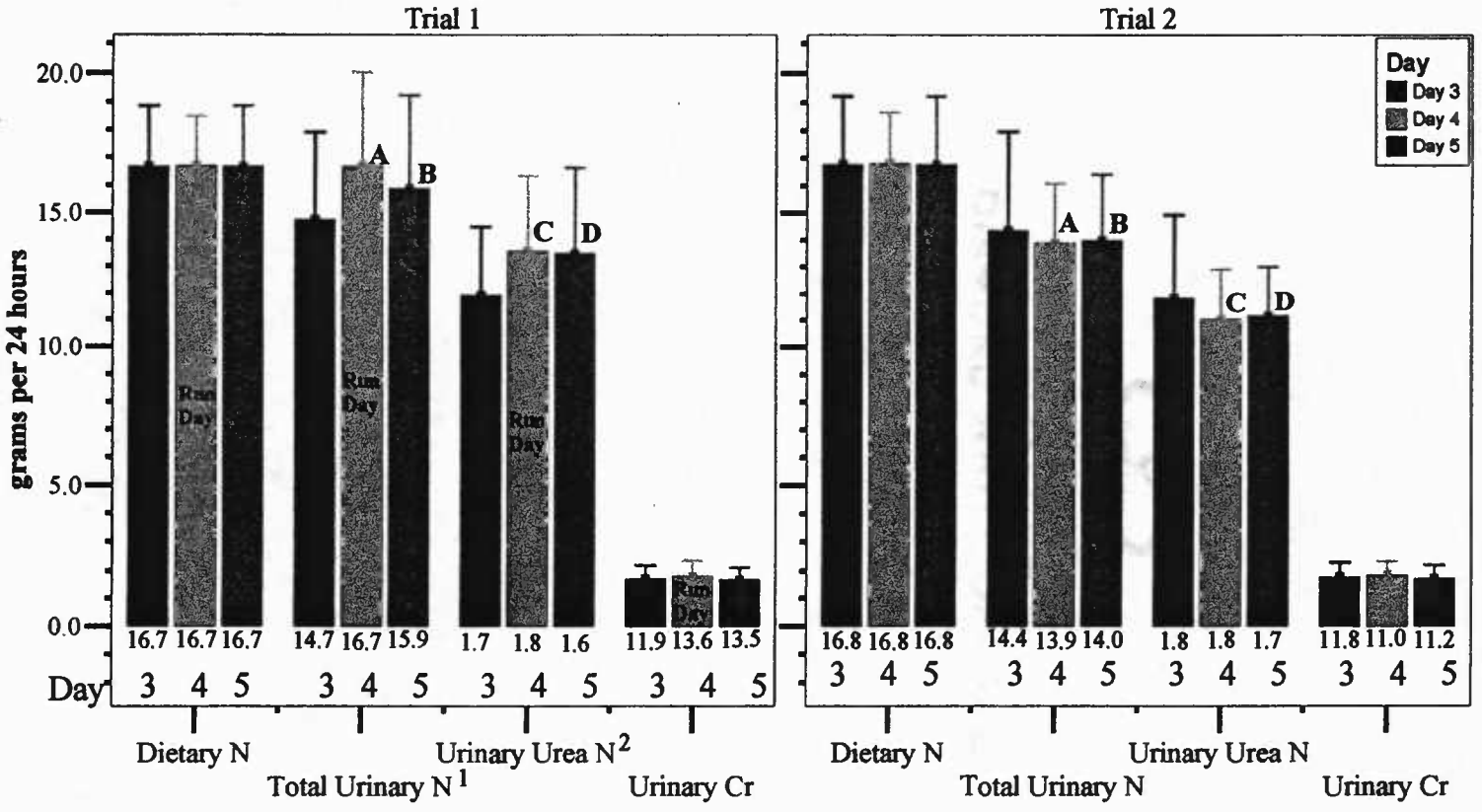

Figure 3.3. Grams per day of dietary nitrogen (N) intake and grams per 24 hours of total urinary nitrogen, urinary urea nitrogen and creatinine excretion for days 3, 4, and 5 during Trial 1 and Trial 2. Like letters indicate a significant ($p<0.05$) between trial difference. [1] For total urinary nitrogen there is a significant univariate trial effect ($p=0.00$) that explains 70% of the observed variability. [2] For urinary urea nitrogen there is a significant univariate trial effect ($p=0.00$) and trial by day effect ($p=0.003$) that explains 67% and 28% of observed variability, respectively.

Table 3.6. Mean (± SD) plasma glucose, lactate, and creatinine concentrations and PUN:Cr before (pre), during (mid), after (post) and one hour after (P-60) an ultramarathon (T1) or inactivity (T2).

		Pre	Mid	Post	P-60
Glucose (mmol/L)	T1	4.6 ± 0.9	5.3 ± 1.1	5.0 ± 0.8^{1}	5.1 ± 1.1^{2}
	T2	4.1 ± 0.8^{a}	5.0 ± 1.2	$5.2 \pm 0.9^{a,1}$	4.1 ± 0.7^{2}
Lactate (mmol/L)	T1	$2.1 \pm 0.5^{A,1}$	2.9 ± 0.8^{2}	$3.3 \pm 1.0^{A,3}$	2.9 ± 1.0^{4}
	T2	1.9 ± 0.6^{1}	2.0 ± 0.9^{2}	1.9 ± 0.6^{3}	1.6 ± 0.5^{4}
Creatinine (μmol/L)	T1	$72 \pm 17^{A,B,C}$	$82 \pm 20^{A,D,E,1}$	$98 \pm 22^{B,D,2}$	$103 \pm 24^{C,E,3}$
	T2	72 ± 16	69 ± 17^{1}	72 ± 20^{2}	70 ± 17^{3}
PUN:Cr (μmol/L)	T1	$80{:}1 \pm 21$	$81{:}1 \pm 21^{A}$	$94{:}1 \pm 35^{1}$	$95{:}1 \pm 27^{A,2}$
	T2	$80{:}1 \pm 17$	$81{:}1- \pm 21^{a}$	$70{:}1 \pm 12^{a,1}$	$69{:}1 \pm 17^{2}$

^{A,B,C,D,E,a} Like letters (upper case for T1, lower case for T2) represent a significant ($p < 0.05$) *within trial* difference.

^{1,2,3,4} Like numbers represents a significant ($p < 0.05$) *between trial* difference.

Discussion

A significant increase in the rate of plasma urea production after two to three hours of moderately intense exercise has not been previously demonstrated. In the present study, compared to the first 2.8 hours of exercise, in the last 3.8 hours there was at least a 3.5 fold increase in the rate of urea accumulation. Furthermore, because this rate of change was not correlated with the rate of change for creatinine, it is unlikely the observed results are attributable only to altered kidney function. The cause of the increase cannot be determined from the present data but it is most likely related to a decrease in liver and muscle glycogen stores, which would increase the use of amino acids as gluconeogenic and oxidative substrates.

In a study covering the same distance as ours, Irving et al (1990) assessed the effect of a 56-km race on pre to post changes in PUN and urinary urea excretion. Compared to blood drawn the day before the race, blood drawn immediately post race showed a 32% increase in PUN, and a 21% increase in plasma creatinine. The mean race time was not given but the range

of duration was 3.7 to 5.4 hours which is shorter in duration than the current study (mean exercise time 6.6 hr), and may account for the smaller increase in PUN they observed. For urinary urea excretion, compared to the day before the race, Irving found on the day of, and the day following the event the excretion of urea increased by 1.4 and 1.7 g/24 hours ($p < 0.05$), respectively. In the present study, on comparative days urea excretion increased by 1.5 and 1.9 g ($p < 0.05$). Because our change in PUN was larger and our event more physically challenging, one might have expected our urinary urea nitrogen loss to be greater. However, Irving did not report data on estimated sweat rate or urea sweat loss and, in the current study, mean temperature was 6°C warmer making it quite likely our subjects lost more urea in sweat than his subjects. Moreover, Irving et al did not provide information on dietary intake prior to or during the event so it is unclear what effect this may have had on the results. In a study covering twice the distance as ours, Decombaz et al (1979) reported a 44% increase in urea production and increased urinary urea excretion following the race. Additionally, he found no change in 3-methylhistidine and concluded that muscle protein catabolism did not occur and the source of the nitrogen was from amino acids made available by a decrease in protein synthesis during exercise.

A decrease in plasma glucose concentration might be an a indicator of glycogen store depletion, however, in this study no significant decrease was found nor was it expected due to the hormonal regulation of plasma glucose concentration during exercise (Holloszy and. Kohrt, 1996). However, in this study between the mid and post there was a small but insignificant decline in plasma glucose concentration that could be related to increased glucose uptake by the muscle as the subjects glycogen stores became limited. Similarly, the slight increase from post to P-60 could be the result of decreased glucose uptake by the muscle secondary to reduced energy requirements. This could suggest that after mid race there was an increased need for

hepatic gluconeogenesis and, thus, increased metabolism of amino acids and would explain the increased rate of PUN accumulation during the last half of the race.

Studies reporting no change in plasma urea nitrogen concentration after less than three hours of exercise are in agreement with our mid (2.8 hr) measure finding of no significant change. Calles-Escandon et al (1984) found no change following 90 minutes at 45% VO_{2max}, Carraro et al (1993) found no change following 3 hours at 40% VO_{2max} or 1 hour at 70% VO_{2max}, and Tarnopolsky et al (1990) reported no change following 90–101 minutes at 65% VO_{2max}. The lack of change in plasma urea concentration during exercise of shorter duration is most likely related to 3 variables: glycogen stores, exercise intensity, and sweat losses. Lemon and Mullin (1980) demonstrated that after 60 minutes of exercise at 61% VO_{2max} compared to carbohydrate loaded subjects, carbohydrate depleted subjects had a significant increase in PUN. Research shows that, not only does exercise increase the activation of branched-chain keto acid dehydrogenase (Kasperek et al 1985), but low glycogen stores may further increase this enzyme's activity and further increase amino acid oxidation. Although we did not directly measure glycogen stores, our subjects consumed a diet containing >60% CHO and tapered training five to six days prior to the event which previous research shows is adequate to maximize glycogen stores (Sherman and Costill, 1984). Exercise intensity, because of its impact on renal function and absolute amounts of amino acids oxidized, is another important factor affecting urea accumulation in the plasma. In our study subjects reported an average RPE of 14 that translates to approximately 75% of their VO_{2max} (Borg 1982, Brooks 1996c) which, according to the research of Sherman and Costill, (1984), would decrease muscle glycogen by 85% in 1.5 hours. It is possible that, due to the extreme physical challenge of this ultramarathon course (elevation change of 3,900 m), subjects may have over estimated their RPE, however, the increase in plasma lactate supports the assertion that exercise intensity was approximately 75% of their VO_{2max}. Wolfe et al (1984) demonstrated that exercise increased the rate of leucine

oxidation confirming the work of Milward et al (1982) who had shown a linear increase in oxidation as exercise intensity increased from 25–89% VO_{2max}, however, neither found an increase in PUN. The lack of plasma urea nitrogen accumulation may have been related to exercise intensity, which was only 30-50% VO_{2max}, and urea loss through sweat, which was not accounted for. At low exercise intensities, urea clearance by the kidneys may not be compromised (Suzuki et al, 1996), and protein synthesis may not decrease (Dohm et al, 1985, 1987), which may allow some urea to be recycled back into protein (Carraro et al, 1993). Calles-Escandon et al (1984) demonstrated that following 90 minutes of exercise there was no change in PUN but sweat urea increased significantly and accounted for 30% of urea excretion. Similarly, Lemon and Mullin (1980) demonstrated that carbohydrate loaded subjects who cycled one hour at 61% VO_{2max} had no change in urine urea output (mg/hr) or PUN concentration but did increase sweat urea nitrogen excretion from 10 mg/hr in a rested state to over 600 mg/hr. In contrast, carbohydrate depleted subjects had a significant increase in PUN concentration and had a sweat urea nitrogen excretion of 1300 mg/hr. This amount sounds impressive, but it is important to note that physiologically it may not be that significant because 600–1300mg of urea nitrogen represents the breakdown of only 4–8 g (16 to 35 kcal) of endogenous protein.

The day of the ultramarathon, total urinary nitrogen excretion was 100% of intake. This loss, combined with dermal and fecal nitrogen loss that averages 2 g/day (Tome and Bos, 2000) indicates the subjects were in negative nitrogen balance. The source of the nitrogen is not known but is likely from a combination of tissue (especially liver protein) breakdown and a decrease in protein synthesis (Dohm et al, 1987). Because urinary creatinine remained constant it appears that there was not a significant amount of muscle damage, but 3-methylhistidine, which we did not measure, would be a better marker to assess muscle damage. Normally, depending on the protein and calorie content of the diet, urea nitrogen constitutes 62% to 88% of

the total urinary nitrogen excretion (Allison and Bird, 1977). In the current study, following the ultramarathon subjects increased the amount of urinary nitrogen excreted as urea from 71% to 82%, and during T2 when no exercise was performed, urea averaged only 68% of total urinary nitrogen excretion. This increase in the percent urea is similar to what occurs during starvation and is indicative of increased amino acid degradation. Gender may also play a role in determining the amount of urea excreted. Tarnopolsky et al (1990) found that in equally trained males and females, following 90–101 minutes of exercise the males excreted 30% more urea and utilized 25% more of their muscle glycogen than females. The present study found the percent of dietary nitrogen intake excreted as urea nitrogen was virtually identical on the day of the run (82 vs 81%) and the day following (83 vs 78%). However, in our study males and females were not matched for fitness and, unlike the Tarnopolsky et al (1990) study, our females were at various points in their menstrual cycle which may also impact substrate utilization (Bonen et al, 1983).

Although highly speculative, using the data from previous research, it is possible to estimate protein utilization and urea sweat loss one might expect during exercise lasting 6.6 hours. Table 3.7 details such an estimate.

Table 3.7. Estimated kcal requirement, percent contribution by protein, and sweat urea nitrogen loss for a 50-km ultramarathon.

	Per Hour		
	Hours 0 to 3 (CHO[2] loaded)	Hours 3 to 6.6 (CHO depleted)	Total (6.6 hours)
Kcal[1]	666	666	4400
Protein contribution to energy needs	5% (equal to 7.7 g protein or 1.3 g N/hr)	10% (equal to 16.7 g protein or 2.7 g N/hr	83 g protein (13.3 g N)
Sweat urea nitrogen loss[3]	600 mg	1300 mg	6.5 g

[1] Estimate of 1.1 kcal/minute cross country running (McArdle, 1999)
[2] CHO = carbohydrate
[3] Based on research by Lemon and Mullin(1980).

As this estimate illustrates, during 6.6 hours of running, 6.5 g of nitrogen is lost via sweat, leaving 6.8 g that would be excreted in the urine. In the present study, compared to T2, there was a 5.5 g total increase in urinary nitrogen on the day of, and day following, the ultramarathon. The purpose of this speculation is to draw attention to the potentially erroneous conclusion that, based on urinary excretion the day of the event, an insignificant amount of protein was catabolized.

The fact that there are increases in both the relative and absolute amount of amino acids oxidized during exercise, may have little or no impact on overall nitrogen balance. Several investigations have confirmed that during exercise protein synthesis decreases (Booth and Watson, 1984; Rennie et al 1981b). Rat studies show the degree of depression in muscle protein synthesis is proportional to the intensity and duration of exercise, and running to exhaustion can decrease synthesis by 70% (Dohm et al, 1980). In humans, using the primed constant infusion technique to measure whole-body protein synthesis during exercise, Rennie et al (1981a, 1981b) and Wolfe et al (1981) both found that incorporation of leucine into protein was decreased. The amount and significance of amino acids added to the pool in this manner remains to be quantified but may be an important point to consider when determining the protein requirements during exercise.

The important aspect of the present study is not that plasma urea nitrogen concentration increased, but that during a real event, well-trained endurance athletes who consumed the recommended 30–60 g/hr of carbohydrate (Coleman, 2000), appeared to increase amino acid oxidation sometime after 2.8 hours. The physiological significance of this remains to be determined but, in the interim, it is inappropriate to estimate the needs of these athletes from research less than three hours in duration. Due to the increasing popularity of ultraendurance events, comprehensive studies involving duration of greater than six hours are needed to evaluate the role of protein during such events. Recommendations for future research includes

tissue sampling (muscle biopsies), an increased frequency of blood sampling, and assessment of sweat urea nitrogen excretion throughout the event.

SUMMARY AND CONCLUSIONS

The purpose of this study was to examine the effect of a 50-km ultramarathon on vitamin B-6 metabolism and plasma and urinary urea nitrogen. Our hypothesis was that greater than six hours of exercise would alter vitamin B-6 metabolism by causing an increase in plasma PLP concentration, followed by a decrease to below pre-event levels. Concurrently, plasma 4-PA and urea concentration would increase and ultimately result in increased urinary 4-PA and urea excretion. The specific objectives of the study were to: 1) determine if running longer than six hours resulted in significant changes in plasma PLP, PL, 4-PA, and urea concentration and to determine if the magnitude and direction of the change was the same during the first three hours of exercise compared to the second three hours 2) determine how much of the change in plasma variables was attributable to food consumed during the event and/or normal daily fluctuations 3) determine if the amount of dietary vitamin B-6 excreted as urinary 4-PA, and dietary nitrogen as urinary urea nitrogen, was greater the day of the ultramarathon compared to the day before and the day after 4) determine if the change in plasma PLP concentration was correlated with the change in plasma glucose, albumin, lactate, and urea concentration or alkaline phosphatase activity.

All 14 subjects completed one exercise and one control trial. With the exception of day four, both five-day trials were identical: during T1 subjects completed an ultramarathon on day four, and during T2 subjects were "inactive" on day four. During both trials, subjects consumed a controlled diet, completed 24-hour urine collections, and had blood drawn before, during, and after exercise or "inactivity". Plasma was analyzed for pyridoxal 5'-phosphate, pyridoxal, 4-pyridoxic acid, urea nitrogen, creatinine, albumin, glucose, and lactate concentration and alkaline phosphatase activity. Urine was analyzed for 4-pyridoxic acid, creatinine, urea nitrogen, and total urinary nitrogen. The SPSS statistical package was used to analyze the data.

Specific statistical tests included analysis of repeated measures, correlation's, and paired t-tests. Null hypotheses were rejected at the 0.05 level of significance.

During T1, midway through the ultramarathon, mean plasma PLP concentration increased by 17%, but by 60 minutes post-race, concentration was 19% below pre-race levels. At the same time, plasma 4-PA concentration increased 135% and, compared to the day before, resulted in an increase in the percent of dietary vitamin B-6 that was excreted as urinary 4-PA. In contrast, during T2, when the subjects were inactive, plasma PLP decreased 13% and plasma 4-PA concentration and urinary 4-PA excretion did not change. Together, these results suggest that exercise alters vitamin B-6 metabolism and the change occurs in at least two distinct phases: the first phase is characterized by an increase in plasma PLP and 4-PA concentration and the second is characterized by a decrease in plasma PLP and a plateau in plasma 4-PA concentration. Moreover, the increase in plasma 4-PA indicates that the liver uptake of PLP is increased during exercise and a greater amount is converted to 4-PA.

Exercise also significantly altered urea production. During the first half of the race, plasma urea increased in the same ratio as plasma creatinine, but during the second half it accumulated at a significantly greater rate indicating an increase in urea production. Further evidence of increased urea production is found in the increase in the percent of dietary nitrogen that was excreted in the urine the day of the ultramarathon compared to the day before. In contrast, during T2 when the subjects were inactive, plasma urea nitrogen decreased 17%, and there was no change in urinary excretion for any of the variables. These data suggest exercise duration is an important factor in determining urea production. After three hours of moderately intense exercise, despite consumption of "adequate" amounts of carbohydrate, urea production significantly increased. This result indicates the protein requirement of ultraendurance athletes may be increased and the amount of the increase may be related to the duration of runs.

Together, the vitamin B-6 findings and the urea findings support the theory that vitamin B-6 is mobilized and redistributed to tissues based on need. Of the current theories, this research suggests that vitamin B-6 metabolism is likely due to a combination of the interstitial store theory, and liver-to-muscle theory. The initial increase in PLP may come from the interstitial space and hepatic tissue, and could be related to an increased need by the muscle for glycogen breakdown. Following three hours of exercise, as both hepatic and muscle stores of glycogen diminish, PLP may be sequestered in the liver to augment gluconeogenic processes. The end result being a decrease in plasma PLP concentration and a decrease in 4-PA production. The homeostatic mechanism for vitamin B-6 metabolism during exercise is not currently known, but may be related to stimulation of the sympathetic nervous system, which causes dramatic changes in circulation and plasma hormone concentrations.

The results of this study should be viewed in light of its limitations. The average age of subjects in this study was 42 years, and twelve of the subjects were experienced ultra-distance runners who reported normal training runs greater than over two to three hours. Therefore, their physiological response to an ultramarathon is likely to be quite different from younger runners who train only 30-60 minutes per session. Additionally, the number of subjects studied was fairly small and the duration of time between measures varied from subject to subject, as did exercise intensity. Perhaps the biggest limitation of this study, and most other studies, is that we did not sample the tissue and, therefore, do not really know what happened to the vitamin B-6 and glycogen content of the liver and muscle. Nonetheless, this study does provide valuable clues to how vitamin B-6 and amino acids are metabolized during exercise in this unique population of ultraendurance runners.

Further research on the effect of exercise greater than six hours is needed to define the phases of vitamin B-6 metabolism and identify homeostatic controls. The use of labeled pyridoxine and/or sampling of tissue would provide valuable information about the source and

fate of mobilized vitamin B-6. Additionally, a study of long duration exercise at varying intensities with frequent evaluation of plasma vitamin B-6 and hormone (insulin, glucagon, epinephrine, norepinephrine, and cortisol) concentration may provide additional insight to the regulation of vitamin B-6 during exercise. To address the issue of an increased vitamin B-6 requirement for endurance athletes, it is important that future studies include determination of the amount of 4-PA lost through sweat. In spite of the preponderance of evidence suggesting greater amounts of 4-PA is produced during exercise, the long term physiological impact and significance of this finding is not known. At this time there is no definitive proof that endurance athletes require more vitamin B-6, although it does appear likely.

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APPENDICES

APPENDIX A

Figure A.1. Health and diet history form

HEALTH / DIET HISTORY(1999 version)-
CONFIDENTIAL

Dr. Jim Leklem
Dept. Nutrition & Food Management
Oregon State University
Code # : _____

Project Name _____
Project Dates _____
Today's Date _____

Age : _____ Date of Birth: _____ Place of Birth _____
Sex : M /F Predominant Place of Residence : _____
Present Employment : _____

Which best describes your racial/ethnic identity? (Please circle all that apply)

_____ White, European American, Non-Hispanic
_____ Asian or Asian American
_____ Black, African American, Non-Hispanic
_____ Middle Eastern or Middle-Eastern American
_____ North African or North African-American
_____ Pacific Islander
_____ Hispanic or Latino American
_____ American Indian or Alaskan Native
_____ If none of the above choices apply to you, please use your own description:_____
_____ Decline to respond

1. HEIGHT / WEIGHT : Height (ft. & in.) _____ Present weight : _____
   Most ever weighed _____ What year _____
   Length of time you have maintained current weight _____

2. DIETARY HISTORY

Dieting : Are you currently on a special diet ? ____ Yes ____ No
If yes, for what purpose ? (please check as many as apply) :

__ 1. weight loss
__ 2. control serum lipids
__ 3. diabetes
__ 4. kidney failure
__ 5. ulcers
__ 6. diverticulitis
__ 7. allergies
__ 8. heart trouble
__ 9. high blood pressure
__ 10. pregnancy
__ 11. breast feeding
__ 12. other (please specify) :

If you are on a diet, was it prescribed by a doctor, dietitian, or nurse ? __ Yes __ No

If you are on a diet, what kind is it ? (please check as many as apply) :

__ 1. low fat
__ 2. low protein
__ 3. high protein
__ 4. low salt
__ 5. low carbohydrate
__ 6. low sugar
__ 7. low calorie
__ 8. low cholesterol
__ 9. high calorie
__ 10. a bland diet
__ 11. other (please specify) :

If you are currently on a diet, for how long have you been on this diet ? _____

Are you a vegetarian ? __ Yes __ No

If yes, circle the type of vegetarian diet you follow :

a. ovo-lacto b. ovo c. lacto d. vegan

Do you take vitamins, minerals or other nutritional supplements? (circle one) :

a. yes, daily
b. yes, frequently (3 to 6 times/wk)
c. often (once or twice/wk)
d. occasionally (less than once/wk)
e. never

If yes what type, how much, and for how long have you taken them ?

Type	Amount	Frequency	How long have you taken?

Do you take any other nutritional supplements [such as iron, calcium, other minerals, amino acids, fiber, supplement drinks (such as Ensure), etc] ? ___ Yes ___ No

Type	Amount	Frequency	How long have you taken?

Please list all foods which you refuse to eat, can not eat, or prefer not to eat :

Please list those foods and beverages that you eat/drink almost every day :

3. HABITS :

A. Smoking :

1) Do you currently smoke ? __ Yes __ No

If yes, please check below what you do smoke, and how much per day :

Cigarettes _____ Packs per day _____

Cigars _____ Number per day _____

Pipe _____ Pipe loads per day _____

At what age did you start smoking ? _____

2) If you do not currently smoke, did you ever smoke ? __ Yes __ No

If yes, at what age did you start ? _____

If yes, when did you quit ? _____

Was this the only time that you have quit ? __ Yes __ No

If you quit, please check below what you did smoke, and how much per day :

Cigarettes _____ Packs per day _____

Cigars _____ Number per day _____

Pipe _____ Pipe loads per day _____

3) Does anyone else in your household smoke ? __ Yes __ No

If yes, please list type and how much per day :

Cigarettes _____ Packs per day _____

Cigars _____ Number per day _____

Pipe _____ Pipe loads per day _____

B. Alcohol:

1) Do you drink alcoholic beverages ? __ Yes __ No

If yes, How many times do you drink per month ? _____

If yes, what do you drink and how many drinks do you consume each time you drink ?

Beer _____ Number of drinks at one time _____
Wine _____ Number of drinks at one time _____
Liquor _____ Number of drinks at one time _____
Other _____ Number of drinks at one time _____

C. Caffeine :
1) Do you drink beverages containing caffeine ? __ Yes __ No
If yes, which of the following beverages do you drink, and how much ?

Coffee _____ Number of cups per day _____
Tea _____ Number of cups per day _____
Soda _____ Number of 12 oz servings per day _____

2) Do you drink any decaffeinated or caffeine-free beverages ? __ Yes __ No
If yes, which of the following beverages do you drink, and how much ?

Coffee _____ Number of cups per day _____
Tea _____ Number of cups per day _____
Soda _____ Number of 12 oz servings per day _____

D. Diet soda pop and other Sugarless Beverages
1) Do you drink any beverages containing artificial sweeteners ? __ Yes __ No
If yes, what do you drink and how many drinks (ounces, servings) per day ?

4. EXERCISE LEVEL : Are you currently involved in a regular exercise program ?
__ Yes __ No If yes, describe :

Type of exercise # Minutes (continuous) Distance covered or repetitions # days/week

Do you monitor your heart rate during exercise ? __ Yes __ No
If yes, what heart rate do you try to maintain while exercising ? _____

5. MEDICAL HISTORY :

Have you ever had a glucose tolerance test ? __ Yes __ No If yes please explain when, the reason, and the results :

Have you ever had a stress electrocardiogram ? __ Yes __ No If yes, please explain when, the reason, and the results :

Have you ever had any health risk screening tests, such as serum cholesterol, blood glucose, or blood pressure ? __ Yes __ No If yes, please explain what tests you had, and what were the results and recommendations you received :

MEDICAL HISTORY (Check any condition for which you have been diagnosed and give AGE at diagnosis) :

Diagnosis	Age at Diagnosis
__ 1. acquired immunodeficiency syndrome (AIDS)	
__ 2. diabetes	
__ 3. hypoglycemia	
__ 4. hypothyroidism	
__ 5. hyperthyroidism	
__ 6. goiter	
__ 7. osteoporosis	
__ 8. hepatitis	
__ 9. cirrhosis	
__ 10. kidney stones	
__ 11. nephritis	
__ 12. cystitis	
__ 13. high blood pressure	
__ 14. angina	
__ 15. ulcer	
__ 16. pancreatitis	
__ 17. ulcerative colitis	
__ 18. recurring gastritis	
__ 19. allergies/hayfever	
__ 20. hypoadrenalism (Addison's disease)	
__ 21. spastic colon/diverticulitis	
__ 22. carpal tunnel syndrome	
__ 23. rheumatoid arthritis	
__ 24. systemic lupus erythematosus	
__ 25. mental depression requiring regular medication	
__ 26. asthma	
__ 27. insomnia requiring frequent medication	
__ 28. emphysema	
__ 29. heart problems	

__ 30. cancer (specify type) _____
__ 31. chronic infection (specify) _____
__ 32. tuberculosis _____
__ 33. chronic headache or other pain (specify) _____
__ 34. hereditary condition _____
__ 35. premenstrual syndrome _____
__ 36. other condition (specify) _____

Comments :

Are you currently suffering from any cold, flu, or allergy symptoms ? __ Yes __ No
If yes, please specify :

Do any of your first-degree relatives (mother, father, brother, sister, son, daughter) have any of the following conditions ? __ Yes __ No If yes, indicate which

__ 1. diabetes
__ 2. heart disease before age 60
__ 3. cancer before age 60
__ 4. high blood pressure before age 60
__ 5. allergies

Have you ever had a nerve condition/muscle stimulation study ? __ Yes __ No
If yes, when, for what reason, and what were the results ?

Have you ever had any other special diagnostic tests (such as special X-ray studies or a CAT-scan) __ Yes __ No If yes, please specify :

SURGICAL HISTORY (Please specify any type of surgery you have had and the date and age when it occurred) :

Operation	Age or Year

MEDICATION HISTORY (Check any which you take on a regular basis and when and how often) :

Medication	Taking Currently ?	How Often ?
__ 1. sleeping tablets		
__ 2. aspirin		
__ 3. cold medications		
__ 4. barbiturates		
__ 5. tranquilizers		
__ 6. diuretics		
__ 7. blood pressure tablets		
__ 8. antibiotics		
__ 9. thyroid hormones		
__ 10. oral contraceptives		
__ 11. insulin		
__ 12. oral hypoglycemics		
__ 13. corticosteroids		
__ 14. estrogens (female hormones)		
__ 15. isoniazid		
__ 16. pain medications		
__ 17. muscle relaxants		
__ 18. theophylline		
__ 19. antiarrhythmatics		
__ 20. ulcer medications		
__ 21. antacids		
__ 22. digoxin		
__ 23. antidepressants		
__ 24. seizure medications		
__ 25. other medications (please specify) :		

How long did you fast prior to having your blood drawn ?

__ more than 12 hours
__ 8-12 hours
__ less than 8 hours

COMMENTS :

Checked by _____ Date _____

Figure A.2. Twenty-four hour activity log

Ultramarathon Research Activity Log
Dept of Food and Nutrition

Name_____

Instructions:

1) Activity: Record all major activities for the day. Use "other" to describe activities other than those listed.

2) Duration: Record the amount of time spent at each activity to the nearest ¼ hour. (Total duration for the day should add up to 24 hours).

3) Intensity: Record the intensity of the activity by the following scale:

6
7 = Very, very light
8
9 = Very light
10
11 = Fairly light
12
13 = Somewhat hard
14
15 = Hard
16
17 = Very Hard
18
19 = Very, Very Hard (Maximal)

4) Accuracy is very important. Please complete the "Activity Log" on a daily basis. Do not wait until the end of the week and try to remember ☺.

Name:

Date	Activity	Duration	Intensity
	Sleeping		N/A
	Sitting		N/A
	Walking		
	Running		
	Other:		
	Other:		
	Comments		
	Sleeping		N/A
	Sitting		N/A
	Walking		
	Running		
	Other:		
	Other:		
	Comments		
	Sleeping		N/A
	Sitting		N/A
	Walking		
	Running		
	Other:		
	Other:		
	Comments		

Figure A.3. Diet study subject daily check in form

Ultramarathon Diet Study Daily Check In

Name:_____ Subject #_____

DATE:_____

- Record all "free" foods in exacts amounts consumed:

Coffee/Tea (cups):_____

Soda Pop (cans)/Kool aid (cups):_____

Candy, Sugar, Margarine:_____

Other (Gu, Hydrafuel, water):_____

- Record any food/beverage that you consumed which was NOT part of the diet:

_____

- How do you feel today? Excellent_____ Good_____ Fair_____ Poor_____
- Unusual Events (exams, injuries):_____
-
- Turned in urine? Yes No Weight today:_____

_____

DATE:_____

- Record all "free" foods in exacts amounts consumed:

Coffee/Tea (cups):_____

Soda Pop (cans)/Kool aid (cups):_____

Candy, Sugar, Margarine:_____

Other (Gu, Hydrafuel, water):_____

- Record any food/beverage that you consumed which was NOT part of the diet:

_____

- How do you feel today? Excellent_____ Good_____ Fair_____ Poor_____
- Unusual Events (exams, injuries):_____
- Turned in urine? Yes No Weight today:_____
-

Figure A.4. Aid station attendant instructions and data collection form

Instructions for Aid Station Attendants:

1) Pick up food, recording form, urine bottles, and urine bottle marker for your station from the kitchen the day of the race. The following foods will be "allowed". Table set up will be Saturday morning. Allow at least 30 minutes for set up (see map for location of your aid station).

Items provided by the race	Items provided by us
Water	Apple slices
Hydrafuel	Oreo Cookies
Orange slices	Vanilla Wafer
Pretzels	Unsalted pretzels
Coke	

The participants are also allowed 1 chocolate chip Clif bar and Gu. They will be carrying these with them (I think... this detail still needs to be worked out).

Please do NOT let them consume bananas, power bars, etc from the "regular race" station

2) Be on the look out for research participants. There will be 14 of them and their race number will have a yellow background instead of white. Please make sure they take foods only from your table. Write down the amount of each item they take or consume. Please be VERY specific. If they add hydrafuel to there water bottles have them do it from the cups on the table so you can record the amount that goes in the bottle.

3) Write down the time each runner passes through your station (even if they do not take anything).

4) You will have unmarked urine bottles at your station. Should a runner need one, record their race number on the bottle and note on the form that they used one. There will be a tarp or other enclosure set up for privacy for the runners. Sue and Tom will come around Sat morning and set it up. Just be aware it is for the runners and their urine bottles only!

5) Please do not leave your aid station until all the racers have passed through. John Morelock #_____ running "sweep" meaning he will be the last person on the course. Once he is through you can leave.

6) Miscellaneous: Please be prepared for rain. Have a jacket, umbrella or anything else you want to keep dry. If you have a little table and chair that you can bring out to set up that would be great. Or if you want to set up a tarp over you....

Aid Station Data Collection Form

Aid Station:_____ Attendant Name:_____

Subject	Time	H2O	Hydra	Orange	Apple	Pretzel	Oreo	Wafer	Coke

APPENDIX B

Table B.1. Individual subject physical characteristics

Subject Data

			AGE	Height (cm)	Weight (kg)	Supplement usage more than 3x/wk ?
GENDER	male	1	57	188.0	91.3	yes
		2	45	172.7	73.7	yes
		3	50	180.3	77.7	no
		4	54	177.8	68.5	no
		5	32	182.9	83.4	yes
		6	30	190.5	79.3	no
		7	47	167.6	65.2	no
		8	50	180.3	85.7	yes
		9	27	177.8	71.9	no
	female	1	43	163.8	57.7	no
		2	47	162.6	59.3	no
		3	25	165.1	59.6	no
		4	38	157.5	51.5	no
		5	50	157.5	59.5	yes

Table B.2. Individual ultramarathon data: weight loss, race time, and fluid intake

				Weight Change (pounds)	Hours to Mid point	Hours to Finsih	Fluid intake during the race (oz)
Gender	Male	1		4.8	3.75	8.75	179
		2		5.2	3.33	7.75	108
		3		7.5	2.75	6.83	124
		4		2.4	2.75	6.75	146
		5		1.3	2.25	5.00	214
		6		3.5	2.08	4.75	112
		7		.0	2.67	6.50	180
		8		.6	2.25	5.50	156
		9		8.0	2.17	6.33	78
		Total	N	9.0	9	9	9
	Female	1		4.1	2.67	6.50	78
		2		1.2	3.00	6.67	124
		3		1.7	2.75	6.50	120
		4		2.9	2.67	6.17	70
		5		2.7	3.58	8.33	136
		Total	N	5.0	5	5	5
	Total	N		14.0	14	14	14

Table B.3. Individual hematocrit, hemoglobin, plasma creatinine, and plasma urea nitrogen concentrations for fasting, pre, mid, post, and P-60 during T1 and T2

Trial	Time	Gender			Hematocrit	Hemoglobin g/dL	Plasma Creatinine umol/L	Plamsa Urea Nitrogen mg/dL
Trial 1	Fasting	Male	1		46.5	16.3	97.9	24.6
			2		48.5	16.2	81.9	23.6
			3		44.5	15.2	86.3	14.7
			4		44.5	15.1	76.3	16.5
			5		47.5	16.1	92.1	16.5
			6		44.0	14.9	80.2	9.3
			7		46.0	15.4	60.9	14.1
			8		51.0	16.4	42.9	11.7
			9		42.5	15.7	52.9	18.9
			Total	N	9	9	9	9
		Female	1		41.0	13.1	67.4	20.4
			2		41.5	13.9	65.8	16.5
			3		43.0	14.0	52.8	17.4
			4		40.5	13.5	76.5	20.1
			5		42.5	14.0	76.2	20.7
			Total	N	5	5	5	5
	Pre	Male	1		47.5	16.4	106.6	21.8
			2		49.5	16.3	79.4	21.8
			3		46.5	15.3	90.9	15.9
			4		46.0	15.9	80.3	18.3
			5		48.0	16.1	87.8	11.7
			6		45.0	15.6	81.3	12.0
			7		48.5	15.9	65.2	14.4
			8		49.5	16.2	44.7	14.4
			9		46.0	15.8	55.2	17.4
			Total	N	9	9	9	9
		Female	1		42.0	13.4	67.4	18.6
			2		42.5	13.9	61.4	13.6
			3		44.5	14.4	49.6	11.4
			4		40.5	13.3	71.6	15.3
			5		43.0	14.7	72.6	12.3
			Total	N	5	5	5	5

Data in this table have not been corrected for plasma volume changes.

(cont.)

Trial	Time	Gender			Hematocrit	Hemoglobin g/dL	Plasma Creatinine umol/L	Plamsa Urea Nitrogen mg/dL
Trial 1	Mid	Male	1		50.0	17.5	136.6	27.8
			2		50.0	17.4	85.2	23.2
			3		48.0	16.5	106.9	19.8
			4		44.5	15.4	95.9	20.4
			5		48.0	17.0	97.2	13.5
			6		44.5	16.0	102.9	17.4
			7		45.5	15.5	58.0	14.4
			8		50.0	16.8	63.6	17.7
			9		46.5	17.0	55.8	19.5
			Total	N	9	9	9	9
		Female	1		42.0	13.8	82.5	21.0
			2		43.5	14.4	68.1	15.3
			3		44.0	14.2	66.9	15.3
			4		43.5	14.3	97.6	21.0
			5		43.5	14.8	88.5	15.6
			Total	N	5	5	5	5
	Post	Male	1		47.5	16.7	143.9	32.8
			2		50.0	17.4	92.0	27.6
			3		46.5	16.1	121.3	35.7
			4		45.0	15.4	78.3	28.2
			5		48.0	16.3	114.2	13.8
			6		43.5	15.2	121.6	19.5
			7		45.5	15.0	72.8	16.5
			8		50.0	17.0	94.2	24.9
			9		45.5	16.8	65.8	30.0
			Total	N	9	9	9	9
		Female	1		40.0	13.2	98.6	26.4
			2		43.5	14.0	70.3	18.9
			3		42.0	13.4	86.4	18.9
			4		42.5	14.2	119.7	29.7
			5		42.5	14.5	97.9	21.6
			Total	N	5	5	5	5

(cont.)

Trial	Time	Gender		Hematocrit	Hemoglobin g/dL	Plasma Creatinine umol/L	Plamsa Urea Nitrogen mg/dL
Trial 1	Post-60	Male	1	46.5	16.0	156.8	31.6
			2	49.0	16.5	102.7	27.2
			3	48.0	15.8	121.8	33.2
			4	45.0	15.5	108.4	28.5
			5	47.0	16.0	109.5	20.7
			6	42.5	14.7	115.5	20.4
			7	43.0	14.0	68.9	16.8
			8	50.0	16.7	85.6	25.5
			9	44.5	15.7	65.3	30.0
			Total N	9	9	9	9
		Female	1	40.5	12.8	94.4	28.2
			2	42.0	13.7	76.3	19.5
			3	42.5	13.4	79.4	19.5
			4	40.5	13.3	107.8	31.5
			5	40.5	13.6	94.4	22.5
			Total N	5	5	5	5
Trial 2	Fasting	Male	1	46.0	15.6	102.9	27.8
			2	50.5	16.8	81.9	15.0
			3	44.0	14.4	103.3	19.5
			4	43.5	14.7	86.5	18.9
			5	48.0	16.1	80.5	15.3
			6	42.5	14.4	82.0	17.4
			7	47.0	15.5	65.8	16.2
			8	47.0	15.3	47.1	20.4
			9	44.0	15.2	49.8	19.2
			Total N	9	9	9	9
		Female	1	39.5	12.4	66.0	19.2
			2	40.5	13.7	66.6	17.4
			3	42.0	13.6	53.0	17.4
			4	42.0	13.5	72.7	10.6
			5	41.5	13.7	67.1	15.0
			Total N	5	5	5	5

(cont.)

Trial	Time	Gender			Hematocrit	Hemoglobin g/dL	Plasma Creatinine umol/L	Plamsa Urea Nitrogen mg/dL
Trial 2	Pre	Male	1		46.5	16.2	105.2	18.6
			2		47.5	16.3	78.0	18.0
			3		43.5	14.0	93.8	18.0
			4		42.5	15.1	80.3	19.2
			5		45.0	15.2	87.7	14.7
			6		45.5	14.9	80.2	15.6
			7		42.0	15.1	59.7	15.0
			8		49.5	15.6	50.9	15.9
			9		42.0	15.0	57.3	17.4
			Total	N	9	9	9	9
		Female	1		39.0	12.4	66.7	15.3
			2		40.0	13.8	64.3	13.8
			3		42.5	14.3	47.9	12.3
			4		39.5	12.7	74.0	11.8
			5		42.0	14.1	69.1	13.8
			Total	N	5	5	5	5
	Mid	Male	1		45.0	15.9	106.5	21.4
			2		48.5	16.2	70.3	13.8
			3		44.0	14.5	87.4	17.1
			4		41.0	13.9	77.7	17.4
			5		45.5	15.8	83.2	10.2
			6		45.5	15.5	73.2	16.2
			7		43.0	14.5	54.7	12.9
			8		48.5	15.6	42.8	14.4
			9		43.0	14.8	51.5	15.9
			Total	N	9	9	9	9
		Female	1		37.5	12.1	69.5	15.9
			2		40.0	13.1	56.9	13.2
			3		40.5	13.0	47.6	11.7
			4		39.5	12.5	69.6	14.7
			5		41.5	13.4	63.9	12.9
			Total	N	5	5	5	5

(cont.)

Trial	Time	Gender			Hematocrit	Hemoglobin g/dL	Plasma Creatinine umol/L	Plamsa Urea Nitrogen mg/dL
Trial 2	Post	Male	1		45.0	15.3	119.7	20.0
			2		46.5	15.8	71.2	11.7
			3		43.5	14.4	87.2	15.6
			4		41.5	14.0	76.7	16.2
			5		45.5	15.3	81.9	10.2
			6		43.5	14.9	72.9	15.0
			7		42.0	13.9	57.5	11.4
			8		47.0	15.0	50.2	11.7
			9		42.0	14.5	50.4	12.9
			Total	N	9	9	9	9
		Female	1		38.0	12.1	68.6	15.0
			2		41.5	13.4	60.4	12.3
			3		41.0	13.3	48.8	11.4
			4		39.5	12.8	65.8	13.2
			5		40.0	13.2	60.1	9.3
			Total	N	5	5	5	5
	Post-60	Male	1		45.5	15.8	104.2	19.8
			2		48.5	15.9	75.2	10.8
			3		43.0	14.0	88.1	15.0
			4		42.0	14.5	76.6	15.9
			5		45.0	15.3	80.2	8.1
			6		43.0	14.7	74.5	10.8
			7		41.5	14.2	56.3	12.9
			8		47.0	15.4	46.3	11.7
			9		41.5	14.8	51.9	12.9
			Total	N	9	9	9	9
		Female	1		38.5	12.1	68.7	15.3
			2		41.0	13.3	57.3	12.3
			3		41.5	13.6	43.7	11.4
			4		40.5	13.2	60.2	12.6
			5		40.5	13.3	76.3	9.0
			Total	N	5	5	5	5

Table B.4. Individual plasma pyridoxal 5'-phosphate (PLP), pyridoxal (PL), and pyridoxine (PN) concentrations for fasting, pre, mid, post, and P-60 during T1 and T2

Trial	Time	GENDER			PLP nmol/L	4-PA nmol/L	PL nmol/L	PN nmol/L
Trial 1	Fasting	Male	1		76.8	53.2	20.0	.0
			2		39.5	28.4	16.3	6.5
			3		43.9	35.7	9.2	4.2
			4		76.6	42.6	25.5	8.7
			5		70.9	61.7	15.0	6.8
			6		76.3	40.4	15.8	11.0
			7		71.4	17.3	12.5	9.1
			8		60.7	43.1	18.6	7.7
			9		57.2	24.5	15.0	.0
			Total	N	9	9	9	9
		Female	1		19.8	23.1	8.0	10.9
			2		46.7	29.5	9.8	13.1
			3		40.4	25.5	13.1	7.1
			4		56.2	37.7	15.2	8.8
			5		82.1	30.1	21.5	2.1
			Total	N	5	5	5	5
	Pre	Male	1		55.1	33.6	23.0	.0
			2		37.3	16.6	12.7	8.0
			3		43.0	21.4	14.5	8.1
			4		54.9	25.6	11.8	4.2
			5		46.7	24.9	13.9	11.1
			6		54.4	19.2	15.2	12.2
			7		70.7	13.1	2.6	5.0
			8		44.5	19.8	24.7	3.7
			9		52.1	21.8	9.3	6.0
			Total	N	9	9	9	9
		Female	1		25.1	15.7	7.5	12.0
			2		32.7	20.5	14.9	11.1
			3		28.7	12.3	10.9	3.8
			4		52.4	17.4	10.9	5.0
			5		53.1	27.0	28.5	5.8
			Total	N	5	5	5	5

Data in this table have not been corrected for plasma volume changes

(cont.)

Trial	Time	GENDER			PLP nmol/L	4-PA nmol/L	PL nmol/L	PN nmol/L
Trial 1	Mid	Male	1		66.4	65.3	19.0	1.5
			2		44.2	29.6	22.3	8.5
			3		55.1	32.1	8.2	4.0
			4		77.2	55.8	21.8	3.0
			5		73.4	53.1	26.6	6.1
			6		65.8	55.6	13.6	23.6
			7		82.8	16.1	10.5	11.5
			8		50.8	82.5	29.9	10.1
			9		44.2	54.5	26.5	8.5
			Total	N	9	9	9	9
		Female	1		42.7	31.2	4.2	12.6
			2		38.9	31.9	4.8	3.5
			3		32.9	52.7	4.0	.0
			4		62.5	36.4	11.1	11.3
			5		51.5	52.3	27.7	.0
			Total	N	5	5	5	5
	Post	Male	1		59.5	71.7	2.7	6.4
			2		34.2	38.3	18.0	7.4
			3		50.2	22.7	8.9	9.2
			4		64.8	68.1	10.0	1.0
			5		71.0	35.1	20.4	14.9
			6		39.3	48.1	14.2	29.1
			7		55.9	19.5	9.5	15.4
			8		32.6	147.9	22.9	5.5
			9		33.3	53.1	12.1	.0
			Total	N	9	9	9	9
		Female	1		21.0	38.6	7.2	5.6
			2		32.3	34.2	999.0	9.3
			3		29.2	22.0	17.6	6.1
			4		50.8	47.0	13.5	21.9
			5		44.6	39.9	33.4	9.9
			Total	N	5	5	4	5

(cont.)

Trial	Time	GENDER			PLP nmol/L	4-PA nmol/L	PL nmol/L	PN nmol/L
Trial 1	Post-60	Male	1		44.4	54.0	13.8	.0
			2		30.2	25.7	21.2	11.0
			3		41.6	20.9	12.3	4.4
			4		43.4	40.0	11.5	2.9
			5		54.1	16.3	22.1	1.1
			6		33.8	24.8	7.5	3.7
			7		55.9	36.7	11.3	18.8
			8		28.8	133.6	24.1	5.1
			9		22.6	48.6	13.0	6.2
			Total	N	9	9	9	9
		Female	1		26.2	39.8	20.2	4.3
			2		18.4	23.7	9.2	7.9
			3		22.3	12.9	7.9	3.7
			4		38.7	24.6	9.6	11.9
			5		36.7	25.6	22.1	10.2
			Total	N	5	5	5	5
Trial 2	Fasting	Male	1		26.2	22.4	25.0	3.2
			2		34.9	25.9	7.6	6.9
			3		43.8	24.7	7.4	12.3
			4		44.1	30.8	10.8	1.0
			5		119.8	57.9	26.9	7.8
			6		47.6	29.8	24.2	15.3
			7		103.3	28.0	23.6	8.1
			8		38.8	31.7	15.4	8.9
			9		59.9	19.7	19.9	3.7
			Total	N	9	9	9	9
		Female	1		21.7	42.3	14.0	9.8
			2		37.2	20.2	10.4	5.1
			3		45.0	20.1	16.8	11.0
			4		56.8	65.3	25.4	19.6
			5		55.0	40.0	13.7	16.5
			Total	N	5	5	5	5

(cont.)

Trial	Time	GENDER			PLP nmol/L	4-PA nmol/L	PL nmol/L	PN nmol/L
Trial 2	Pre	Male	1		28.9	22.1	5.2	4.4
			2		33.8	14.2	7.6	6.3
			3		36.0	12.3	6.7	2.6
			4		48.6	22.2	24.5	.0
			5		62.8	34.8	14.6	3.1
			6		34.7	20.2	9.7	15.1
			7		93.6	19.2	23.7	13.1
			8		31.9	20.2	4.8	5.4
			9		44.1	19.5	2.8	4.2
			Total	N	9	9	9	9
		Female	1		20.7	10.8	7.4	17.1
			2		28.9	14.8	6.0	1.1
			3		35.6	12.4	7.7	11.7
			4		43.5	14.8	14.8	8.2
			5		47.8	13.7	23.7	6.2
			Total	N	5	5	5	5
	Mid	Male	1		23.9	16.8	11.5	4.5
			2		22.4	17.3	5.1	3.5
			3		22.9	16.7	7.7	3.0
			4		36.4	17.0	23.8	.0
			5		63.9	32.3	9.1	9.9
			6		35.8	24.0	13.2	7.4
			7		80.6	16.5	27.4	7.0
			8		36.7	20.0	4.6	4.1
			9		47.6	21.0	2.6	7.0
			Total	N	9	9	9	9
		Female	1		27.4	13.2	5.8	9.5
			2		25.2	18.1	6.7	7.5
			3		24.3	12.4	3.8	3.7
			4		31.4	15.4	2.9	11.0
			5		34.9	17.4	36.3	7.3
			Total	N	5	5	5	5

(cont.)

Trial	Time	GENDER			PLP nmol/L	4-PA nmol/L	PL nmol/L	PN nmol/L
Trial 2	Post	Male	1		27.7	16.0	10.0	3.9
			2		21.0	20.5	13.3	4.7
			3		28.2	17.7	4.6	.0
			4		34.5	21.8	16.0	.0
			5		55.6	30.4	18.2	4.1
			6		39.1	20.7	20.2	9.8
			7		80.1	18.0	13.9	8.2
			8		32.5	19.7	999.0	4.3
			9		43.3	18.7	2.9	7.3
			Total	N	9	9	8	9
		Female	1		23.8	16.5	8.8	15.8
			2		27.2	16.6	6.9	.0
			3		21.6	10.5	6.3	6.1
			4		37.0	15.0	4.0	6.9
			5		36.6	17.2	14.4	8.7
			Total	N	5	5	5	5
	Post-60	Male	1		28.9	20.8	4.8	1.3
			2		24.1	18.4	11.0	10.2
			3		30.4	17.0	.0	4.2
			4		35.0	23.7	8.6	.0
			5		55.1	27.0	11.8	9.2
			6		42.8	21.4	11.0	6.7
			7		73.2	17.9	12.8	4.4
			8		27.7	20.0	5.5	6.0
			9		41.9	20.4	2.7	6.2
			Total	N	9	9	9	9
		Female	1		19.9	13.7	9.9	16.5
			2		22.8	16.2	3.3	7.2
			3		21.1	12.0	2.7	6.5
			4		32.0	15.9	2.7	9.2
			5		31.1	26.9	24.4	5.9
			Total	N	5	5	5	5

Table B.5 Individual plasma glucose, lactate, albumin concentrations and alkaline phosphatase activity for fasting, pre, mid, post, and P-60 during T1 and T2

Trial	Time	GENDER			Glucose mg/dL	Lactate mg/dL	ALP U/L	Albumin g/dL
Trial 1	Fasting	Male	1		105.4	14.50	19.56	5.9
			2		80.3	8.10	31.44	5.2
			3		71.7	17.70	27.69	5.0
			4		87.5	12.60	13.15	5.1
			5		86.4	9.40	18.00	5.7
			6		91.5	11.90	24.36	5.9
			7		89.2	13.40	16.23	5.6
			8		87.4	9.80	19.64	5.1
			9		90.6	10.60	24.03	5.7
			Total	N	9	9	9	9
		Female	1		86.4	5.90	17.68	4.9
			2		77.4	14.00	16.39	4.6
			3		77.9	6.10	18.18	5.4
			4		89.2	12.30	14.44	5.2
			5		81.5	10.20	13.79	5.2
			Total	N	5	5	5	5
	Pre	Male	1		86.0	16.70	19.40	5.4
			2		127.1	20.00	30.19	4.8
			3		66.1	17.20	26.44	4.8
			4		70.2	25.50	13.46	5.0
			5		67.6	14.80	18.00	5.3
			6		73.7	11.70	23.71	6.2
			7		77.4	19.80	16.72	5.5
			8		80.2	20.60	18.51	4.9
			9		99.2	25.30	21.92	5.6
			Total	N	9	9	9	9
		Female	1		73.7	11.40	18.15	4.9
			2		86.5	23.30	16.72	4.7
			3		71.5	20.40	17.41	5.4
			4		81.5	21.20	14.12	5.0
			5		96.0	22.20	14.44	5.2
			Total	N	5	5	5	5

999 = missing data

Data in this table have not been corrected for plasma volume changes.

(cont.)

Trial	Time	GENDER			Glucose mg/dL	Lactate mg/dL	ALP U/L	Albumin g/dL
Trial 1	Mid	Male	1		119.4	20.05	21.90	6.2
			2		137.3	25.90	35.35	5.7
			3		90.5	24.60	30.35	5.5
			4		79.8	30.80	13.46	5.1
			5		126.1	19.50	19.09	5.6
			6		86.9	25.10	24.36	6.5
			7		89.7	44.60	16.72	5.7
			8		104.2	27.90	19.81	5.5
			9		67.9	14.10	24.20	6.2
			Total	N	9	9	9	9
		Female	1		100.7	25.50	19.56	5.4
			2		122.3	29.60	17.86	5.0
			3		82.9	38.10	18.67	5.8
			4		68.4	22.20	15.91	5.6
			5		109.1	23.20	15.26	5.7
			Total	N	5	5	5	5
	Post	Male	1		118.2	16.49	19.72	6.2
			2		83.9	23.50	37.70	5.9
			3		84.9	42.40	30.19	5.7
			4		62.0	38.60	14.71	5.5
			5		90.5	22.70	19.87	5.5
			6		75.8	33.80	25.66	6.4
			7		92.8	25.70	16.88	6.0
			8		112.3	32.80	21.43	5.7
			9		82.4	16.50	24.52	6.1
			Total	N	9	9	9	9
		Female	1		82.9	28.90	20.34	5.5
			2		85.1	37.30	18.83	5.3
			3		83.3	45.20	17.86	5.6
			4		106.4	32.00	16.72	5.8
			5		95.1	25.40	17.04	5.6
			Total	N	5	5	5	5

999 = missing data

(cont.)

Trial	Time	GENDER			Glucose mg/dL	Lactate mg/dL	ALP U/L	Albumin g/dL
Trial 1	Post-60	Male	1		131.7	16.22	19.87	6.1
			2		78.3	29.20	35.04	6.0
			3		90.0	50.40	30.19	5.7
			4		68.6	35.10	14.40	5.1
			5		70.7	23.40	20.03	5.5
			6		87.5	27.90	24.36	6.3
			7		92.8	27.00	16.56	5.7
			8		86.5	26.10	21.76	5.5
			9		81.1	13.40	22.90	6.0
			Total	N	9	9	9	9
		Female	1		91.5	15.80	19.09	5.3
			2		73.8	18.90	17.69	5.2
			3		78.3	31.00	18.02	5.7
			4		119.1	22.80	15.89	5.3
			5		81.5	18.90	14.93	5.3
			Total	N	5	5	5	5
Trial 2	Fasting	Male	1		93.7	10.05	18.15	5.8
			2		72.5	15.70	34.42	5.5
			3		76.6	11.00	27.38	5.6
			4		84.3	10.50	13.93	4.8
			5		84.3	9.70	16.28	5.7
			6		91.4	6.80	24.36	6.2
			7		82.0	8.80	16.88	5.8
			8		87.4	7.90	17.86	4.8
			9		90.6	18.40	20.13	5.7
			Total	N	9	9	9	9
		Female	1		86.3	5.20	19.25	4.7
			2		79.2	14.80	15.58	4.6
			3		80.6	7.40	16.39	5.2
			4		86.0	18.90	15.26	5.2
			5		81.5	8.60	14.93	5.1
			Total	N	5	5	5	5

999 = missing data

(cont.)

Trial	Time	GENDER			Glucose mg/dL	Lactate mg/dL	ALP U/L	Albumin g/dL
Trial 2	Pre	Male	1		70.2	12.12	17.84	5.5
			2		91.5	21.50	29.26	5.0
			3		61.5	13.30	25.19	4.8
			4		66.1	22.40	13.15	4.9
			5		89.0	11.40	15.81	5.6
			6		84.9	13.40	23.06	5.5
			7		93.7	20.80	16.88	5.5
			8		80.2	20.50	17.86	4.7
			9		81.5	27.50	18.67	5.7
			Total	N	9	9	9	9
		Female	1		48.8	9.70	17.21	4.6
			2		59.3	20.80	16.72	4.6
			3		65.7	11.80	16.72	5.2
			4		91.5	20.60	15.09	4.9
			5		53.0	16.80	14.28	5.0
			Total	N	5	5	5	5
	Mid	Male	1		46.8	7.43	16.75	5.2
			2		90.0	30.40	30.98	5.0
			3		108.8	14.60	26.28	5.1
			4		108.3	16.70	13.15	4.8
			5		76.3	13.30	15.81	6.1
			6		93.6	22.40	23.06	5.9
			7		66.6	15.30	16.23	5.8
			8		101.4	14.80	18.02	4.7
			9		86.5	14.50	19.16	6.0
			Total	N	9	9	9	9
		Female	1		89.5	9.60	16.28	4.7
			2		94.2	15.10	16.72	4.8
			3		115.9	15.70	15.91	5.0
			4		90.6	23.90	15.09	5.1
			5		67.9	35.50	14.44	5.1
			Total	N	5	5	5	5

999 = missing data

(cont.)

Trial	Time	GENDER			Glucose mg/dL	Lactate mg/dL	ALP U/L	Albumin g/dL
Trial 2	Post	Male	1		53.8	9.97	17.53	5.5
			2		69.6	15.50	30.19	5.2
			3		66.5	26.00	25.03	5.2
			4		70.1	10.90	12.84	5.0
			5		94.0	20.40	16.12	6.2
			6		62.5	16.90	22.41	5.6
			7		81.9	24.20	15.26	5.6
			8		79.2	23.80	18.34	4.8
			9		81.4	14.60	18.51	6.1
			Total	N	9	9	9	9
		Female	1		78.7	7.73	17.06	4.9
			2		81.4	14.90	18.18	5.0
			3		80.1	18.90	15.58	5.5
			4		81.0	14.80	14.12	5.1
			5		67.1	17.80	15.42	5.3
			Total	N	5	5	5	5
	Post-60	Male	1		76.1	9.69	18.47	5.7
			2		75.3	27.20	30.82	5.3
			3		67.6	10.20	25.97	5.2
			4		91.5	11.30	13.46	5.2
			5		61.0	14.40	15.81	6.2
			6		66.1	18.20	21.76	5.7
			7		67.5	12.50	15.91	5.6
			8		51.6	12.70	18.99	4.9
			9		92.8	16.30	18.67	6.1
			Total	N	9	9	9	9
		Female	1		79.3	9.70	17.21	5.0
			2		72.5	11.10	16.72	4.7
			3		47.5	13.00	16.39	5.5
			4		86.0	11.00	14.93	5.3
			5		78.3	12.40	13.79	5.1
			Total	N	5	5	5	5

999 = missing data

Table B.6. Individual daily urine volumes and urinary creatinine, urea, total nitrogen, and 4-pyridoxic acid excretion for days one through five during T1 and T2

Trial	Day	Gender			Urine Volume (mL)	Urinary Creatinine g/24 hr	Total Urinary Nitrogen g/24 hr	Urinary Urea Nitrogen g/24 hr	Urinary 4-PA umol/day
Trial 1	Day 1	Male	1		2120	2.4	999	13.6	16.0
			2		1500	1.6	999	12.3	7.0
			3		4200	1.8	999	12.7	12.0
			4		3800	1.5	999	12.5	11.8
			5		4300	2.4	999	19.0	18.7
			6		3140	2.3	999	14.8	11.9
			7		2620	1.6	999	11.2	7.7
			8		2200	.8	999	9.6	13.0
			9		1700	2.0	999	13.0	9.7
			Total	N	9	9		9	9
		Female	1		2700	1.3	999	11.4	6.9
			2		5000	1.3	999	10.2	7.0
			3		1900	1.2	999	11.1	5.7
			4		1300	1.3	999	11.9	7.9
			5		3000	1.1	999	11.2	8.8
			Total	N	5	5		5	5
	Day 2	Male	1		2500	2.4	18.8	16.4	14.1
			2		2000	1.6	16.4	13.6	7.4
			3		3300	1.9	14.3	11.4	9.7
			4		4000	1.4	17.2	14.3	10.9
			5		4200	2.4	19.2	16.1	14.8
			6		2700	2.3	13.2	12.9	8.6
			7		2900	1.5	13.2	11.6	6.1
			8		2200	1.6	15.1	12.0	9.5
			9		1800	1.5	14.8	11.8	6.7
			Total	N	9	9	9	9	9
		Female	1		4100	1.3	13.0	11.1	6.3
			2		5200	1.3	12.7	10.3	7.0
			3		2600	1.2	13.3	11.1	4.6
			4		1500	1.0	10.3	8.5	5.8
			5		3600	1.2	11.9	10.5	8.9
			Total	N	5	5	5	5	5

999 = missing data

(cont.)

Trial	Day	Gender			Urine Volume (mL)	Urinary Creatinine g/24 hr	Total Urinary Nitrogen g/24 hr	Urinary Urea Nitrogen g/24 hr	Urinary 4-PA umol/day
Trial 1	Day 3	Male	1		3100	2.2	17.0	14.0	11.2
			2		3300	1.7	17.6	13.7	7.1
			3		2900	1.8	13.3	10.0	8.4
			4		3100	1.5	15.9	13.1	9.3
			5		5600	2.6	19.1	16.0	13.4
			6		2700	2.1	13.5	10.7	9.8
			7		2800	1.8	15.8	11.6	7.2
			8		3300	2.0	18.1	14.4	11.3
			9		2900	2.0	18.9	15.6	9.0
			Total	N	9	9	9	9	9
		Female	1		5000	1.4	12.9	10.7	6.8
			2		6300	1.3	10.6	10.0	6.1
			3		3900	1.2	11.6	8.9	3.8
			4		2000	1.0	9.4	7.7	4.7
			5		3700	1.2	12.6	10.8	8.8
			Total	N	5	5	5	5	5
	Day 4	Male	1		3600	2.8	20.4	17.5	10.3
			2		2100	1.5	12.8	10.7	6.8
			3		2300	1.9	13.8	11.1	7.7
			4		4300	1.5	19.6	16.0	7.7
			5		6200	2.6	21.6	17.9	11.8
			6		4400	2.8	22.9	17.6	11.0
			7		4800	1.7	18.0	14.3	6.0
			8		1600	1.7	14.1	11.7	9.2
			9		1400	1.9	18.0	15.5	9.5
			Total	N	9	9	9	9	9
		Female	1		1800	1.3	15.1	11.7	5.9
			2		4600	1.3	14.2	11.3	5.4
			3		3700	1.4	13.4	10.7	3.9
			4		1600	1.6	15.2	12.7	7.2
			5		2200	1.3	14.3	11.4	7.4
			Total	N	5	5	5	5	5

999 = missing data

(cont.)

Trial	Day	Gender			Urine Volume (mL)	Urinary Creatinine g/24 hr	Total Urinary Nitrogen g/24 hr	Urinary Urea Nitrogen g/24 hr	Urinary 4-PA umol/day
Trial 1	Day 5	Male	1		1600	2.2	20.3	17.7	12.9
			2		1700	1.8	17.3	14.2	7.6
			3		2000	1.7	15.1	12.3	7.5
			4		2500	1.5	16.8	15.6	9.4
			5		4000	2.4	19.4	15.3	12.6
			6		2400	2.1	18.9	14.8	9.8
			7		1800	1.7	15.4	12.7	8.0
			8		1600	1.9	20.2	17.6	11.6
			9		1700	1.9	17.8	15.3	7.9
			Total	N	9	9	9	9	9
		Female	1		2200	1.3	14.6	15.9	6.3
			2		6100	1.2	13.1	10.7	5.7
			3		3700	1.3	12.3	9.7	5.0
			4		1500	1.0	9.6	7.7	4.3
			5		2500	1.2	11.5	9.5	8.0
			Total	N	5	5	5	5	5
Trial 2	Day 1	Male	1		3000	2.4	999	18.0	7.7
			2		2100	1.4	999	10.6	5.7
			3		3600	1.9	999	13.2	8.2
			4		3800	1.5	999	12.8	9.6
			5		3300	2.3	999	17.2	18.6
			6		1900	2.1	999	14.6	10.1
			7		2300	1.7	999	12.9	11.3
			8		999	1.9	999	12.8	9.0
			9		2000	1.9	999	13.5	6.8
			Total	N	9	9		9	9
		Female	1		1700	1.2	999	9.7	5.7
			2		5100	1.2	999	9.5	4.7
			3		2500	1.3	999	10.2	5.9
			4		2000	1.4	999	9.7	6.7
			5		2400	.9	999	7.0	6.8
			Total	N	5	5		5	5

999 = missing data

(cont.)

Trial	Day	Gender			Urine Volume (mL)	Urinary Creatinine g/24 hr	Total Urinary Nitrogen g/24 hr	Urinary Urea Nitrogen g/24 hr	Urinary 4-PA umol/day
Trial 2	Day 2	Male	1		3800	2.2	19.4	16.8	6.5
			2		2800	1.7	15.8	12.5	6.6
			3		3100	1.8	13.6	11.5	7.3
			4		3300	1.5	13.8	11.5	7.4
			5		4300	2.3	17.0	13.5	14.7
			6		2700	2.3	19.2	14.5	9.3
			7		1600	1.5	14.0	12.3	8.0
			8		2100	.9	8.8	7.1	3.9
			9		1400	2.1	16.9	14.4	6.8
			Total	N	9	9	9	9	9
		Female	1		2900	1.4	15.6	12.3	6.6
			2		5600	1.3	12.2	9.7	4.8
			3		1800	1.1	10.0	8.8	4.9
			4		1400	1.2	10.5	8.6	4.8
			5		3300	1.3	11.9	9.3	8.4
			Total	N	5	5	5	5	5
	Day 3	Male	1		3400	2.3	16.3	14.6	7.6
			2		1500	1.5	12.9	10.6	6.3
			3		3200	1.9	14.3	11.4	6.9
			4		3400	1.5	14.9	12.5	7.9
			5		3900	2.4	17.3	13.1	13.7
			6		2600	2.0	14.6	12.4	7.6
			7		2100	1.6	13.2	11.6	8.2
			8		3800	3.0	24.7	20.4	12.5
			9		1200	1.8	15.8	13.5	6.7
			Total	N	9	9	9	9	9
		Female	1		2300	1.3	11.4	9.2	5.5
			2		5300	1.3	12.5	9.9	5.0
			3		2900	1.2	10.7	8.4	5.8
			4		1800	1.3	10.8	9.2	5.0
			5		2600	1.5	11.7	9.1	9.8
			Total	N	5	5	5	5	5

999 = missing data

(cont.)

Trial	Day	Gender			Urine Volume (mL)	Urinary Creatinine g/24 hr	Total Urinary Nitrogen g/24 hr	Urinary Urea Nitrogen g/24 hr	Urinary 4-PA umol/day
Trial 2	Day 4	Male	1		5400	2.7	15.4	13.0	7.6
			2		4600	1.8	13.6	11.3	5.7
			3		5800	2.0	15.4	12.0	6.1
			4		3700	1.5	14.9	12.6	7.7
			5		6100	2.4	15.5	10.9	12.0
			6		4000	2.6	16.3	13.3	9.2
			7		4600	1.7	14.2	10.5	7.7
			8		4200	2.0	15.8	12.7	8.9
			9		3700	2.1	16.0	12.8	7.9
			Total	N	9	9	9	9	9
		Female	1		2900	1.4	13.1	10.8	5.8
			2		5700	1.2	11.0	8.7	4.2
			3		4200	1.4	11.5	8.8	4.3
			4		1700	1.2	9.0	7.0	4.2
			5		3800	1.5	12.9	9.9	8.3
			Total	N	5	5	5	5	5
	Day 5	Male	1		3700	2.5	17.2	13.0	7.6
			2		1800	1.6	12.3	9.8	5.1
			3		2700	1.8	13.9	10.9	6.0
			4		3500	1.4	14.4	12.4	6.7
			5		4800	2.5	18.8	13.6	11.8
			6		1900	2.1	15.3	12.2	7.4
			7		2300	1.7	15.1	12.4	7.3
			8		2100	1.8	13.5	11.0	9.1
			9		2100	2.1	15.0	12.1	6.4
			Total	N	9	9	9	9	9
		Female	1		2400	1.5	14.4	12.3	5.9
			2		6800	1.3	13.1	10.7	4.5
			3		2500	1.3	12.4	9.9	4.1
			4		2100	1.3	11.8	9.8	5.1
			5		2700	.9	8.7	6.3	4.9
			Total	N	5	5	5	5	5

999 = missing data

Table B.7. Vitamin B-6 content of the diet

Vitamin B-6 Content of Menu 1 and Menu 2, for Trial 1 and Trial 2

		Female Menu 1 (mg/day)	Female Menu 2 (mg/day)	Male Menu 1 (mg/day)	Male Menu 2 (mg/day)
TRIAL	1	1.69	1.48	2.07	1.85
	2	1.47	1.47	2.02	1.93

Table B.8. Nitrogen content of the diet

Nitrogen Content of Menu 1 and 2, for Trial 1 and Trial 2

		Female Menu 1 (g/day)	Female Menu 2 (g/day)	Male Menu 1 (g/day)	Male Menu 2 (g/day)
TRIAL	1	13.8	14.4	18.3	18.0
	2	13.6	14.4	18.5	18.1

APPENDIX C.

Figure C.1. Menu 1 and Menu 2 (foods items during T1 and T2).

Menu 1(Wednesday, Friday, and Sunday)

* Amount in ( ) indicates portion size for females

	Amount	Food Item
Breakfast....		
	200g	Orange Juice
	230 g	Milk, 1%
	40g (27 g)	Oatmeal (uncooked)
	15g	Brown Sugar
	120g (100g)	Blueberries (frozen)
	60 g (30g)	Bread, whole wheat
	4 tsp (2 tsp)	Margarine
Lunch...		
	1	Tuna Sandwich --Males: 60g bread, 80g tuna, 35g mayo, 15g relish, 15g lettuce --Females: 60g bread, 55g tuna, 25g mayo, 15g relish, 15g lettuce
	44g (33g)	Oreo Cookies
	1 svg	Jello with Peaches (100g peaches, 200g Jello)
Dinner		
	1 svg	Spaghetti --Males: 85g noodles (dry), 100g chix, 175g sauce --Females: 65g noodles (dry),40g chix, 150g sauce
	30g (20g)	Parmesan Cheese
	50g	Sourdough Roll
	10g	Margarine
	100g (80g)	Brocccoli
	1	Green Salad with Ranch Dressing (60g lettuce, 50g cucumber, 15g cabbage, 20g dressing)
	120g	Vanilla Ice Cream (low fat)
Snack....		
	50g (40g)	Graham Crackers

Menu 2 (Thursday and Saturday)

* Amount in ( ) indicates portion size for females

	Amount	Food Item
Breakfast....		
	200g	Orange Juice
	230g	Milk, 1%
	60g (40g)	Puffed Wheat Cereal
	100g	Bagel, whole wheat
	30g	Jelly
	30g (20g)	Peanut Butter
Lunch...		
	1	Roast beef /cheese sandwich --Males: 70g beef, 30g cheese, 10g mayo, 5g mustard, 15g lettuce --Females: 45g beef, 30g cheese, 10g mayo, 5g mustard, 15g lettuce
	50g	Chocolate Chip Cookies
	100g	Pineapple
	70g (20g)	Carrot Sticks
	70g (20g)	Celery Sticks
Dinner		
	120g (65g)	Sliced Turkey Breast
	85g (55g)	Steamed Rice (dry wt)
	100g	Green Beans
	40g	Whole wheat roll
	10g	Margarine
	1	Green Salad with French Dressing (60g lettuce, 50g cucumber, 15g cabbage, 20g dressing)
	120g (90g)	Rainbow Sherbet
Snack....		
	50g (40g)	Vanilla Wafers

Figure C.2. Instructions for metabolic kitchen personnel

Menu #1

- Unless otherwise indicated, prepare 14 portions
- All labeling should include the subjects initials and wt of the product

Breakfast (For consumption Wed, Fri, and Sun)

Orange Juice—Make up 2 12 oz cans per day. Empty on thawed 12 oz can into a 1000 ml graduated cylinder. Add water to make 1000 ml (be sure to rinse can and lid with some water). Pour into plastic pitcher. Add another 450 ml water and stir well. Weigh out 200 grams into a 250 ml Nalgene containers with screw top. Label and refrigerate. Take 2 12 oz cans from freezer to thaw for next day.

Milk—Weigh out 230 grams of milk into a 250 ml Nalgene container with screw top. Label and refrigerate.

Oatmeal—Pre-weighed. 40g for males; 27g for females

Blueberries—Pre-weighed in freezer (males 120g, females 100g). Transfer to 400ml round screw top container. Label with initials and wt.

Bread—Weigh 10 portions @ 60 grams (2 slices) and 4 portions @ 30 grams (1 slice). Place in baggie and label.

Margarine—Pre-weighed (males 20g, females 10g)

- Assemble each subjects breakfast items into one sack and label with meal, menu number, and subject name.

Lunch Menu #1 (for consumption Wed, Fri, Sun)

Tuna Sandwich preparation—

--Males (make 10):

- Place small plastic mixing bowl on scale and tare. Add 35 g low cal mayo. Tare. Add 15 g relish. Tare. Add 80 g tuna. Mix together thoroughly.
- Weigh out 60 g of bread (2 slices). Spread tuna mixture on bread. (Scrape out bowl with spatula to ensure complete transfer of contents). Add 15 g Romaine lettuce and place in baggie.
- Label (initials and total wt)

--Females (make 4):

- Place small plastic mixing bowl on scale and tare. Add 25 g low cal mayo. Tare. Add 15 g relish. Tare. Add 55 g tuna. Mix together thoroughly.

- Weigh out 60 g of bread (2 slices). Spread tuna mixture on bread. (Scrape out bowl with spatula to ensure complete transfer of contents). Add 15 g Romaine lettuce and place in baggie.
- Label (initials and total wt)
- Jello with Peaches (14 servings)
- Weigh 100 g peaches (drained) into 400 ml round plastic container.
- Prepare jello. While still warm and liquid, weigh out 200 g and pour over peaches.
- Cover, label with subject initials and refrigerate.

Oreo Cookies: Pre-weighed (males 44g, females 33g)

- Assemble each subjects lunch items into one sack and label with meal, menu number, and subject name.

Dinner Menu #1

Milk: Weigh out 230g into glass. Cover, label, refrigerate.

Spaghetti

- Spaghetti Noodles: (Noodles will be cooked in 3 batches):

--Weigh out 425g of spaghetti (to feed 5 males). Bring 1 ½ gallons of water to a boil (add ½ tsp salt and 1 Tsp oil). Break spaghetti noodles in half and add to boiling water. Bring water back to boil and cook on medium for 10 minutes *exactly*. Drain noodles in colander and immediately rinse for 5 minutes with cold water. Get out 5 plates and one plastic weighing dish. Weigh out 5 170 g portions and place each on a plate. Weigh the remaining noodles and divide into 5 equal portions. Add one portion to each of the 5 plates. (End result is to have 425g of dry noodles in 5 equal cooked portions). Repeat this process.

--Weigh out 260g of spaghetti noodles (to feed 4 females). Bring 1 ½ gallons of water to a boil (add ½ tsp salt and 1 Tsp oil). Break spaghetti noodles in half and add to boiling water. Bring water back to boil and cook on medium for 10 minutes *exactly*. Drain noodles in colander and immediately rinse for 5 minutes with cold water. Get out 5 plates and one plastic weighing dish. Weigh out five 120g portions and place each on a plate. Weigh the remaining noodles and divide into 5 equal portions. Add one portion to each of the 5 plates. (End result is to have 260g of dry noodles in 4 equal cooked portions).

- Spaghetti Sauce:

--Males: Into a small plastic container, weigh 175g of sauce and pour over a "males" portion of noodles (approx 200g). Scrape bowl with a spatula to ensure complete transfer of contents.

--Females: Into a small plastic container, weigh 150g of sauce and pour over a "females" portion of noodles (approx 150g). Scrape bowl with a spatula to ensure complete transfer of contents.

Parmesan Cheese: Pre-weighed 30g (male) and 20g (female) portions.

Broccoli (thawed and "drained in colander for 10 minutes):

--Males: Weigh out 100g into bowl. Label and refrigerate.
--Females: Weigh out 80g into bowl. Label and refrigerate.

Sourdough roll: Weigh out 50g portions. Label

Margarine: Pre-weighed @10g

Salad preparation: Prepare ingredients for all salads before weighing individual portions.
--Iceberg Lettuce: Each day, wash 2 pounds, drain, then tear into bite size pieces.
--Red Cabbage. Weigh out 220g shredded cabbage.
--Cucumber: Wash and slice 800g cucumber.

- Assembly. Place serving bowl on scale. Tare. Add 60 g lettuce. Tare. Add 15 g cabbage. Tare. Add 50g sliced cucumber. Tare. Add 20g ranch dressing. Cover, label and refrigerate.

Ice-cream: Weigh out 120g servings into serving bowls. Label and freeze.

Graham Crackers: Pre-weighed in 50g (male) and 40g (female) portions.

- As subjects arrive, microwave spaghetti and broccoli.

Menu #2

Breakfast Menu # 2 (For consumption Thur and Sat)

Orange Juice: Make up 2 12 oz cans per day. Empty on thawed 12 oz can into a 1000 ml graduated cylinder. Add water to make 1000 ml (be sure to rinse can and lid with some water). Pour into plastic pitcher. Add another 450 ml water and stir well. Weigh out 200 grams into a 250 ml Nalgene containers with screw top. Label and refrigerate. Take 2 12 oz cans from freezer to thaw for next day.

Milk: Weigh out 230 grams of milk into a 250 ml Nalgene container with screw top. Label and refrigerate.

Bagel: Weigh out 100 grams of bagel. Trim or add if necessary. Place in baggie and label.

Peanut butter—Pre-weighed: (Males 30 g ; females 20 g).

Jelly—Use 2 "ready serve" packages. (jelly wt 30g)

Puffed wheat—Pre-weighed (Males 40 g; females 27 g)

- Assemble each subjects breakfast items into one sack and label with meal, menu number and subject name.

Lunch Menu #2 (for consumption Thur and Sat)

Roast Beef sandwich preparation—
--Males (make 10):
- Place small paper plate on scale and tare. Add 60 g of bread. Tare. Spread on35 g low cal mayo. Tare. Add 15 g relish. Tare. Add 80 g tuna. Mix together thoroughly.
- (2 slices). Spread tuna mixture on bread. (Scrape out bowl with spatula to ensure complete transfer of contents). Add 15 g Romaine lettuce and place in baggie.
- Label (initials and total wt)

--Females (make 4):
- Place small plastic mixing bowl on scale and tare. Add 25 g low cal mayo. Tare. Add 15 g relish. Tare. Add 55 g tuna. Mix together thoroughly.
- Weigh out 60 g of bread (2 slices). Spread tuna mixture on bread. (Scrape out bowl with spatula to ensure complete transfer of contents). Add 15 g Romaine lettuce and place in baggie.
- Label (initials and total wt)

Pineapple Chunks: Drain pineapple in colander. Into 200 ml round bowl with screw top lid, weigh 100g portions. Label and refrigerate.

Carrot and Celery Sticks (already peeled and cleaned in refrigerator).
Males: Weigh out 70 g carrots and 70g celery. Place in baggie. Label
Females: Weigh out 20g of carrots and 20g of celery. Store in baggie. Label

Chocolate Chip Cookies: Pre-weighed (50g). Label.

- Assemble each subjects lunch items into one sack and label with meal, menu number, and subject name.

Dinner Menu #2

Milk: Weigh 230g into glass. Cover, label, refrigerate.

Rice (Preheat 3 ovens to 350 degrees)
--Males: Place 85 g rice in glass casserole dish. Add 230ml water. Cover and bake 20 minutes (6 casseroles per oven)
--Females: Place 55 g rice in glass casserole dish. Add 150 ml water. Cover and bake 20 minutes.

Turkey: Pre-sliced and weighed into 120g (male) and 65g (female) portions.

Whole wheat roll: Weigh into 40g portions>

Margarine: Pre-weighed 10g portions.

Green Beans. Drain green beans and weigh out 100g into each serving bowl.

Rainbow sherbet: Weigh 120g (male) or 90g (female) into serving dishes. Cover, label, freeze.

Vanilla Wafers: Pre-weighed into 50g (male) and 40g (female) portions.

Figure C.3. Instructions for lab personnel for urine processing and storage

Urine Processing and Storage Procedures

1. Take bottles from the refrigerator in Rm 106. Sort by subjects. Make sure all bottles or the day have been brought in. Take to Rm 6 and place in refrigerator.

2. For each subject (one at a time):
- Remove urine from refrigerator.
- Transfer to a glass 2 liter graduated cylinder. Rinse each bottle and lid and include in total urine volume.
- Add water to bring volume up to the nearest 100-ml mark.
- Record urine volume in lab book. Also record the number of urine bottles.
- If volume is greater than 2000 ml transfer to a plastic bucket. (Be sure to keep track of total urine volume in the bucket).
- MIX, MIX, MIX. Mix well by inverting at least 8 times.
- Aliquot urine into prelabeled containers as follows:

Container	Number	Assay
50 ml (fill ONLY 2/3 full!)	1	Extra
20 ml plastic vial (10-15 ml/vial)	2	4-PA
10 ml glass tube (No more than 4-5 ml)	1	creatinine

- To each label add Total Volume.

3. Store all bottle/tubes in appropriate freezer.

4. Rinse graduated cylinder and beakers with tap water then purified water before doing next urine sample.

5. When finished with all urines, wash graduated cylinder, bucket, etc with soapy water. **Rinse well.**

Figure C.4. General instructions for participants: meals and urine collections

General Instructions for Diet Study

Urine Collection Procedures:

1. Collect ALL urine in containers provided (24-hour urine collection). You will receive new containers each evening.

2. Ensure all containers are labeled with your initials and the correct date.

3. Each day:
   Urine collections will be made on a 24-hour basis. For example, from 7 am the 21st to 7 am the 22nd. Therefore, the collection made on rising in the morning belongs with the urine collected the previous day. (The 24-hour collection is all urine *produced* in a 24-hour period. The first urine in the morning was *produced* during the night so belongs in the previous day's collection).
   It is important that the collection made on rising is done at the same time each day. In other words, for each day of the diet study get up at the same time each morning.

4. Urine collection will start the morning of the first day of the diet study.

5. Store urine in a cool place and protected from the light.

6. Urine should be delivered to Milam Hall, room 106 every day. You may bring it in the morning (preferred), during the day, or bring it with you to the evening meal.

7. Urine collections are a critical pat of this study. Please be careful not to spill or lose any urine. If you make a mistake please let us know so we can make a note of it.

8. Drink approximately the same amount of fluids each day if possible.

Diet Procedures:

1. Eat ALL food given to you each day. All foods have been weighed to the nearest 0.1 gram. It is important that you consume it all. Use the "spatula" to scrape out your food containers the best you can. Rinse all containers with a small amount of water and consume this water. Bring back your breakfast and lunch containers each evening. Keep the spatula for the duration of the study.

2. NO alcoholic beverage.

3. No vitamin or mineral supplements.

Figure C.5. Detailed daily participant instructions

April....Detailed Participant Schedule

(Questions or conflicts??? Call me! Ann Grediagin 754-1396)

Version 3

Date	Time	Task Description
Apr 5		Race Registration
		Health History Completed
		Informed Consent Completed
Apr 12	7 am	Stop all "normal" vitamin and mineral supplements
	8 am	Begin "activity log" (everyday for the next 2 weeks)
Apr 20	8 pm	Do not eat or drink (except H_20) until after your "fasting" blood draw tomorrow.
Apr 21	8-9 am	Come to the Nutrition Lab to have your blood drawn
	8-9 am	Begin "diet". Pick up breakfast and lunch from Nutrition Lab.
	8-9 am	Begin urine collection. Pick up urine bottles from Nutrition Lab.
	5-7 pm	Eat dinner (kitchen upstairs)
	5-7 pm	Pick up urine bottles for the 22nd
	5-7 pm	Weigh in, compete food record and activity/sleep log
	5-7 pm	Pick up breakfast and lunch for the 22nd
Apr 22	7 am	Complete 1st 24 hour urine collection
	701 am	Begin 2nd 24 hour urine collection (new bottle!!)
	5-7 pm	Deliver 1st 24 hour urine collection to the lab (downstairs)
	5-7 pm	Pick up urine bottles for the 23rd
	5-7 pm	Eat Dinner (kitchen upstairs)
	5-7 pm	Weigh in, complete food record and activity/sleep log
	5-7 pm	Pick up breakfast and lunch for the 23rd
Apr 23	7 am	Complete 2nd 24 hour urine collection
	701 am	Begin 3rd 24 hour urine collection (new bottle)
	5-7 pm	Deliver 2nd 24 hour urine collection to the lab (downstairs)
	5-7 pm	Pick up urine bottles for the 24th
	5-7 pm	Eat Dinner (kitchen upstairs)
	5-7 pm	Immediately **after** your dinner, take 1 Vitamin E supplement
	5-7 pm	Weigh in, complete food record and activity/sleep log
	5-7 pm	Pick up breakfast for the 24th
Apr 24	5 am	Eat your bagel, jelly, and OJ (water, coffee, tea O.K. too)
	7 am	Complete 3rd 24 hour urine collection
	701 am	Begin 4th 24 hour urine collection...be sure to take your bottle to the race with you!!
	730-750am	Pre race blood draw
	800-???	Have fun running. At the aid stations be sure the "recorder" writes down what you eat and drink. If you have to pee during the race there will be urine bottles at each aid station...please wait and use one!!
	Mile 15	The midpoint blood draw will take place at the bottom of Extendo. We will have a tent set up at the aid station. While you are giving blood we will fill your water bottles for you!!
	Post race	Within 5 minutes of race completion, have your blood drawn.

		remain "inactive" (desk work is O.K.)
	Post inactivity	After your final blood draw you can eat... we will have your lunch ready!
	5-7 pm	Deliver 3rd 24 hour urine collection
	5-7 pm	Pick up urine bottles for the 21st
	5-7 pm	Eat Dinner (kitchen upstairs)
	5-7 pm	Weigh in, complete food record and activity/sleep log
	5-7 pm	Pick up breakfast and lunch for the 21st
May 21	700 am	Complete 4th 24 hour urine collection
	701 am	Begin 5th urine collection
	5-7 pm	Deliver 4th 24 hour urine collection to the lab (downstairs)
	5-7 pm	Eat Dinner (kitchen upstairs)
	5-7 pm	Weigh in, complete food record and activity/sleep log
May 22	7 am	Complete 5th urine collection
	701 am	Eat what ever you desire!! Pee where ever you want!!!!!
	8-10 am	Deliver final urine collection to the lab...hand over the urine and we will hand over the check!!

MAY....Detailed Participant Schedule

(Questions or conflicts??? Call me! Ann Grediagin 754-1396)

Version 3

Date	Time	Task Description
May 8	8 am	Stop all "normal" vitamin and mineral supplements
	8 am	Begin "activity log" (everyday for the next 2 weeks)
May 16	8 pm	Do not eat or drink (except H_20) until after your "fasting" blood draw tomorrow.
May 17	8-9 am	Come to the Nutrition Lab to have your blood drawn
	8-9 am	Begin "diet". Pick up breakfast and lunch from Nutrition Lab.
	8-9 am	Begin urine collection. Pick up urine bottles from Nutrition Lab.
	9 am	Begin same "activity pattern" you had Apr 21 to 25th
	5-7 pm	Eat dinner (kitchen upstairs)
	5-7 pm	Pick up urine bottles for the 18th
	5-7 pm	Weigh in, compete food record and activity/sleep log
	5-7 pm	Pick up breakfast and lunch for the 22nd
May 18	7 am	Complete 1st 24 hour urine collection
	701 am	Begin 2nd 24 hour urine collection (new bottle!!)
	5-7 pm	Deliver 1st 24 hour urine collection to the lab (downstairs)
	5-7 pm	Pick up urine bottles for the 19th
	5-7 pm	Eat Dinner (kitchen upstairs)
	5-7 pm	Weigh in, complete food record and activity/sleep log
	5-7 pm	Pick up breakfast and lunch for the 19th
May 19	7 am	Complete 2nd 24 hour urine collection
	701 am	Begin 3rd 24 hour urine collection (new bottle)
	5-7 pm	Deliver 2nd 24 hour urine collection to the lab (downstairs)
	5-7 pm	Pick up urine bottles for the 20th
	5-7 pm	Eat Dinner (kitchen upstairs)
	5-7 pm	Immediately **after** your dinner, take 1 Vitamin E supplement
	5-7 pm	Weigh in, complete food record and activity/sleep log
	5-7 pm	Pick up breakfast for the 20th
May 20	630-730am	Eat your bagel, jelly, and OJ (water, coffee, tea O.K. too)
	7 am	Complete 3rd 24 hour urine collection
	701 am	Begin 4th 24 hour urine collection
	930-1030	Blood Draw (nutrition lab)
	0930-???	At the same intervals as race day, have your blood drawn. Additionally, consume ONLY the foods you did during the race (same foods, same times). During this time you must

		Don't eat anything for the next 60 minutes (500 ml water is O.k.). 60 minutes after race completion have your blood drawn again. Now you can eat…we will have your lunch ready!
	5-7 pm	Deliver 3rd 24 hour urine collection
	5-7 pm	Pick up urine bottles for the 25th
	5-7 pm	Eat Dinner (kitchen upstairs)
	5-7 pm	Weigh in, complete food record and activity/sleep log
	5-7 pm	Pick up breakfast and lunch for the 25th
Apr 25	700 am	Complete 4th 24 hour urine collection
	701 am	Begin 5th urine collection
	5-7 pm	Deliver 4th 24 hour urine collection to the lab (downstairs)
	5-7 pm	Eat Dinner (kitchen upstairs)
	5-7 pm	Weigh in, complete food record and activity/sleep log
Apr 26	7 am	Complete 5th urine collection
	8-10 am	Deliver final urine collection to the lab…now you are done until May!!

Figure C.6. Informed consent

Department of Nutrition and Food Management
Oregon State University

Informed Consent

A. Title: Plasma Vitamin B-6, Coenzyme Q, and Vitamin E changes following a 50 K-ultramarathon

B. Investigators: James E. Leklem, PhD., Maret G. Traber, PhD, and Ann Grediagin, MS

C. Purpose: You are being asked to participate in a research study. The purpose is to examine the effect an ultramarathon has on the metabolism of vitamin B-6, Coenzyme Q (CoQ), vitamin E, and cellular antioxidant mechanisms in men and women. Blood plasma, red blood cells and urine will be collected and analyzed. The information sought will help give nutrition researchers and councilors a better understanding of the metabolism and requirements these nutrients during lengthy endurance events.

D. Procedures: I have received an oral and written explanation of this study and I understand that as a participant in this study the following things will happen:

1. ***Screening***: I will fill out a medical history and health questionnaire prior to becoming a subject in the study. Also, during this interview I will give my age, weight, height, & training habits. After the prescreening, I am aware that I may or may not be asked to participate in the study.

2. ***Diet.*** I will be asked to consume a controlled diet that will be provided for me for 5 days (3 days prior to the race, race day, and 1 day postrace) which will contain 15-20% protein, 55-65% carbohydrate, 15-30% fat and adequate calories. During the race I will eat and drink only the allowed foods and beverages which do not contain significant amounts of vitamin B-6. After the race, except for up to 500 ml of water, I will not consume any food or beverages for an hour. Other than the vitamin E supplement provided in the study. My weight will be taken at the beginning and end of the race.

3. ***Supplements***: After dinner on April 23rd I will be asked to consume a vitamin E supplement that has been specially "tagged" (deuterated) to make it heavier so it can be measured. I understand that the vitamin E is NOT radioactive, is not harmful in any way, and except for the "tagging" is identical to the vitamin E available in drug stores.

4. ***Urine Collection:*** I will be required to collect all my urine for the 5 days I consume the controlled diet. I will use the containers provided and deliver them to the laboratory on a daily basis.

5. ***Blood Draws***: I will have 19 milliliters (slightly over 1 tablespoon) of blood drawn, by a trained phlebotomist, from the anticubital vein in my forearm, under sterile conditions, 3 days prior to the race, 30-60 minutes prior to the race, between miles 15 and 20 during the race, 5 minutes after the race, and 60 minutes after the race. In total, blood will be drawn from me 5 times. For the fasting blood draw 3 days prior to the race, I am aware that I am not to eat or drink anything except water after 8 pm the night before. The total blood drawn will be 95 ml ($1/3^{th}$ of a cup) or about 15% of what you might donate for the Red Cross.

6. ***Activity Log***. For the 2 weeks before both the ultramarathon and the postrace control study I will record on a daily basis the amount, type, and intensity of exercise performed each day.

7. ***Postrace Control Study:***
Diet, urine, blood: During the week of May 17^{th} I will be asked to complete a postrace control study. During this 5 day study I will consume the same diet (provided to me), collect my urine, and have my blood drawn the same as I did during the ultramarathon portion of the study. On day four of the study (when the ultramarathon occurred) I will eat my specified breakfast 2 hours prior to reporting to the Nutrition Lab. I will report to the lab at a specified time between 0730 and 0930, have blood drawn at the same intervals as race day, consume the same foods as on race day and remain inactive for a duration equivalent to my race time. During this inactive time, if my job is inactive I will be able to return to work but will return to the Nutrition Lab at the times specified to have my blood drawn.
Activity: During the 2 weeks prior to the postrace control study, I will maintain the same activity level as during the 2 weeks prior to the ultramarathon. For this 2 week period I will record on a daily basis the amount, type, and intensity of exercise performed each day

E. Risks.

The only potential risks or discomforts to me as a subject in this research project are the possibility of slight discomfort when the needle enters the vein and a small bruise or slight bleeding after the blood is drawn.

F. Compensation.

If I complete everything asked of me, at the end of this study I will receive in a lump sum of $200.00. If I am unable to complete the study I will be compensated $5.00 for each blood draw and each urine collection I complete. Also, I may request the results for all of the lab analyses done on my samples.

G. Confidentiality.

Any information obtained from me will be kept confidential. A code number will be used to identify any test results or other information that I provide. The only persons who will

have access to this information will be the investigators and no names will be used in any data summaries or publications.

H. Costs.

I understand that Oregon State university does not provide a research subject with compensation or medical treatment in the event the subject is injured as a result of participation in the research project.

I. Voluntary participation.

I understand that my participation in this study is completely voluntary and that I may either refuse to participate or withdraw from the study at any time. I understand that if I withdraw from the study before it is completed, the amount of money or other compensation that I receive for participating may be less than the full amount.

J. Questions.

I understand that any questions I have about the research study and/or specific procedures should be directed to Dr. James E. Leklem, at Oregon State University, (541) 737-0969. Any other questions that I have should be directed to Mary Nunn, Sponsored Programs Officer, OSU Research Office, (541) 737-0670.

Understanding and Compliance. My signature below indicates that I have read and that I understand the procedures described above and give my informed and voluntary consent to participate in this study. I understand that I will receive a signed copy of this consent form.

Signature of subject (or subject's legally authorized representative)	**Name of Subject**
Date Signed	
Subject's Present Address	**Subject's Phone Number**
Signature of Principal Investigator	**Date Signed**

You will be given a signed and dated copy of this form to keep.

Figure C.6. Recruiting flyer

Attention McDonald Forest Ultramarathoners

(Race April 24th 1999)

Want to know more about how running effects ***YOUR*** antioxidant defense system and fuel metabolism???

We are looking for people to participate in a nutrition-exercise research project.

The Department of Nutrition and Food Management at Oregon State University is carrying out an exercise study, looking at blood changes of vitamin B-6 and antioxidants before and after an ultra-marathon. There is very little data available for these nutrients for an endurance event of this length.

The study will involve taking small amounts of blood before, during, and after the race, collecting urine, consuming a supplement, and consuming a specified diet.

You will be compensated $200 for completion of the study. You must be signed up by 5 April to participate.

If you are interested please contact Ann Grediagin, MS, RD at (541) 754-1396, or e-mail at grediaga@ucs.orst.edu.

Don't miss this chance to learn more about nutrition and the effect of exercise.

Thank you

Dr. Jim Leklem
Nutrition & Food Management
Oregon State University, Milam #101
Corvallis, OR 97331