THE EFFECTS OF BORON SUPPLEMENTATION ON BONE MINERAL DENSITY, BLOOD AND URINARY CALCIUM, MAGNESIUM, PHOSPHOROUS AND URINARY BORON IN FEMALE ATHLETES

by

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(ABSTRACT)

The effects of boron supplementation on blood and urinary minerals were studied in 17 female college athletes and 11 sedentary college control subjects. The subjects were similar in age (19.8 ± 1.4 and 20.3 ± 1.1 years for athletes and sedentary groups, respectively) and weight (61.8 ± 9.1 and 59.6 ± 10.5 kgs for athletes and sedentary groups, respectively). The athletic subjects had lower percent body fat averages (20.6 ± 5.6 and 25.8 ± 6.5 %, respectively, ($p<0.05$)) and higher aerobic capacities (2.9 ± 0.5 and 2.1 ± 0.4 L O_2*min_{-1}, respectively, ($p<0.05$) than sedentary controls. No differences in dietary intake were observed. Serum phosphorous levels were lower in boron supplemented subjects than in subjects receiving placebos ($p<0.05$) and were lower during final analysis than during baseline analysis. A group-supplement interaction was noted with serum phosphorous also ($p<0.05$). In the sedentary group, boron supplementation lowered serum phosphorous and placebo supplementation elevated serum phosphorous. In the athletic group, no changes in serum

phosphorous were observed due to supplementation. Serum magnesium was greatest in the sedentary controls supplemented with boron and increased with time in all subjects ($p<0.05$). Again, a group-supplement interaction was observed with serum magnesium; exercise in boron supplemented subjects lowered serum magnesium ($p<0.05$). In all subjects, calcium excretion increased over time ($p<0.05$) and in boron supplemented subjects boron excretion increased over time ($p<0.05$). In conclusion, boron supplementation affected serum phosphorous and magnesium and the excretion of boron in the urine. The significance of these findings in relation to overall mineral status, bone mineral density, and exercise in college female athletes needs further investigation. It is possible that increasing the intake of foods high in boron may be found to be beneficial in the treatment and/or prevention of metabolic bone disorders such as those related to excessive exercise.

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CHAPTER I

INTRODUCTION

In order to improve health, women have altered dietary patterns and incorporated exercise into their daily routines. The benefits of exercise are defined in popular communications in both the lay press and in medical journals. Exercise is described as having numerous benefits ranging from improved lipid profiles and decreased cardiovascular risk for some, to the amorphous "runner's high" for others (Morgan, 1985; Henley and Vaitukaitis, 1988). There are an estimated 18 million joggers in the United States alone (Henley and Vaitukaitis, 1988). In 1982, the number of women in the United States who participated in regular vigorous exercise had increased dramatically over the past decade and was estimated to be over 7 million; about 80% of these women were premenopausal (Speroff et al., 1982; Lloyd et al., 1986). This enormous increase in participation by women in physical recreation and competitive sports since the early 1970s has been paralleled by an increase in research into the effects of strenuous exercise on female body composition, endocrine profiles, and the reproductive system (Highet, 1989).

For a minority of women who exercise to excess, intense physical training may have adverse health consequences, including menstrual dysfunction, i.e., delayed menarche, altered pubertal progression, defective luteal phase, anovulation, amenorrhea, and infertility (Baker, 1981). There are physiological

similarities between young female athletes experiencing menstrual dysfunction and postmenopausal women. One of the most important of these is reduced bone mineral density (Cann et al., 1984; Drinkwater et al., 1984; Lindberg et al., 1984; Marcus et al., 1985), suspected to be a consequence of the abnormal reproductive functioning and/or loss of estrogen.

Of primary concern is the failure of young females to ever develop adequate bone mineral density prior to the postmenopausal years, increasing the susceptibility of these individuals to osteoporosis and bone fractures. These individuals are also actively accruing bone and typically are consuming diets that are inadequate to support this growth (Jowsey, 1976). For these reasons young females in particular are targeted for dietary intervention with the objective of retarding or preventing osteoporosis later in life.

Boron supplementation has recently been proposed as a possible treatment modality for osteoporosis in postmenopausal women. Supplementation with boron has recently been reported to elevate serum levels of both estrogen and testosterone, conditions consistent with the treatment of reduced bone mineral density in postmenopausal women (Nielsen et al., 1987a,b).

In plant metabolism a boron-calcium interaction has been well documented (Brenchley and Warington, 1927; Marsh and Shive, 1941; Jones and Scarseth, 1944; Reeve and Shive, 1944; Shive,1945). Possibly, in human

metabolism, a similar interaction exists that is important in maintaining normal bone mineral density under normal dietary and hormonal conditions.

The objective of this study was to observe the effects of boron supplementation on bone mineral density and mineral status of college female athletes. The following measures were taken: bone mineral density, dietary, serum and urinary calcium, phosphorous, and magnesium. Following experimentation with sample preparation methodologies and detection instruments, boron concentrations were determined in urine samples.

Definitions

The following definitions will apply to the terms used throughout this review:

Osteopenia is generalized bone loss. Osteomalacia is characterized by normal bone matrix that is not completely mineralized. Osteoporosis is defined as a decrease in the amount of bone, leading to fractures after minimal trauma. Bone matrix, bone mineral and bone protein are lost; yet, the remaining bone is normal in composition (Sowers, 1990).

Type I osteoporosis primarily affects early menopausal women and is related to estrogen deficiency, not lack of calcium. This condition is manifested by loss of trabecular bone leading to vertebral, distal radial, and os calcis fractures. Type II osteoporosis is a slow bone loss beginning around age 30 where both trabecular and cortical bone are lost as a consequence of chronic

calcium deficiency leading to hip fractures later in life (Einhorn et al., 1990). Each bone in the body is composed of two types of bone, but the relative proportions differ and the rates of synthesis and resorption between the two types differ. Cortical bone is dense and the turnover time slow. It forms the outer shell of a bone and is prominent in the shafts of the long bones. Trabecular bone (cancellous bone) is spongy in appearance and has a high turnover rate. Trabecular bone forms the internal support network for cortical bone (White and Hergeroeder, 1990).

Bitner (1985) has described different types of menstrual dysfunction. Primary amenorrhea refers to a failure to menstruate by age 16. Secondary amenorrhea is defined as the cessation of menstruation for six months or three cycles, after having previously menstruated. Oligomenorrhea is infrequent, irregular episodes of bleeding, usually occurring at intervals greater than 40 days. Loucks and Horvath (1985) state that eumenorrhea refers to women who have menstrual cycles that recur consistently at intervals of from 25-38 days. The regularity of this occurrence is more important than interval length.

An athlete is anyone who exercises regularly, whether competitively or not.

CHAPTER II

REVIEW OF LITERATURE

The following provides background material related to osteoporosis, female athletes and menstrual dysfunction. Although osteoporosis includes the loss of both bone matrix and bone mineral, this discussion will focus on the major bone minerals, calcium, phosphorous, and magnesium and an ultratrace mineral, boron, which has recently been implicated as an element involved in bone metabolism.

Osteoporosis

In elderly females. Osteoporosis is a major health concern in the United States today and will continue to be a growing concern as the proportion of elderly individuals continues to grow. Today, as many as 20 million Americans suffer from osteoporosis. The reduction in bone due to aging in all individuals, especially postmenopausal women, weakens the skeleton, increasing the risk of fractures with increasing age. Current studies report that six months after a hip fracture, only 25% of patients are fully recovered, 50% are in need of assistance with activities of daily living, and 25% need nursing home care (Aloia, 1989).

The annual costs for the medical treatment of fractures and their complications range from 6 to 10 billion dollars (Aloia, 1989; White and Hergenroeder, 1990). The U.S. Bureau of the Census has estimated that by the

year 2050, 21.7% of the American population will be older than 65, a figure almost double that in 1981. The economic impact of potentially twice as many hip fractures in 2050 as in 1981 is difficult to predict. The pain, disability, depression, and secondary death incurred by osteoporotic patients will be even harder to endure (Aloia, 1989).

In young females. The attainment of peak bone mass, the amount of bone present before the onset of bone loss due to aging, is the best protection against osteoporosis. Peak bone mass is also a major determinant of bone mass later in life (Aloia, 1989; Einhorn et al., 1990). Most people reach their peak bone mineral density in their teens, with 45% of cortical and trabecular bone mass accruing during the adolescent years under the influence of gonadal hormones (Aloia, 1989). Another 10 to 15% of bone mass continues to accumulate between the ages of 25 to 35 years (White and Hergenroeder, 1990). Thereafter, an equilibrium is maintained for a number of years, where bone formation and bone resorption rates are equal. Later, age-related bone loss begins, generally at a rate of 0.5% per year, with the exception of 5-10 years in postmenopausal women when the rate of bone loss is accelerated. The reported rate of bone loss due to estrogen deficiency varies. A rate of 2% per year has been reported by some authors (Aloia, 1989; Einhorn et al., 1990). Others have reported up to 3 to 6% per year during the postmenopausal years (Weinerman and Bockman, 1990).

A lifelong difference in bone mass between males and females exists. Females begin life with a lower bone mass than do males (Aloia, 1989) and adolescent and adult females have a lower bone mass than do males (White and Hergenroeder, 1990). Throughout life the net loss of bone in females is about 35% of their cortical bone mineral and more than 50% of their trabecular bone mineral (White and Hergenroeder, 1990). Due to the lower bone mass and accelerated bone mineral loss associated with hypoestrogenic states, females are more susceptible to developing osteoporosis than males.

Other differences in bone mineral density have been identified in various groups and reported in recent reviews. Blacks have a greater bone mineral density during childhood, adolescence and adulthood than do whites (White and Hergenroeder, 1990; Einhorn et al., 1990). The drinking of milk in children is associated with higher peak bone mass later in life. Women who have taken oral contraceptives, which have a very high estrogen content, may be protected against osteoporosis later in life. Estrogen replacement, eumenorrhea, thiazide diuretics, physical activity, moderate obesity, fluoride (1 to 6 mg), and dietary vitamin D, calcium, magnesium and boron supplementation have also been implicated as having a positive influence on bone mineral metabolism (Baer, 1988; Aloia, 1989; Abraham and Grewal, 1990; Einhorn et al., 1990; Nielsen, 1990a,b; White and Hergenroeder, 1990).

Factors associated with lower bone mass include hypoestrogenic states, i.e., menopause, ovariectomies, amenorrhea, and extensive lactation for three or

more children. Other associations with bone loss include smoking, glucocorticoid use, hyperparathyroidism, hyperthyroidism, alcoholism, cigarette smoking, slender figure, bed rest (months) or immobilization, weightlessness, dietary fiber intake and strenuous exercise (Weinerman and Bockman, 1990; White and Hergenroeder, 1990).

Bone mineral density in young female athletes. The risks of strenuous exercise are becoming an issue and researchers are actively pursuing studies that have confirmed a positive correlation between intensive exercise programs and menstrual dysfunctions (Feicht et al., 1978; Dale et al., 1979; Baker et al., 1981; Boyden et al., 1983; Lloyd et al., 1986; Dalsky, 1990). The menstrual abnormalities associated with strenuous exercise range from abnormal bleeding (Lloyd et al., 1988), and primary and secondary amenorrhea (Erdelyi, 1976; Baker, 1981) to complete loss of menses (Bullen et al., 1985; Olsen, 1989). Anovulation and infertility (Bonen et al., 1981) are also physiological consequences reported in females engaged in these activities. In addition, stress fractures are frequently reported resulting from intensive exercise. Also, osteoporosis is a frequently reported risk resulting from hormonal changes due to intensive exercise (Lloyd et al., 1986). Other factors implicated in the development of secondary amenorrhea include rapid weight loss, a low percentage of body fat, emotional and physical stress, prior menstrual dysfunction, altered pubertal development (Warren, 1980), a shortened luteal phase (Shangold et al., 1979; Prior et al., 1982), age at onset of training, amount

of energy expended during exercise, diet and exercise-associated neuroendocrine changes (Henley and Vaitukaitis, 1988).

Researchers have systematically investigated the etiology of what has been termed "athletic amenorrhea", a form of hypothalamic amenorrhea. Many researchers have attempted to delineate the etiology of menstrual dysfunction in terms of one or two isolated physiological abnormalities. Recently, a number of thorough reviews on amenorrhea have been published (Loucks and Horvath, 1985; Henley and Vaitukaitis, 1988; Highet, 1989; Olsen, 1989). They have covered some of the existing knowledge surrounding amenorrhea in adolescent and premenopausal athletes and discussed the numerous theories associated with amenorrhea, eumenorrhea and oligomenorrhea.

The three basic factors that are believed to predispose the female athlete to secondary or "athletic" amenorrhea are physical, hormonal and psychological (Bitner, 1985). Baker (1981) contends that the incidence of athletic amenorrhea appears to vary directly with the degree of physical effort and the endurance required. The work of Bullen et al. (1985) supports this hypothesis. They performed a prospective study of 28 initially untrained college women, with documented ovulation and luteal adequacy, to determine whether strenuous exercise, spanning two menstrual cycles, would induce menstrual disorders. Only four subjects had a normal menstrual cycle during the 8 weeks of training (progressing from 4 to 10 miles per day, plus 3 1/2 hours of moderate-intensity sports daily). A higher percentage of abnormalities proved to be detectable by

hormonal means (89%) than by clinical assessment (60%, $p<0.02$). They concluded that vigorous exercise, particularly if compounded by weight loss, can reversibly disturb reproductive function in women.

Frisch, as principal investigator in numerous studies for over fifteen years, contends that a critical minimum weight appears to be necessary for the onset and maintenance of normal menstrual cycles in the human female (Frisch and McArthur, 1974; Frisch et al., 1980; Frisch, 1987). Low body weights and low body fat percentages have been reported repeatedly in those athletes participating in gymnastics (Sinning, 1978), ballet (Cohen et al., 1982), running (Frisch and McArthur, 1974; Speroff and Redwine, 1980; Boyden et al., 1982; Lindberg et al., 1984; Bullen et al., 1985; Glass et al., 1985; Marcus et al., 1985), and in college athletes on university teams (Lloyd et al., 1987; Lloyd et al., 1988). Speroff and Redwine (1980) found that menstrual irregularities and amenorrhea were most common in runners who weighed less than 115 pounds and who lost more than 10 pounds after beginning training. Ballet students, with primary and secondary amenorrhea, had lower body weights than did the regularly menstruating ballet dancers, 43.5 kg, 44.9 kg, and 47.0 kg respectively (Frisch et al., 1980).

Cann et al. (1984) were the first to report a decrease in bone content in amenorrheic women. Twenty-five amenorrheic athletic women, age 19-49 years, had secondary amenorrhea due to exercise, had hyperprolactinemia secondary to prolactinomas, or primary ovarian failure (premature menopause). The authors

concluded that the combination of decreased bone mass and the increased skeletal stress of exercise may increase the rate of stress fractures in amenorrheic women during exercise or later in life.

Drinkwater et al. (1984) reported decreased bone density in young amenorrheic athletes. The bone mass of 14 amenorrheic athletes (no more than one period in the previous year) was compared to that of 14 regularly menstruating athletes to determine whether skeletal mass is compromised by a hypoestrogenic status. The two groups of athletes were matched for age, height, weight, sport, and frequency and duration of daily training. Single-photon and dual photon absorptiometry were used to measure regional bone mass at the distal radius and lumbar vertebrae. Bone mineral density at the radius did not differ between groups; however, the mineral density of the lumbar vertebrae was significantly lower in the amenorrheic athletes (1.12 ± 0.04 g/cm^2) compared to the regularly menstruating athletes (1.30 ± 0.03 g/cm^2) ($p<0.01$); in fact, it was comparable to the bone density of 52 year old postmenopausal women.

Lindberg et al. (1984), in a brief report, also stated that exercise induced amenorrhea decreased bone mineral density. There was a significant ($p<0.05$) reduction in radial bone density in both trabecular bone and cortical bone and in trabecular bone mineral content in amenorrheic runners when compared with values in normal controls and eumenorrheic runners using single photon absorptiometry. The lumbar spinal bone mineral content, assessed using dual photon absorptiometry, yielded values for the amenorrheic runners that were

lower than normal (1.078 ± 0.346 g/cm^3 vs. normal average values of 1.2 to 1.6 g/cm^3).

Marcus and coworkers (1985) studied bone mass in 17 women distance runners, 11 of whom had secondary amenorrhea for one to seven years. The remaining six had maintained regular menses since menarche. The bone mineral density of the lumbar spine (151 ± 8 mg/cm^3) in the amenorrheic runners was lower than that in the normally cycling women (182 ± 5 mg/cm^3, $p<0.02$). The values for bone mineral density for the amenorrheic runners were greater than values obtained on less active amenorrheic runners (121 ± 6.9 mg/cm^3) ($p<0.05$) in previous studies in the same laboratory.

Amenorrheic athletes have also been reported to experience higher rates of musculoskeletal injury. Lloyd et al. (1986) performed a retrospective study to evaluate the effect of menstrual status upon musculoskeletal injuries in women athletes. In one phase of the study the menstrual and running histories were collected, via a questionnaire, from women participating in a regional 10-km footrace. Some of the respondents, 61%, reported a continuous running program and 39% reported an interruption, primarily due to injury (14 of 39 cases), of at least 3 months in their running program. Those who had interrupted their running due to injury were more likely to have had irregular or absent menses before beginning their running program (47% vs 7%, $p<0.001$), had started running at an earlier age (22 vs. 26 years, $p<0.02$), had been running

longer (5.2 vs 3.7 years, $p<0.02$) and were less likely to have been using oral contraceptives (1 vs 30) than the group of continuous runners.

This study also obtained information on the relationship between bone injury and menstrual status of 207 collegiate women athletes by reviewing their sports medicine records. In 9% of the women athletes, with regular menses, and in 24% of women athletes, with irregular or absent menses, X-ray documented fractures were reported ($p<0.025$). Specifically, stress fractures occurred in 4% of the regularly cycling females and in 15% of the athletes with irregular or absent menses ($p<0.025$) (Lloyd et al., 1986).

Marcus et al. (1985) also reported more frequent bone fracture occurrences in amenorrheic women (6 vs 1) as compared to normally cycling women ($p<0.05$). Of those 6 reporting running related fractures, 4 had several tibial and metatarsal stress fractures.

In another study, Howat and coworkers (1989) observed university women, most of them members of the gymnastics team, 18 to 26 years of age, and compared them to 10 eumenorrheic controls. The eumenorrheic athletes had a significantly higher ($p < 0.01$) mean bone mineral density (1.37 ± 0.04 gm/cm^2) than the eumenorrheic controls (1.20 ± 0.08 gm/cm^2) and the oligomenorrheic/amenorrheic athletes (1.17 ± 0.03 gm/cm^2). The authors of this study stated that their results confirm that activity strengthens the bone density of women with normal menses. However, women who experience menstrual

dysfunction should receive attention from a physician to correct the problem before bone is affected.

To summarize, osteoporosis is an increasing concern. Therapy for increasing numbers of individuals will be increasingly in demand. Preventative measures may be the most advantageous approach. Young female athletes are the principal target audience for education on the preventative measures available today to retard or prevent bone mineral loss due to aging.

Major Mineral Metabolism in Bone

As stated earlier, osteoporosis is the loss of both bone matrix and bone mineral. However, the following discussion will focus only on the major bone minerals; calcium, phosphorous, and magnesium and the ultratrace mineral, boron, recently implicated as an element involved in bone metabolism. Extensive work, particularly with regard to calcium, has been conducted related to mineral balance in bone. Dietary mineral intake, absorption, regulation, excretion and other nutrient interactions have all received considerable attention (Einhorn et al., 1990). Numerous dietary calcium studies, involving nutrient excesses and deficiencies have also been reported. Dietary programs utilizing single nutrient supplements, such as calcium, for preventative and therapeutic uses for osteoporotic persons have also been proposed (Weinerman and Bockman, 1990). Rather than attempt to review all of these works, a general overview of the

minerals of interest will be presented here, with attention focused on dietary, serum and urine values as they pertain to mineral balance and bone metabolism.

Calcium. Calcium is the fifth most abundant element in the human body. It is the most abundant inorganic component of the skeleton and is an important cofactor for neural transmission, enzyme activity, blood coagulation, and other intracellular and extracellular functions. Calcium also affects the transport function of cell membranes and influences the transmission of ions across the membranes of intracellular organelles (Boden and Kaplan, 1990).

The extracellular to intracellular calcium gradient is on the order of 10^4:1 (Boden and Kaplan, 1990). Serum calcium is maintained at a normal level of 8.5 to 10.5 mg/dl in adults and normal urinary excretion levels of calcium are 23 mg/day (Free and Free, 1975) or 0.001-0.28 moles/kg body weight/day (Ross and Neely, 1983). These levels are all tightly regulated via parathyroid hormone, vitamin D, and calcitonin, affecting intestinal absorption, bone resorption and synthesis, and renal resorption (Boden and Kaplan, 1990).

Calcium is also related to the formation and resorption of bone. Of the 1,000,000 mg of calcium in an average adult, 99% of this mineral is sequestered in the skeleton and 1%, 10,000 mg, is found in extracellular fluids. Renal filtration and reabsorption is extremely efficient, only 1-2% of this extracellular calcium being lost in the urine per day (200 mg/day). Plasma calcium is readily available for exchange and regulation of mineral homeostasis. Less than 1% of bone calcium is part of a readily exchangeable pool (Boden and Kaplan, 1990);

however, in chronic deficiency situations, calcium may be resorbed from bone to maintain serum calcium concentrations at the desired levels.

Calcium intakes are now being recommended at levels difficult to achieve through diet alone, up to 1500-2000 mg/day. These recommendations are still being made although there is evidence that massive intakes of calcium do not prevent bone loss (Gordon and Vaughn, 1986) and population groups that consume relatively low amounts of calcium have low incidences of osteoporosis (Hegsted, 1986).

Calcium appears to operate as a threshold nutrient in that, above a certain intake, further increases in intake do not cause further increases in bone mass (Heaney, 1987). Large single nutrient supplements may create nutrient imbalances, e.g. calcium supplements may interfere with iron absorption. This is particularly important to consider in young athletes who, because of their activity and low total dietary intake, may be at risk for other nutrient deficiencies (Highet, 1989).

Dietary factors also influence serum and urine calcium levels. Dietary deficiencies in calcium, phosphorous, magnesium, or vitamin D will upset the normal serum calcium balance (Boden and Kaplan, 1990). High dietary protein has been shown to be associated with increased urinary calcium excretion. High protein diets may ultimately lead to calcium loss sufficient to induce osteoporosis. However, high protein diets as a result of high meat intakes, such as the normal Western diet, are accompanied by high phosphorous intakes. The hypercalciuric

effects of protein are, generally, offset by the hypocalciuric effects of phosphorous (Einhorn et al., 1990).

Although calcium metabolism is classically associated with osteoporosis in adults a number of other factors can be responsible for subnormal calcification (McDonald et al., 1980). This deficiency disease may also be produced by a deficiency of phosphorous or vitamin D, or an abnormal calcium:phosphorous ratio (Ca:P). In animal nutrition an abnormal Ca:P is considered to be as harmful as a deficiency of either element in the diet. The Ca:P considered suitable for most animals other than poultry is within the 1:1 to 2:1 range (McDonald et al., 1980). A number of studies have addressed the Ca:P in human diets. These will be discussed later in relation to phosphorous and bone metabolism.

Adequate nutrition early in life plays a major role in reducing the risk of osteoporosis (Matkovic et al., 1979; Anderson and Tylavsky, 1984; Sandler et al., 1985; Arnaud and Sanchez, 1990). Results of many investigations support a strong relationship between an adequate calcium intake during periods of bone mineralization and the development of peak bone mass (Monsen, 1989). This concept is reflected in the recent increase in the Recommended Dietary Allowance (RDA) for calcium for 19-24 year old females from 800 to 1200 mg per day (National Research Council, 1989).

Phosphorous. Like calcium, the RDA for phosphorous is 1200 mg per day for females 19-24 years of age (National Research Council, 1989). Not only will this level of phosphorous intake assure a desirable Ca:P ratio but it will also

supply adequate phosphorous for the multitude of functions it performs in the body. Phosphorous has more functions in the body than any other mineral element (McDonald et al., 1980). Most of the phosphorous, 85%, is found closely associated with calcium in bone, as calcium phosphate [$Ca_3(PO_4)_2$] and hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_6$]. Also, like calcium, phosphorous plays a central role in the structure of soft tissues in addition to such regulatory functions as neuromuscular transmission, cellular secretion, blood clotting, oxygen transport, and enzymatic activity. Only 15% of plasma phosphate is bound to proteins. The rest is ultrafiltratable and consists mainly of free HPO_4^- and $NaHPO_4^-$(85%), with free $H_2PO_4^-$ making up the remainder. Plasma phosphate is expressed in terms of the amount of elemental phosphorous measured (Arnaud and Sanchez, 1990).

Serum phosphate concentration has a wide range of normal values, 0.8-1.0 mmol/l. Increases or decreases in dietary phosphorous are promptly reflected in changes in the same direction in serum phosphorous and urinary phosphorous excretion. There are also marked diurnal variations in serum and urinary phosphorous excretion, as much as double in the afternoon and evening, even during a fast (Arnaud and Sanchez, 1990). The amount of inorganic phosphorous found in urine ranges from 0.3 to 0.48 moles/kg body weight/day (Ross and Neely, 1983). Free and Free (1975) reported urinary phosphorous levels to be 9 mg/day of organic and 840 mg/day of inorganic phosphorous (100 mg/dl). The commercially reported expected values of inorganic phosphorous

are 2.5-4.8 mg/dl (0.8-1.6 mmol/l) in serum and 0.34-1.0 g/24 hr (11-32 mmol/24 hr) in urine for adults (Sigma Chemical Co., 1985).

Unlike calcium, the concern today is over the high dietary intake of phosphorous among adolescents and young adults (Wyshak et al., 1989; Calvo et al., 1990). The typical diet of today's young females may be high in phosphorous primarily due to the high phosphorous content of soft drinks. High dietary phosphorous may limit calcium absorption and an altered Ca:P may contribute to the increased risk of bone fractures (Jowsey, 1976; Wyshak et al., 1989). High phosphorous, low calcium diets have been shown to induce osteoporosis and alter parathyroid function in animals (Saville and Krook, 1969). Recently, Calvo et al. (1990) reported that a high phosphorous (1700 mg/d), low calcium (400 mg/d) diet increased serum parathyroid secretion in a human study. After four weeks on the test diet the maximal serum IPTH and intact PTH levels increased approximately 26% ($P<0.002$) and 36% ($P<0.004$), respectively, over the level of the basal dietary period. The authors concluded that this common dietary pattern in young adult women causes persistent alterations in calcium-regulating hormones that could be unfavorable to achieving maximal positive bone balance.

Other authors have stated that typical phosphorous intakes and Ca:P were found to have no effect on calcium balance or bone maintenance (Heaney and Recker, 1982). These factors were studied in 168 premenopausal, white, Roman Catholic nuns in the age range of 36 to 45 years. Changes in dietary

phosphorous intake and the Ca:P exhibited no deleterious effect on calcium balance in these osteoporosis-prone subjects. However, these authors did relate that dietary phosphorous affected calciuria through changes in serum phosphorous levels. The researchers concluded that nitrogen, phosphorous and caffeine intakes do, in fact, have small but real effects on certain components of calcium balance.

Magnesium. Magnesium also plays an essential role in a very wide range of fundamental cellular reactions, including interrelations of this ion with other electrolytes, second messengers, hormone receptors, parathyroid hormone secretion and action, vitamin D metabolism, and bone formation. Magnesium is the most common enzyme activator in the body and is particularly important in activating phosphate transferases, decarboxylases and acyl transferases (McDonald et al., 1980; Lassiter and Edwards, 1982). Many of these functions can be directly related to the functions of calcium and phosphorous.

The average 70 kg adult human contains 20-28 mg of magnesium. About 60-65% of total body magnesium is present in bone, 27% in muscle, 6-7% in other cells, and 1% in extracellular fluid. The large amount of magnesium in the bones appears to be more tightly bound in the mature animal than in the young growing animal. A large fraction of bone magnesium is an integral part of the bone crystal and not the bone matrix (Shils, 1990).

In plasma, magnesium is found in several forms; the approximate percentages are 55% free, 13% complexed with citrate, phosphate, and other

ions, and 32% bound to protein (Shils, 1990). Normal urine and serum magnesium concentrations vary somewhat depending on analytic methods. Atomic absorption spectrophotometry is currently the procedure usually employed in clinical chemistry laboratories. Free and Free (1975) report a normal urinary excretion rate for magnesium of 100 mg/day. Normal serum magnesium is reported in the range of 0.7-0.75 mmol/l, or 1.7-2.8 mg/dl (Perkin-Elmer, 1982).

Magnesium deficiency in the U.S. population is presumably very uncommon. This is based on the data obtained in the first (1971-1974) National Health and Nutrition Examination Survey where 95% of adults (18-74 yrs of age) had serum concentrations in the range of 0.75-0.96 mmol/l. Serum magnesium concentrations are thought to be very good indicators of magnesium status (Shils, 1990).

Magnesium deficiency in animals is quite common in acute cases such as that seen in postpartum dairy cattle. The metabolic disorder resulting from magnesium deficiency is interrelated with other elements such as calcium, phosphorous, potassium and sodium. This condition, known as "grass tetany", is characterized by convulsions. However, the dietary restriction of magnesium in healthy humans was, until recently, believed not to cause any disorders of concern (McDonald et al., 1980; Shils, 1990). However, Nielsen (1990f) recently reported that detrimental changes can occur in healthy postmenopausal women if they consume a diet low in magnesium. These changes, such as low plasma ionized calcium, may adversely affect bone health and cardiovascular function.

Abraham and Grewal (1990) are of the opinion that the RDA for magnesium, based on short-term balance studies, probably is the minimum daily intake of magnesium that the body can adjust to but at the cost of increased susceptibility to stress (Seeling, 1981) and probably primary postmenopausal osteoporosis. Long term studies on magnesium balance have reported much greater needs for magnesium; intakes greater than 1,000 mg/day are sometimes required to maintain a positive balance under stressful conditions (Seeling, 1981). Shils (1990) states that there is some question as to the reliability of the 1989 RDA for magnesium. The RDA for magnesium for females 19-24 in this country is 280 mg/day (National Academy of Sciences, 1989). The RDA for magnesium for Soviet women varies from 500 to 1250 mg, depending on physiologic conditions (Lederer, 1984).

Dalderup (1960) was the first to report a possible role for magnesium in osteoporosis therapy. Abraham (1982) has since postulated that primary postmenopausal osteoporosis is a skeletal manifestation of magnesium deficiency. Abraham and Grewal (1990) have proposed a total dietary program emphasizing magnesium instead of calcium for women on hormonal therapy.

This proposed dietary program was tested on 19 postmenopausal women on hormonal replacement therapy and 7 control postmenopausal women. The women were supplied with micronutrients in the form of a complete multivitamin, multimineral tablet containing 500 mg of calcium as the citrate salt and 200 mg of magnesium as the oxide. Seven patients received dietary advice but chose

not to take the tablet. The other 19 subjects received the dietary advice and ingested the tablet daily. In addition, these 19 subjects were supplemented with magnesium oxide at a daily dosage of 400 mg of elemental magnesium. Therefore, the 19 patients received 50% of RDA for calcium and 200% of the RDA for magnesium for women (Abraham and Grewal, 1990).

A significant ($P<0.1$) 11% increase in mineral bone density of the calcaneous bone was observed within one year, increasing from 0.303 ± 0.048 to 0.337 ± 0.052 g/cm^2 in the 19 women taking the tablets and supplements. Before treatment, 15 of the 19 women had bone mineral density values below the spine fracture threshold; within one year, only 7 of them still had bone mineral density values below that threshold. The seven women not taking the tablets or supplements displayed a nonsignificant increase, (0.7%), in bone mineral density changing from 0.303 ± 0.041 to 0.305 ± 0.50 g/cm^2 within one year. The authors concluded that the effect of the magnesium-emphasized program on calcaneous bone density was 16 times greater than that of dietary advice alone in postmenopausal women on hormonal replacement therapy (Abraham and Grewal, 1990).

Magnesium metabolism is affected by the levels of calcium and phosphorous consumed in the diet. In a recent study by Kim and Schuette (1991) five healthy, young, adult males participated in a 60-day metabolism trial. Increasing calcium intake to 2400 mg, with 900 mg of phosphorous intake, resulted in a significant decrease in magnesium retention ($p<0.01$) caused by a

decrease in magnesium absorption ($p<0.01$). Increased phosphorous intake had no effect on magnesium balance, yet both magnesium absorption and urinary magnesium decreased.

Additional magnesium research has been done studying the interactions of this mineral with boron, an ultratrace mineral, recently implicated to be involved in calcium and/or bone metabolism.

The expected dietary intakes, and blood and urine values of calcium, phosphorous, magnesium and boron are reported in Table 1.

Boron - An Ultratrace Mineral

The previously described elements calcium, phosphorous, and magnesium are all major constituents of bone mineral and are generally recognized as playing a major role in normal bone metabolism. Because the etiology of osteoporosis is still elusive, researchers are continually searching for new factors that may be involved in this metabolic disorder. Improved analytical techniques have allowed for more in depth study of the trace and ultratrace minerals.

The clinical significance of trace and ultratrace element research can be illustrated in the following situation where a home parenteral, nutrition-associated bone disease has been detected. The etiology of this condition remains speculative. Howard and Michalek (1984) proposed more than half a dozen theories related to this condition where no identifiable metabolic or biochemical features distinguished those patients with symptoms from those with subclinical

Table 1
Reference concentrations of calcium, phosphorous, magnesium, and boron in the diet, blood and urine

	Calcium	Phosphorous	Magnesium	Boron
Diet (mg/d)	1200[1]	1200[1]	280[1]	1-3[2]
Serum (mg/dl)	9.0-11.0[3]	2.5-4.8[4]	1.7-2.8[3]	([5])
Urine (mg/d)	23[6]	840[6]	100[6]	(.1-.2ppm)[7]

[1] National Research Council, 1989
[2] Nielsen, 1990e
[3] Perkin-Elmer, 1982
[4] Sigma Diagnostics, 1985
[5] recent data not available
[6] Free and Free, 1975
[7] Hunt, 1991

disease. One of the theories presented proposed that current, home parenteral, nutrition solutions may contain an unrecognized trace element excess or deficiency. The researchers were recommending that further research into the following minerals be conducted with regard to this metabolic disease: aluminum, cadmium, strontium, fluoride, vanadium, silicon, copper, manganese, magnesium, zinc, selenium, chromium, molybdenum, and iodine. These authors, at this time, did not consider boron.

Recently, boron, an ultratrace mineral, has been implicated as a possible treatment/preventative modality for postmenopausal women susceptible to osteoporosis (McBride, 1987; Nielsen et al., 1987a,b). Boron is also being considered for essentiality in human nutrition primarily because of its role in normal bone maintenance (Nielsen, 1988a; McBride, 1989; Nielsen, 1990b,c,e). Until this time boron has attracted very little attention in the field of human nutrition. Hence, there is very little information easily obtainable in relation to boron and human nutrition. Because of this obscurity the following discussion is an extensive review of boron, its chemistry, healthful benefits, and role in microorganism, plant, animal and human nutrition. The work from the USDA Human Nutrition Research Laboratory at Grand Forks, North Dakota, under the direction of Dr. Forrest H. Nielsen will also be summarized as it attempts to establish the essentiality of boron in human nutrition.

Boron - a general overview. Historically, boron was a commercially important element long before chemists were able to identify it. The Babylonians

in 2000 B.C. may have been the first to import the boron compound, borax ($Na_2B_4O_7*10H_2O$), from the Far East for use in the working and welding of gold. In Egyptian and Roman times it was used to prepare hard (borosilicate) glasses. Later, Marco Polo brought the first authentic specimens of borax, then called tincal, from Mongolia to Europe in the thirteenth century (Greenwood, 1973).

In a review by Shive (1945), a wide variety of situations in which boron was commercially important were mentioned. For example, boron was used in the preparation of raw silks for weaving, the manufacture of rayon fabrics, and the tanning of hides. Boron was an ingredient in the glazes on pottery and ceramics, in chemically resistant glass, and was used in the production of alloys, and was present in antiseptic solutions, medicated bandages, and cosmetics (Shive, 1945). Boric acid was also used as an antibacterial agent. Aromatic organoboron compounds were excellent insecticides and borates were widely used as wood preservatives (Greenwood, 1973).

Boron in the form of its compounds, never as the element, constitutes about 0.001% (3-10 ppm) of the earth's crust and is present in most soils. California has been, and most likely will remain, the world's largest producer of boron ores (Muetterties, 1967; Greenwood, 1973). Three large regions in the United States have been identified as regions that are likely to be deficient in boron for plant needs. These regions are the Atlantic Coastal Plain extending westward to the Appalachian Mountains, a region across northern Michigan, Wisconsin, and Minnesota, and the Pacific coastal region and the Pacific

Northwest (Shive, 1945). In sea water, boron is present in amounts of only a few parts per million (Greenwood, 1973).

Boron in plant metabolism. Plants differ in their ability to absorb boron from soils and water; monocotyledonous plants have a much lower capacity for absorbing boron than have the dicotyledonous plants. Soils which formed under normal rainfall were found to contain from 4 to 88 ppm and averaged 17.1 ppm. Except in arid regions, where soluble boron salts may accumulate in the soil, natural boron toxicity in plants is unlikely (Shive, 1945).

Despite the sparse research and literature pertaining to boron in human nutrition, it is known that boron is an essential element for the normal growth and development of higher plants (Agulhon, 1910; Warington, 1923, 1926; Greenwood, 1973; Lovatt and Dugger, 1984). Agulhon (1910) determined the optimal concentration of boron for plant growth to be 5 to 10 mg per liter (5,000-10,000 ppm) of liquid culture. This concentration improved growth 50% in the broad bean plant over that shown when no boron was added. Warington (1923, 1926), in several publications, confirmed the essentiality of boron in higher plants with 1:12,500,000-1:25,000 (.08-40 ppm) concentrations of boric acid (H_3BO_3) in water being beneficial to the broad bean plant. Brenchley and Warington (1927) continued to observe the growth of plants under various conditions including altered nutrient solution pH; varied concentrations, and total supplies of H_3BO_3; interchanging boron with other elements; and testing various plant species. All borates were effective and no element was capable of replacing boron. These

investigations revealed that a constant, yet small, supply of boron was necessary throughout growth for normal metabolism; the results indicated that boron was being utilized in metabolism and was not merely acting as an ordinary catalyst.

In 1927, Brenchley and Warington also showed that boron was essential for leguminous plants and melons, and not necessary, but beneficial, for the development of other plant species, such as the cereals. The authors admitted that it may be possible that this latter group of plants may have a boron requirement that is so low that a sufficient supply may be stored in their seeds. However, Sommer and Lipman (1926) stated that the need for boron appears to be general for the higher green plants.

Although Warington (1926) discussed the all-important role which boron plays in the metabolic activities of certain plants, there remained an uncertainty; was the action of boron on plant metabolism direct or indirect? Sommer and Lipman (1926) also stated that they had no good clues relative to the manner in which this element functioned in cell metabolism. Shive's review (1945) refers to the hypotheses mentioned in the studies of his time as being only suggestive and mere conjectures about the possible role for boron in plant metabolism. These hypotheses presented possible roles for boron in protein synthesis and degradation (Wadleigh and Shive, 1939), sugar accumulation (Jacob and White-Stevens, 1941) and respiratory breakdown (Johnston and Dore, 1929).

Even the most recent reviews admit that despite the considerable amount of research designed to determine the role(s) of boron in vascular plant

metabolism, definitive evidence to support a specific role has not been elucidated (Lovatt and Dugger, 1984). A wide array of observed plant responses to boron deficiency indicates that the element is probably involved in a number of metabolic pathways or via a cascade effect, thereby, regulating metabolic processes in a manner similar to that proposed for plant hormones, such as the auxins. A relationship has been emphasized between boron and auxins; many investigators agree that boron is essential for the normal growth and functioning of the apical meristems, tissues responsive to auxins.

Boron-calcium interactions in plants. It is interesting to note that in early papers dealing with boron metabolism in plants, references to a boron-calcium interaction were made. Brenchley and Warington (1927) noted that even abundant supplies of calcium cannot be efficiently utilized in the absence of boron, with the injury being localized in the rapidly growing meristematic apices. The authors concluded that this strongly suggested that boron was associated with the absorption or utilization of calcium. The authors suggested that further work should be performed directed towards the elucidation of this phenomenon, in order to determine how boron influences the utilization of calcium in plants. Brenchley and Warington (1927) had observed plants grown with the omission of various nutritive elements, including chlorine, sodium, sulphur, iron, nitrogen, magnesium, potassium, phosphorous, and calcium. Without phosphorous, growth of the bean plants was severely checked; however, the omission of calcium produced such a drastic effect that very little growth was achieved before

the plants turned black and died, long before the symptoms of a boron deficiency could be manifested. Shive (1945), in his historical survey of the literature on boron in plant life, cited several investigators who addressed the relationship between boron and calcium. Marsh and Shive (1941) found that the metabolically effective calcium, which is maintained in the soluble state in active plant tissues, is directly correlated with the supply of available boron in the same tissues. Jones and Scarseth (1944) have shown that a plant will grow normally only when a certain balance between the intake of calcium and boron exists. Reeve and Shive (1944) reported similar relationships between boron and calcium in plant metabolism. They also reported that potassium in the growth substrate influenced boron accumulation in the tissues.

Boron-health benefits. Reports have mentioned the benefits of boron compounds in relation to human health. Newnham (1981) claimed that the trace mineral balance of our food supply has been upset by modern agricultural methods and that this is a major contributing factor towards the increasing incidence of arthritis and rheumatism. He stated that a daily supplement of a few mg of boron in a properly balanced tablet corrects and prevents these crippling diseases. From observations over a three year period he noted that 25,000 people had taken 70,000 bottles of boron and magnesium tablets; 90% of these experienced a cure of all or most of their arthritic troubles. An initial analysis showed that arthritic bone contained less boron than normal bone. These claims have not been validated by scientific investigations. In a later

study, research based on over 300,000 people who had used a boron supplement showed that a daily intake of 5-6 mg of boron would prevent arthritis and probably osteoporosis. An intake of 8-10 was considered a therapeutic dose that will correct most arthritis (Newham, 1989).

Animal studies have reported on the use of boron as an antidote to fluoride toxicity. It was concluded that boron enhances the sequestration of fluoride from bones, its excretion through the kidneys and, possibly, from the intestinal tract. Boron also corrected the radiographic changes of the skeleton and the secondary effect of excess fluoride on calcium-phosphorous metabolism (Elsair et al., 1980).

Boron toxicity and boron deficiency. Newnham (1981) states that most of the boron research prior to the 1980s was designed to test its toxicity which has been described as mild (Adams, 1964). Boron, when administered orally, has a low order of toxicity; signs generally occur only after dietary boron concentrations exceed 110 ug/g. In rats, when boron exceeded 150 mg/l in drinking water, the animals exhibited lack of incisor pigmentation, aspermia, impaired ovarian development, depressed growth, and prepubescent fur. When the boron concentration in water increased to 300 mg/l, plasma triglycerides, proteins and alkaline phosphatase were depressed, as were total bone, fat and calcium. In humans the signs of acute toxicity are well known, and include nausea, vomiting, diarrhea, dermatitis, and lethargy. High boron ingestion also increases urinary excretion of riboflavin (Nielsen, 1990a).

The consumption of amounts of boron, larger than those necessary to prevent a deficiency, is not likely to cure any disorder, including osteoporosis or arthritis. Moreover, contrary to the claims of some marketers of supplements, boron is not likely to change steroid metabolism in a manner to build more muscle mass. Nonetheless, supplements containing boron have become available to the public. Nielsen states that this is a concern because, like all mineral elements, boron can be toxic if ingested in excessive amounts (McBride, 1987; Nielsen, 1988a; Nielsen, 1990a,e). The lowest reported lethal dose of boric acid is about 45 grams (1.6 ounces) for an adult and only 2 grams (0.07 ounce) for an infant. (McBride, 1987).

In the past, the most common health hazards were the accidental ingestion of household chemicals such as boric acid or borax, and absorption of boric acid applied to wounds and burns; absorption through intact skin is too slight to result in systemic toxicity (Greenwood, 1973). Boric acid and borates, years ago, had been used for burn treatment, as a mild disinfectant, as an emmenagogue and as a slimming agent. As an infant pacifier, boron was sometimes used too freely and infant deaths occurred. However, its internal use had almost ceased since the 1920s (Solis-Cohen, 1928). Even with animal studies, researchers have been unable to induce boron deficiencies even when the diets used apparently contained only 155-163 ng of boron/g (Hove, 1939; Orent-Keiles, 1941; Teresi et al., 1944). This early research with animal models demonstrated that it was difficult to obtain diets for laboratory animals, low

enough in boron concentration, to produce deficiency situations. Differing dietary boron concentrations also complicated collaborating research efforts. This was evident when researchers were able to report that supplemental dietary boron was beneficial in potassium-deficient rats (Skinner and McHargue, 1945); however, the findings were not confirmed by Follis (1947) who used a different diet with an unknown boron content, and different levels of boron supplementation. As a result of these studies boron was generally accepted as being essential only for plants, not for animals.

However, recently Uhrich et al. (1984) reexamined the possibility that boron might be an essential nutrient for the rat. Boron deprivation depressed growth, hematocrit, hemoglobin, and kidney weight/body weight ratio, and elevated spleen weight/body weight ratio. Boron deprivation tended to depress hemoglobin more in magnesium inadequate than in magnesium adequate rats. These findings suggest that boron is essential for the rat and that boron may have a physiologic role that influences magnesium metabolism.

Recently, the lack of boron in the human diet has been acknowledged as a concern because it may adversely affect calcium metabolism, which in turn could lead to bone loss under certain conditions, as will be discussed further in relation to postmenopausal women and young female athletes experiencing menstrual dysfunction. Consuming 2 to 3 mg of boron/day should assure a normal calcium metabolism, and, thus, should prevent any loss of bone mineral because of a boron deficiency (Nielsen, 1990e).

Boron in the diet. In the early 1900s, boron compounds were added to foods as preservatives (Wiley, 1907). By 1942, borax and boric acid were being experimentally added to cattle feedstuffs to determine their preservative action.

In the 1940s it was generally accepted that boron was a constituent of all vegetable tissues (Kent and McCance, 1941). Consequently, it was an inevitable component of the daily diet of humans in addition to its use as a food preservative. Wiley (1907) studied the excretion of boric acid after giving doses of 3 g, and upwards, to humans and concluded that 82-100% of the boron was quickly excreted in the urine. The author also stated that the feces were never an important avenue of excretion and, although small amounts were found in sweat, this also was quantitatively unimportant.

Kent and McCance (1941) studied two normal women, who each took 352 mg of boron as boric acid during the 3rd, 4th and 5th days of a 28 day metabolism experiment. During the first week, 93-94% of the boron was recovered reflecting its rapid absorption and excretion. Naturally occurring boron was metabolized in much the same way. However, only 40% of a dose of boron ingested by dogs was found in a 24 hour urine collection.

The greater part of the boron found in the diet seems to be introduced by the large amounts of fruit, particularly plums and greenages, which are consumed. Accurate estimates of daily boron intakes are still unavailable. Nielsen (1990e) stated that a diet containing an abundance of these items was likely to supply 2 to 6 mg of boron/day. Nielsen estimated that normal boron

intakes, in persons eating well balanced diets, are 3 to 5 mg per day (1989). In another report, Nielsen (1990a) stated that normal average intakes of boron range from 0.5 to 3.1 mg per day. Yet, in another report he stated that dietary intakes of 1.7 to 7 mg of boron/day are average (Nielsen, 1990c).

In a review Nielsen (1986) cited reports by others stating daily boron intakes ranging from 0.35-0.42 mg to 10-20 mg. Finnish diets reportedly contain 1.7 mg boron/day; English diets, 2.8 ± 1.5 mg. Newnham (1981) also reviewed reported boron intakes and cited a number of studies reporting normal daily boron intakes. In Australia the daily boron intakes are reported to range from 0.3 to 41 mg. Studies in France and America indicate that normal, daily, boron consumption rates are about 36 mg in France, and about 8 mg in America. It is apparent that the daily intake of boron by humans can vary widely depending on the proportions of various food groups in the diet, geographic locations, and cultural differences. Additional work is needed before a RDA or a safe and adequate intake for boron can be determined.

Thus, the current position on boron in human nutrition is that it is considered a nutrient, not a pharmaceutical or drug, and that it is found in all sustaining foods (Newnham, 1981; Nielsen, 1990e;). Nielsen (1990e) is of the opinion that it is best to consume boron through a balanced diet containing an abundance of non-citrus fruits (apples, pears, cherries, etc.), leafy vegetables (cabbage, broccoli, beet greens, etc.), nuts, and legumes (dried beans, lentils, etc.). As stated earlier, supplements containing boron have become available to

the public which is a concern because boron can be toxic if ingested in excessive amounts (McBride, 1987; Nielsen, 1990a,e).

Is boron an essential nutrient? Nielsen (1990a,b,c,e) recently stated that findings from animal and human experiments strongly suggest that the mineral element, boron, is an essential nutrient for health and well-being. Presently, boron is categorized as a nonessential ultratrace element in human nutrition. All elements designated as ultratrace (arsenic, boron, bromine, cadmium, fluoride, lead, lithium, nickel, silicon, tin, and vanadium) have estimated dietary requirements that are usually less than 1 ug/g, and often less than 50 ng/g dry diet (Nielsen, 1984, 1988b). However, recent research has established that boron meets some of the criteria for essentiality in humans, including: It is a low molecular weight element which forms chelates and, thus, is chemically suitable for biological function. It is ubiquitous on the earth's crust and in sea water; therefore, it has been generally available to animals during their evolution. It is present in significant quantity in animals (in all tissues at concentrations of <1 ppm) (Greenwood, 1973). It is toxic to animals orally only at relatively high levels. Homeostatic mechanisms for boron in animals are implied by serum levels, excretion rates, and lack of excess accumulation (Hunt, 1988). However, the major criterion for essentiality in animals not fulfilled by boron is the demonstration that its deficiency reproducibly impairs a biological function (Nielsen, 1974; Klevay, 1987).

At present, as in plants, no known physiological role for boron in higher animals has been described and sufficiently supported. However, Dr. Forrest H. Nielsen, Research Nutritionist, USDA, Grand Forks, North Dakota, believes boron has human nutritional significance (Nielsen, 1988a,b). Nielsen has stated that the results support the possibility that boron has physiological action.

Boron's physiological role. The support for the possibility that boron has physiological action is derived from a number of human, animal, plant and microorganism studies. In vitro studies attempted to identify a role for boron in normal metabolism. Hunt and Nielsen (1981) reviewed these studies and summarized the observations reporting that borate, or its derivatives, competitively inhibits the activity of in vitro oxidoreductase enzymes which require pyridine or flavin nucleotides, and serine protease enzymes. Oxidoreductase enzymes, inhibited by borate, include yeast alcohol dehydrogenase, aldehyde dehydrogenase, xanthine oxidase, and cytochrome b5 reductase. Borate possibly inhibits these enzymes by competing for the NAD-, or flavin, co-factor. Borate apparently complexes with ribosyl hydroxyl groups of NAD. Serine protease enzymes, inhibited by borate, include chymotrypsin, subtilisin and protease-3 from Streptomyces griseus. Borate and boronic acid derivatives apparently inhibit these enzymes by forming transition state analogues. When injected intraperitoneally into rats fed diets containing less than 1 mg of boron/g, boron stimulated hepatic RNA synthesis.

Just recently, a number of abstracts have appeared in the literature studying boron and its relation to calcium and phosphorous metabolism. Elsair et al. (1980) found that boron alleviated the negative effects of fluoride toxicity on calcium and phosphorous metabolism in rabbits. For example, boron corrected the fluoride-induced, negative calcium and phosphorous balances caused by depressed intestinal absorption of calcium and phosphorous and depressed renal reabsorption of phosphorous. Boron also partially alleviated the fluoride-induced secondary hyperparathyroidism signs of hypercalcemia, hypophosphatemia, and depressed renal absorption of phosphorous. Signs of hyperparathyroidism are characteristic of magnesium deficiency in rats (Shils, 1976).

McCoy et al. (1990) recently reported on the effects of boron supplementation on bones from rats fed low-calcium diets. The break force for femurs in rats on low calcium (30% of American Institute of Nutrition 1976 recommendations) was reduced. Adding boron increased load to break as compared to that for low calcium alone.

Bock et al. (1990) studied the fecal and urinary excretion of calcium, magnesium and manganese in female rats fed high and low levels of calcium and boron. The preliminary data indicate that urinary calcium loss tended to be greater in normal females, fed low boron diets, regardless of the calcium level fed. Boron in the diet tended to be associated with lower levels of manganese

in the feces; in addition, urinary magnesium losses were greatest in normal animals fed low boron diets.

Boron studies at Grand Forks, North Dakota. The USDA Human Nutrition Research Center at Grand Forks, North Dakota has, by far, published more information on boron in human and animal nutrition than any other institution. In 1981, Hunt and Nielsen reported that boron deprivation depressed growth and elevated plasma alkaline phosphatase activity in chicks fed inadequate cholecalciferol (Nielsen et al., 1988c). Subsequent experiments suggested that cholecalciferol deficiency enhanced the need for boron and that boron might affect cholecalciferol metabolism, which in turn affected calcium, phosphorous, or magnesium metabolism (Uhrich et al., 1984; Brossart and Nielsen, 1986; Shuler and Nielsen, 1986). Additional studies have been performed relating boron to numerous other inorganic and organic compounds; i.e., cholesterol and erythrocytes (Nielsen, 1990d), aluminum (Shuler and Nielsen, 1984; Nielsen, 1986), molybdenum (Hunt and Nielsen, 1986), methionine (Shuler and Nielsen, 1987), cholcalciferol (Hunt and Nielsen, 1987), and potassium (Nielsen et al., 1988a).

The initial experiment identifying boron as a factor in bone metabolism was conducted in chicks (Hunt and Nielsen, 1981). Day-old chicks were assigned to groups of 25 in a 2x2 factorial design experiment with a completely randomized factorial arrangement for treatments. The treatments were the supplementation of the basal diet (containing 0.28 ug boron/g) with boron (as boric acid) at 0 and

3 ug boron/g, cholecalciferol at either 250 or 2500 IU/kg (Exps. 1 and 2), or 125 and 2500 IU/kg (Exp. 3). The chicks were fed their respective diets for 28-32 days. Hemoglobin and plasma cholesterol, alkaline phosphatase activity and uric acid were determined.

The findings confirmed an interrelationship between boron and cholecalciferol. The interrelationship was a reflection of contrasting effects of dietary boron on cholecalciferol-deficient or cholecalciferol-adequate chicks. In one experiment, growth was 38% greater in boron supplemented chicks as compared to growth in boron deprived chicks, all fed a cholecalciferol-deficient diet. When the diet contained adequate cholecalciferol, growth was only 11% greater in the boron-supplemented chicks as compared to the boron-deprived chicks. Similarly, plasma alkaline phosphatase activity and heart weight/body weight ratios were apparently unaffected by dietary boron in cholecalciferol-adequate chicks. Also rachitic long bones were found in 17 of 21 boron-deprived chicks and in 9 of 22 boron supplemented chicks, all fed the cholecalciferol-deficient diet.

The researchers hypothesized that cholecalciferol deficiency enhanced the need for boron and that boron might interact with cholecalciferol metabolism, which in turn would affect calcium, phosphorous, or magnesium metabolism. Later experiments confirmed a boron-magnesium interaction, but did not confirm interactions between boron and calcium or phosphorous. Boron-deprived chicks, consuming a diet marginal in magnesium content (250 mg/kg) and

cholecalciferol (250 IU/kg), exhibited depressed growth and rachitic-like long-bone histology. Those abnormalities were not found in boron-deprived chicks fed adequate dietary magnesium (500 mg/kg) and cholecalciferol (2500 IU/kg) (Nielsen, 1984 and 1985).

Another study, by Shuler and Nielsen (1986), supported the hypothesis that boron is an essential nutrient involved in major mineral metabolism. Boron occasionally modified the response to dietary aluminum in the bone and plasma of rats.

Hunt and Nielsen (1987) reported that low physiological amounts of dietary boron either enhance or inhibit maturation of growth cartilage in response to the dietary status of Vitamin D_3. He had previously observed that dietary boron either inhibits or enhances the initiation of growth cartilage calcification depending upon magnesium status of the cholecalciferol deficient chick.

Recently, a dietary supplement of 3 mg boron/day has been suggested as a potential aid in the prevention and cure of osteoporosis in humans (McBride, 1987). Nielsen et al. (1987a,b) housed thirteen postmenopausal women in a metabolic unit and fed them a diet of conventional foods supplying about 600 mg calcium, 870 mg phosphorous, 116 mg magnesium, and 0.25 mg boron per day. After 23 days of equilibration, during which the basal diet was supplemented with 200 mg of magnesium per day, all women participated in four dietary treatment periods of 24 days each. These treatment periods were: 1) basal diet, 2) basal diet + 1000 mg aluminum per day, 3) basal diet + 200 mg magnesium per day

and 4) basal diet + 1000 mg aluminum and 200 mg magnesium per day. Twelve women continued on two more, 24-day, trials in which the treatment diet was supplemented with 3 mg of boron per day. Seven women were fed 1) the basal diet only, and 2) basal diet + 1000 mg aluminum per day. The other five were fed: 1) basal diet + 200 mg magnesium per day and 2) basal diet + 200 mg magnesium per day and 1000 mg aluminum day.

Within eight days after the supplement was introduced, all subjects lost 40% less calcium, one-third less magnesium, and slightly less phosphorous through the urine. Blood levels of 17B estradiol doubled to levels found in women on estrogen replacement therapy. Blood levels of testosterone, precursor to estradiol, more than doubled also. Additionally, boron supplementation elevated serum ionized calcium and decreased the urinary excretion of calcium. The effect of boron seemed to be more marked in the magnesium-deprived, than in the magnesium-adequate, postmenopausal women. Boron supplementation induced changes in postmenopausal women consistent with the prevention of calcium loss and bone demineralization. These findings indicated that dietary boron affected major mineral metabolism and that magnesium status apparently affected the response to dietary boron (McBride, 1987; Nielsen et al., 1987a,b; Nielsen, 1988a).

In a subsequent human study Nielsen (1989) discovered that dietary boron affects the metabolism of copper. Blood levels of copper dropped during the low-boron period as did blood levels or red blood cell levels of copper containing

enzymes, indicators of copper status. Findings from these two human studies indicated that an adequate intake of boron resulted in more available calcium and vitamin D. With boron supplementation subjects had higher plasma ionized calcium and vitamin D precursor concentrations than unsupplemented subjects. Unsupplemented subjects had higher calcitonin and higher concentrations of the bone breakdown indicator, osteocalcin. However, in the second study estrogen and testosterone levels of the ten women in the study did not increase as dramatically during the supplemental period as they had in the initial study. Nor did the boron supplement reduce the loss of calcium and other minerals. Nielsen says the design of the second study could explain the differing results, with a lower magnesium intake and a shorter period of boron depletion. Also six of the ten women in the second study were either on estrogen therapy or premenopausal.

The data from a third study, 6 months in duration and under more controlled conditions, has been collected and is awaiting publication (McBride, 1989).

Pendland et al. (1989, 1990), a psychologist working in the same laboratory, reported that relatively short periods of magnesium and boron depletion affected brain function in the healthy, older men and women. He also found substantial differences in brain wave patterns when the subjects in the second human study ate a low-boron diet (0.23 mg boron /day) compared with when they took a daily 3 mg boron supplement (McBride, 1989). This was the

first boron study to show that boron depletion alters the function of an organ system.

To summarize, experiments conducted in the laboratory of Nielsen and collaborators have shown that when the diet was manipulated to cause possible changes in cellular membrane integrity (potassium or magnesium deficiency) or in hormone responsiveness, a large number of responses to dietary boron occurred. However, when the animal was fed a diet apparently optimal in all respects, the response to dietary boron was not very marked (Nielsen, 1988a,b, 1990a,c).

To conclude: (1) More studies are needed to help delineate the role of boron, particularly with regard to bone metabolism. (2) Boron is known to be essential for growth and cell division in plants. Its exact role is still uncertain but the latest theory is that it is involved in RNA/DNA metabolism. If correct, this might apply to humans and animals (Newnham, 1981). (3) Boron possibly participates in the hydroxylation, or extends the half life, of cholecalciferol through its known affinity for hydroxyl groups (Johnson and Smith, 1976) and, thus, may be important in bone diseases such as osteoporosis. (4) Nielsen (1990b,c,e) states that the evidence to date strongly suggests that boron is an essential nutrient for human health. However, more studies are needed before a RDA or a safe intake for boron is determined. Hunt, not as emphatic, states that no study has shown that boron is unequivocally essential for either humans or animals, but, that there is more than a 50% chance that it is (McBride, 1989).

Boron - concentrations in foods and tissues. Few studies exist reporting boron concentrations in foods or tissues. A comprehensive mineral study performed in 1980 in the United Kingdom observed 50-60 chemical elements present in various healthy human tissues and the quantitative results were reported for about forty elements (Varo et al., 1980). The methodologies used were spark mass spectrophotometry, X-ray fluoresence, and various radioactivation methods. The data from the United Kingdom are comparable to data elsewhere with only small differences. However, Hunt et al. (1991) contend that these values are not reliable for comparison with American food intakes due to regional differences.

Recently, Hunt et al., (1991) completed a comprehensive study of the concentration of boron and other elements in human foods and personal-care products. They found that boron concentrations are typically higher in plant tissues than in animal tissues. Boron is unequally distributed among the class of plants most commonly consumed in human diets, the Angiospermae; the subclass Dicotyledoneae (fruits, vegetables, tubers, legumes) have much higher concentrations of boron than do species from the subclass Monocotyledoneae (grasses, i.e., rice, wheat, corn). Animal products, grain products, and confections were found to contain negligible to low levels (<0.015 and 0.100-0.500 ug boron per g wet food, respectively). The boron concentrations of a variety of foods and human health care products are reported in Table 2.

Table 2
Boron concentration in various foods and human health care products.*

Item	Boron concentration (ug/g)
beef, round, ground, raw	≤0.015
eggs	≤0.015
milk, 2%	≤0.015
bread, white enriched	0.202 ± 0.070
noodles, egg, dry, enriched	0.371 ± 0.054
spaghetti, dry, enriched	≤0.015
applesauce, bottled	2.828 ± 0.120
onions, flaked, dried	6.573 ± 3.228
cinnamon, ground	10.370 ± 0.661
green beans, frozen	0.461 ± 0.133
parsley flakes, dried	26.878 ± 1.778
amoxicillin	1.03
denture cleaner	184.0
antacid/anti-gas	34.7
milk of magnesia, plain	52.0
lipstick	11.5

* Hunt et al., 1991.

Boron concentrations have been reported in a variety of substances and organisms. Because of the recent association of boron with osteoporosis, Massie et al. (1990) reported on the whole body changes in boron concentration during development and ageing of *Drosophila*. They found that the highest concentration of boron was found during the egg stage (82.6 ppm boron). The concentration declined during the larval stages, reaching a low of 31.3 ppm during the third instar larval stage. Newly emerging flies contained 35.5 ppm boron. During the adult stage the boron concentration increased by 52% at 9 weeks of age. Adding excess dietary boron during the adult stage decreased the median life span by 69% and 21% when concentrations of 0.01 M and 0.001 M sodium borate, respectively, were administered. However, lower concentrations gave small but significant increases in life span. When young and old mouse tissues were examined they were similar to those of *Drosophila* and human samples. The authors concluded that moderate levels of dietary boron may have a general protective effect in biological systems (Massie et al., 1990). The boron concentrations of various human, mouse, and *Drosophila* tissues are reported in Table 3.

Methodology Review

Today atomic absorptiometry (AA) and inductively coupled argon plasma spectroscopy (ICP) are popular methods for mineral determinations. Other techniques available for the quantitative analysis of several elements at one time

Table 3
Boron concentrations in various tissues

Material	Boron Concentration
Human Tissues	
muscle	0.1 ug/g fresh weight[1]
blood	0.4 ug/g[1]
serum	0.18-0.21 ug/g[1]
plasma	0.5-0.6 ug/ml at birth[1]
	0.2-0.3 ug/ml within 5 days[1]
milk	0.06-0.08 ug/ml[1]
bone	10.2 ± 5 ug/g ash, hard water areas[1]
	6.0 ± 2 ug/g ash, soft water areas[1]
teeth enamel	18.2 ± 2.65 ug/g[1]
kidney	0.6 ug/g[1]
kidney (56 yr)	43.0 ppm[2]
heart (56 yr)	33.0 ppm[2]
heart (76 yr)	25.2 ppm[2]
liver	0.2 ug/g[1]
liver (49 yr)	27.4 ppm[2]
liver (76 yr)	22.9 ppm[2]
liver (98 yr)	32.0 ppm[2]
Mouse tissues[2]	
lung, young mouse	70.6 ug/g
kidney	63.1 ug/g
liver	42.7 ug/g
heart	45.8 ug/g
brain	65.5 ug/g
lung, old mouse	72.6 ug/g
kidney	64.9 ug/g
liver	50.6 ug/g
heart	41.1 ug/g
brain	64.8 ug/g
Drosophila[2]	
eggs	82.6 ppm
third instar larval stage	31.3 ppm
early emerging	35.5 ppm
ageing adults (8 wks)	56.9 ppm

[1] Nielsen, 1986
[2] Massie et al., 1990

are neutron activation, spark source mass spectrometry, energy dispersion x-ray fluorescence, and several forms of emission spectrometry. These procedures are still limiting due to availability, high initial and operating costs, and various problems of sample size and preparation (Williams et al., 1986).

Mineral analysis using atomic absorptiometry. Atomic absorptiometry relies on measuring the amount of light absorbed, as energy is applied to a specific atom, absorbed and an outer electron promoted from "ground state" to "excited state", a less stable configuration. The "ground state" atom absorbs light energy of a specific wavelength as it enters the "excited state". As the number of atoms in the light path increases, the amount of light absorbed also increases; thus, the concentration of a specific chemical element in a sample can be quantitatively determined. However, AA has a comparatively limited linearity, following Beer's law with a 1-2 order of magnitude; computer processing can accommodate some nonlinearity; dilutions are needed beyond this range. The atomic absorption spectrophotometer, with a graphite furnace, still has greater sensitivity for a few selected elements than does the inductively coupled argon plasma spectrophotometer; also, ICP procedures require more skill in preparation of samples (Williams et al., 1986).

Inductively Coupled Argon Plasma Spectroscopy. The fastest developing multielement technique of choice for the analysis of many trace metals is ICP (Williams et al., 1986). Inductively coupled argon plasma spectroscopy maintains an argon plasma within the torch assembly of the instrument. The plasma, a gas

in which a significant fraction of its atoms are ionized, is created when the argon is made conductive by exposing it to an electrical charge. Argon is also a coolant gas and carries the nebulized sample (Williams et al., 1986).

The argon plasma, initiated by a spark source that ionizes the argon, reaches temperatures of 9000 K. Energy from a radio-frequency range induction coil forms an oscillating magnetic field around the flowing gas and influences the movement of the ions and electrons. This results in ohmic heating and the high temperature plasma formed is sustained by the radio-frequency energy. A nebulizer changes the liquid sample into an aerosol which is carried by the argon gas into the very hot plasma region, where various elements are excited to emission temperatures. This changes most of the molecular species into their atoms or ions (Williams et al., 1986).

The intensity of the light emitted is proportional to the amount of each element present and is measured by a spectrometer at selected wavelengths, characteristic to each element. The various optical systems offered fall into two distinct types, simultaneous and sequential. Simultaneous system instruments place individual photomultiplier tubes at various slit positions along the spectrum to register the light intensity for an individual element. Sequential system instruments feature a moving grate which focuses the selected wavelength onto a single photomultiplier. The sequential systems provide more flexibility, but, have had greater problems with optical interferences.

Inductively coupled argon plasma spectroscopy has several advantages over AA. (1) This technique has an extended linearity, in the order of 10^5. All simultaneous analysis can then be obtained with a single dilution. 2) Inductively coupled argon plasma spectroscopy has greater sensitivity for phosphorous, boron, molybdenum, and aluminum. This procedure also has the capability of analyzing the more difficult to ionize elements which can not be energized by AA techniques. (3) Finally, because of their high energy level, almost all elements are analyzed in their ionic state by ICP. The chemical modification needed for AA to prevent various phenomena of ionization, recombination, and molecular species formation are eliminated. Thus, the chances of error due to manipulation or chemical contamination of the samples are reduced. Inductively coupled argon plasma spectroscopy can use samples of less than 0.1 g to yield accurate data for up to 11 elements. This does not require dilutions or modification, even though element concentrations may vary a thousand-fold.

Analysis of Boron. Impure boron preparations, containing less than 50% boron, were first isolated in 1808; not until 1909 were pure preparations obtained (Greenwood, 1973). In 1857, the presence of boron in *Maesa picta* seeds was detected (Nielsen, 1988a). The method of analysis used for boron, by the 1940s, involved the esterification of boron to methyl borate, which was removed by distillation with methyl alcohol (Hove et al., 1939; Owen, 1944). The distillate was condensed into caustic soda. The borate, so formed, was determined colorimetrically by its reaction with quinalizarin in concentrated H_2SO_4. The

percentage of borate recoverable by this method is never complete, and varies with the substance examined. Analyses showed that the percentage recovery was 72% with urine and 83% with feces. With milk, where the boron content is extremely low, the recovery varied from 70 to 120%. However, the borate excreted in the milk never exceeded 3-5% of the total daily output, so that these variations were of no significance in calculating the total boron balances. A mean recovery of 100% was assumed.

Boron is one of the least sensitive elements to AA. Boron can be determined only in a nitrous oxide/acetylene flame and has a characteristic concentration limit of 1 mg/l. Numerous researchers have attempted to improve upon the determination of boron using the atomic absorption spectrometer by using various extraction procedures and volatilizing boron to increase the sensitivity (Welz, 1985). Recently, graphite furnace techniques have increased the sensitivity of the method to the parts per billion (ppb) range for some elements (Williams et al., 1986).

Boron is a difficult mineral to analyze due to: 1) its low concentration in biological tissues, 2) volatilization of this ultratrace mineral at temperatures required in traditional ashing procedures, and 3) the presence of boron in borosilicate glass which leads to contamination of the samples (Herbel et al., 1989; Hunt and Shuler, 1989).

Early detection methodologies relied on colorimetric procedures preceded by conventional wet ashing for sample digestions. Presently, newer sample

preparation and detection methodologies are being employed for improved boron analysis. Hunt and Shuler (1989) propose that the best technique for the determination of boron is ICP.

Sample preparation for boron determinations. Conventionally, biological samples are either wet ashed or dry ashed to remove all organic matter for mineral determinations. Gorsuch (1970) has discussed the advantages and disadvantages of these methods for the analysis of biological material.

The characteristic properties of boron, the low levels in samples being analyzed, the low temperature of volatilization and the ease with which contamination occurs have necessitated the development of sample preparation techniques specifically suited for boron determinations. Traditionally, a nitric-perchloric acid mixture has been utilized for the preparation of samples for mineral analysis. However, perchloric acid rapidly corrodes Tygon tubing (used for solution delivery to the nebulizer), is potentially hazardous during digestion of biological materials, requires constant operator supervision and a special hood to handle fumes, and can cause loss of boron due to volatilization.

Microwave digestions. Numerous laboratories have developed microwave digestion procedures that afford advantages over traditional procedures (Abu-Samra et al., 1975; Barrett et al., 1978; Nadkari, 1984; Fischer, 1986; Borman, 1988; Perry and Early, 1990). The problems of conventional sample dissolution can be minimized by adapting an inexpensive commercial microwave oven for wet ashing. Acid mixtures are heated internally, resulting in very rapid, safe, and

efficient ashing. Abu-Samra et al. (1975) report that twelve small biological samples, 0.5 g or less, can be digested in about 15 min. Seven to 10 ml of the acid mixtures, nitric and perchloric and nitric and hydrogen peroxide, were used. No foaming, frothing or bumping occurred using Erlenmeyer flasks or test tubes. A possible problem with this procedure is that the containers are not covered or capped and boron volatilization could result in the loss of boron from these samples.

Fischer (1986) has developed a microwave dissolution procedure that results in a more complete and efficient, (one-fifth the time requirement), dissolution, utilizing pressurized vessels within a microwave oven. Element volatility decreased due to the use of pressurized containers; in addition, enhanced sample-acid contact, generated by microwave action, improved boron determinations compared to other microwave dissolution procedures. Thus, microwave digestions in capped vessels may be feasible for sample preparation for boron analysis.

Nitric acid digestions. A second possibility for preparing samples for boron determinations has been presented by Zarcinas et al. (1987); this method involves wet ashing with nitric acid in conditioned glassware. Wet ashing temperatures below 140° C are required to prevent the loss of boron from samples due to volatilization. Since perchloric acid boils at 225° C, a simple, quick and accurate procedure for digesting plant materials, using only nitric acid, in conditioned glassware has been demonstrated to allow the simultaneous

determination of at least 14 elements, including boron, by ICP. The accuracy and precision of the proposed digestion and analytical procedure were confirmed by an interlaboratory quality assurance program coordinated by the Department of Agriculture, Victoria, Australia (Zarcinas et al., 1987).

Wet-ash, low-temperature, Teflon tube digestions. A third digestion procedure for the determination of boron has been proposed by Hunt and Shuler (1989). They describe a novel open-vessel, wet-ash, low temperature, Teflon tube (WALTTT) digestion procedure to prepare samples for analysis by ICP. A sandbath was used to heat the samples digested in nitric acid and hydrogen peroxide. Teflon tubes were used to eliminate the suspected contamination of the samples due to borosilicate when glass tubes are used. The WALTTT procedure was validated for boron by the recovery (99.7%) of boron from spiked standards.

CHAPTER III

METHODOLOGY

Subjects and Study Design

Subjects. The protocol for the recruitment and treatment of human subjects was reviewed and monitored by the Institutional Review Board for Research Involving Human Subjects at the University (Appendix A). Twenty-eight college females, ages 18 to 25 years, from Virginia Tech, Blacksburg, Virginia and Ferrum College, Ferrum, Virginia were selected to participate as subjects and completed the 10 month study. The subjects were classified as either athletes or sedentary, depending on the level of activity they engaged in. Athletic subjects (n=17) were recruited from basketball, volleyball, tennis, triathlete, and track and field programs at Virginia Tech and Ferrum College. Sedentary controls (n=11) were selected from among their contemporaries. Subjects were excluded if they had a history of smoking, previous pregnancies, eating disorders, orthopedic problems, use of recreational drugs, oral contraceptives or anabolic steroids within the previous six months, or a cumulative oral contraceptive use of greater than six months. Menstrual status was classified as eumenorrheic if menses occured every 25-35 days, or 10-13 menses per year.

All subjects received an oral and written explanation of the purpose of the study and procedures to be followed. All gave written consent (Appendix B). Each subject completed a medical history and exercise questionnaire which

included information regarding her exercise regimen and menstrual history (Appendix C). Written consent was also obtained from each subject prior to every blood collection (Appendix B). Subjects also completed monthly menstrual history records in order to accurately assess menstrual status. They were given calendars in which to record the dates and symptoms of their periods (i.e., headaches, bloating, back or breast aches) and daily gauge menstrual flow as heavy, medium or light (Appendix D). An additional questionnaire was completed at the end of the study to ascertain that their health, exercise and dietary habits were consistent throughout the year (Appendix E). The subjects were provided with their own individual data at the completion (Appendix F) of the study and received two undergraduate research credits for participating in the 10 month study (Appendix G).

Experimental design. In a single blind, random assignment, the 28 college students, were given either daily boron supplementation (Tri-Boron, Twin Laboratories, Inc., Ronkonkoma, NY) or placebos consisting of cornstarch in gelatin capsules (Revco Pharmacy, Blacksburg, VA). Tri-Boron is a combination of three highly utilizable, 100% natural, chelated sources of the trace mineral boron. Each capsule contains 3 mg of pure elemental boron derived from boron citrate, aspartate and glycinate chelates. This boron supplement dosage was determined by Nielsen et al. (1987a,b) and was equivalent to the amount of boron found in diets high in fruits and vegetables. The subjects were instructed to take one capsule per day. No other dietary restrictions were imposed on

either the athletes or the controls during the study. Table 4 displays the assignment of subjects to treatments.

The present study was part of a larger study, in which blood samples collected from these same subjects were also analyzed for various hormones, alkaline phosphatase, cholesterol and vitamin D. A complete listing of all the measures taken on these subjects can be found in Appendix H. Table 5 summarizes all of the parameters measured for the present study. Table 6 summarizes the analytical procedures utilized in the study described here.

Analytical Procedures

Aerobic Work Capacity. Aerobic work capacity was assessed to verify that the athletes differed from the sedentary controls in physical condition. Subjects with higher aerobic capacities can exercise longer and achieve greater workloads than subjects with lower aerobic capacities. A Physical Work Capacity 170 Test (PWC_{170}) (Wahlund, 1948) was performed. The PWC_{170}, a submaximal exercise test, is designed to predict subjects' maximal oxygen consumption (VO_2max) by assessing their aerobic work capacity when a submaximal heart rate of 170 beats per minute is obtained (Appendix I).

A Monark bicycle ergometer (Monark-Cresent AB, Vargerg, Sweden) was calibrated and the seat height adjusted before each subject's testing. Subjects were instructed to keep a constant cadence, 50 revolutions per minute (rpm) by watching the cycle speedometer. Throughout the test, the workloads were

Table 4
Assignment of subjects to treatment groups

Group	Boron	Placebo
athletes	n = 10	n = 7
sedentary	n = 6	n = 5

Table 5
Summary of the experimental design

Parameter	Baseline	10 months
Bone mineral density (g/cm^2)	*	*
Percent body fat (%)	*	*
Aerobic capacity (1 O_2 * min^{-1}	*	
Weight (kg)	*	*
Nutrient Intake:		
(laboratory food analysis)		
• Energy (kcal)	*	
• Protein (%[1])	*	
• Fat (%[1])	*	
• Carbohydrate (%[1])	*	
• Acid Detergent Fiber (g/day)	*	
• Minerals		
• Calcium (mg/day)	*	
• Phosphorous (mg/day)	*	
• Magnesium (mg/day)	*	
(computer diet analysis)		
• Energy (kcal)	*	*
• Protein (%[1])	*	*
• Fat (%[1])	*	*
• Carbohydrate (%[1])	*	*
• Dietary Fiber (g)	*	*
• Minerals		
• Calcium (mg/day)	*	*
• Phosphorous (mg/day)	*	*
• Magnesium (mg/day)	*	*
Blood minerals (mg/dl):		
• plasma		
• Ionized Calcium	*	*
• Total Calcium	*	*
• Normalized Calcium	*	*
• serum		
• Calcium	*	*
• Phosphorous	*	*
• Magnesium	*	*
Urine minerals (mg/day):		
• Calcium	*	*
• Phosphorous	*	*
• Magnesium	*	*
• Boron	*	*

[1] percentage of total calories consumed per day

Table 6
Summary of analytical methods

Measure	Method
Aerobic capacity	$PWC_{(170)}$[1]
Percent body fat	skinfold thickness[2]
Height and weight	physicians' scale
Bone mineral density	dual photon absorptiometry
Nutrient Intake:	
• Moisture	freeze drying, Nutritionist III[3]
• Protein	Kjeldahl[4], Nutritionist III
• Fat	Soxhlet[5], Nutritionist III
• Fiber	acid detergent[6], Nutritionist III
• Minerals	
• Calcium	AA[7], Nutritionist III
• Phosphorous	colorimetric[8], Nutritionist III
• Magnesium	AA, Nutritionist III
Plasma minerals:	
• Ionized calcium	NOVA 7[9]
• Total calcium	NOVA 7
• Normalized calcium	NOVA 7
Serum minerals:	
• Calcium	AA
• Phosphorous	colorimetric
• Magnesium	AA
Urine minerals:	
• Calcium	AA, ICP[10]
• Phosphorous	AA, ICP
• Magnesium	AA, ICP
• Boron	ICP

[1] Physical Work Capacity 170 Test (Wahlund, 1948)
[2] Jackson, Pollock and Ward (1980)
[3] Nutritionist III computer data base, (N-squared Company, Analytic Software, Silverton, OR
[4] A.O.A.C., 1970
[5] Randall, 1972
[6] A.O.A.C., 1970
[7] Model 2100 Atomic Absorption Spectrometer (Perkin-Elmer Corp., Norwalk, CT)
[8] Inorganic Phosphorous Procedure No. 670, (Sigma Diagnostics, St. Louis, MO)
[9] NOVA 7 Electrolyte Analyzer (NOVA Biomedical, Waltham, MA)
[10] Inductively Coupled Argon Plasma Spectroscopy (Soil Testing and Plant Analysis Laboratory, Soil Testing Laboratory, Virginia Tech)

increased incrementally every four minutes. The workload for the first stage of the test was equal to 50 watts, the second stage workload was 100 watts, the third stage workload was 150 watts, etc. until the submaximal heart rate, 170 beats per minute, was reached. The subjects were required to cool down by pedaling at a lower intensity for several minutes following the test.

Resting and exercising heart rates were obtained by radial or carotid palpitation. Exercising heart rates were taken during each minute of the test, immediately post exercise, and for four minutes post exercise.

Subjects described how the exercise felt at each minute during the exercise test using the Borg's Rating of Perceived Exertion (RPE) scale (Borg, 1978) (Appendix J).

Percent Body Fat. Percent body fat was determined using skinfold thicknesses obtained with Lange calipers (10/mm, constant pressure) (Cambridge Scientific Industries, Inc., Cambridge, MD) (Appendix I). The average of five measurements was used as the representative score for each site tested, the triceps, suprailiac, and mid-thigh. The equation developed by Jackson, Pollock, and Ward (1980) was used to calculate the percent fat for each subject:

$$\text{Percent Fat} = \left(\frac{4.92}{BD} - 4.5\right) \times 100$$

$$\text{Body Density (BD)} = 1.0994921 - 0.0009929(x) + 0.0000023(x)2 - 0.001392(y)$$

x = sum of triceps, suprailiac, and thigh skinfolds (mm)

y = age, in years

Height and Weight. All subjects were weighed on a physicians' scale in light clothing without shoes. The weight was recorded to the nearest 0.5 kg; height was recorded to the nearest cm.

Bone Mineral Density. Trabecular bone mineral density of the vertebral column, (lumbar spine L2-L4) was determined by dual photon absorptiometry at Montgomery Regional Hospital, Nuclear Medicine Division, using a Lunar Model DPA (Lunar Radiation Corporation, Madison, WI) (Sample data sheet is included in Appendix K). Subjects were asked to void, lie down on the examining table, elevate their legs on the cushion provided and remove any metal, i.e. belts, zippers, snaps from the lumbar region for approximately 25 minutes while the photomultiplier tube moved in a rectilinear pattern over the spine region. The same technician positioned all subjects, calibrated the instrument daily and performed all the tests throughout the study.

Food Collection. Prior to supplementation, the subjects collected duplicate plates of all food and beverages consumed for three days, including one weekend day. The subjects collected a serving of everything they consumed during the day, meals, snacks, beverages, etc. The subjects were provided with plastic 1 gallon containers (Wilson Enterprises, Disputanta, VA) double lined with small plastic bags for the food and beverage collections. Total food intake was recorded in grams. The total intake was homogenized using a 4 quart Waring blender and 5% composites were made in duplicate, stored in labeled freezer bags and frozen until later nutrient analysis (Appendix L).

Subjects also kept records of their daily food intakes over these three days. Dietary records were also kept over three days at the end of the study (Appendix M).

Diet Analysis. Duplicate samples were analyzed in the Forage Testing Laboratory, Department of Dairy Science, Virginia Tech, for (1) moisture by first freeze drying for 5-7 days and then oven drying overnight at 90° C, (2) protein (A.O.A.C., 1970), (3) fat (Randall, 1972, Appendix N) and (4) acid detergent fiber (A.O.A.C, 1970). Analyses were done in duplicate with duplication in the range of 1% for protein, 2% for fat, and 5% for moisture and acid detergent fiber. Food samples were digested using a nitric-perchloric wet ash procedure and analyzed for calcium and magnesium using a Model 2100 Atomic Absorption Spectrometer (Perkin-Elmer Corp., Norwalk, CT) (Appendix N). Phosphorous was determined using a colorimetric procedure (Sigma Diagnostics, #670-C, St. Louis, MO) (Appendix P). Analyses were done in duplicate with duplication in the range of 10%. The dietary intakes recorded at months 0 and 10 were analyzed using the Nutritionist III computer program (N-squared Company, Analytic Software, Silverton, Oregon, Appendix Q) for total kilocalories, protein, fat, carbohydrates, calcium, magnesium, and phosphorous.

Blood collection. Twelve to 14-hour fasting blood samples, (30 ml) taken from the subjects' antecubital vein, were drawn by qualified personnel at 0 and 10 months. Samples were taken on the same day of the week for four weeks during each collection period. The blood was collected in prelabeled vacutainers

and kept on ice. The samples for ionized calcium determinations were collected in sodium heparinized vacutainers, centrifuged, and the plasma filtered and transferred via plastic pipets to capped polyethylene storage tubes; samples were analyzed within one hour of being drawn (Appendix T). Two mineral free vacutainers were used for serum collections for determination of other minerals. These samples were allowed to sit until clot formation was completed; serum was then separated from the red cells using a plastic pipet or automatic pipet and plastic tip. Duplicate aliquots of serum were frozen at -20° C in polycarbonate plastic tubes for later analysis of other minerals (Appendix U).

Blood analysis. Blood analyses were done on blood samples drawn during one week of the baseline month and on samples drawn during one week of the final collection month. Plasma total and ionized calcium were determined on the day of collection using a NOVA 7 Electrolyte Analyzer (NOVA Biomedical, Waltham, MA). Ionized calcium, total calcium, normalized calcium and pH were determined for each subject. Serum total calcium was analyzed in duplicate using a Model 2100 Atomic Absorption Spectrometer (Perkin-Elmer Corp., Norwalk, CT). The samples were diluted 1:25 with 0.3% (w/v) lanthanum diluent, as chloride. Serum magnesium was determined on stored serum samples, collected and frozen as reported above. The thawed samples were diluted 1:50 with deionized water and analyzed in duplicate using a Model 2100 Atomic Absorption Spectrometer (Perkin-Elmer Corp., Norwalk, CT). Serum phosphorous was determined in duplicate using the procedures and materials

obtained from Sigma Diagnostics. The Fiske and Subbarow (1925) method was utilized for the quantitative, colorimetric determination of inorganic phosphorous in serum on a spectrophotometer at 660 nm (Sigma Diagnostics, St. Louis, MO). Analyses were done with duplication in the range of 10%.

Urine collection. Urine was collected in labeled, polyethylene, wide-mouth containers during the same three-day period during which food collections were made and food records kept, during month 0; a single day collection was made during month 10 on a day coinciding with one day of the three-day dietary recall. The collection for a 24-hr period was defined as beginning with the second voiding of one day and including the first voiding of the following day. Duplicate aliquots were taken from the 3-day urine composites at month 0 and from the 24 hr urine collection made at month 10. Urine composites were frozen at -20° C in plastic screw top bottles and stored until analysis.

Urine analysis: Urinary calcium and magnesium were determined in duplicate using a Model 2100 Atomic Absorption Spectrometer (Perkin-Elmer Corp., Norwalk, CT) and an appropriate lamp for each element. The urine samples were diluted 1:40 with 0.3% lanthanum as chloride for urinary calcium determinations; urinary magnesium was determined on samples diluted 1:200 with deionized water. Inorganic phosphorous was determined following the quantitative colorimetric technique described by Sigma Diagnostics (Sigma Diagnostics, St. Louis, MO). For urinary boron determinations, samples were digested using the WALTTT procedure developed by Hunt and Shuler (1989)

(Appendix R) and analyzed using ICP with the assistance of Nancy Phillips (Soil Testing and Plant Analysis Laboratory, Soil Testing Laboratory, Virginia Tech) (Appendix S). Analyses were done in duplicate, with duplication in the range of 10%.

Statistical analysis: Data distributions are expressed as means ± standard deviations (SD). Factorial analysis of variance procedures were used to test for between treatment differences and within treatment changes in the various experimental parameters (SAS, 1982). Student's t-test was employed to locate differences when significant interactions were encountered (Appendix T). The level of significance was set at 0.05.

CHAPTER IV

RESULTS

Twenty-eight subjects, ages 18-25 years, completed the 10 month supplementation period and baseline and final, treatment effect, testing protocols. It was assumed that subjects complied with the daily supplementation regime. Subject characteristics are shown in Table 7. The athletic subjects (n=17) did not differ from the sedentary group (n=11) in age and body weight. Uneven distribution of subjects was the result of withdrawal of subjects prior to completion of the study due to factors such as time constraints and oral contraceptives. The athletic subjects differed from the sedentary group in body fat as assessed by skinfold thickness measurements. The baseline percent body fat for athletes was lower than that for the sedentary group, 20 ± 5% and 25 ± 6%, respectively ($p<0.05$). Physical fitness, assessed by the PWC_{170} test, differed between subject groups. The athletic subjects had greater estimated work capacities. The baseline estimates of maximal oxygen consumption were VO_2max = 2.9 ± 0.5 l O_2*min^{-1} for the athletes and VO_2max = 2.1 ± 0.4 l O_2*min^{-1} for the sedentary group ($p<0.05$).

Dual photon absorptiometry measurements of bone mineral densities in the lumbar vertebrae were higher for athletes than for sedentary subjects ($p<0.05$) (Table 8). The greatest difference was observed final between athletes and sedentary subjects receiving placebos, where their bone mineral densities

Table 7
Subjects' age, body weight, body fat, and VO_2max.*

	Athletes (n=17)	Sedentary (n=11)
Age (yrs)	19.8 ± 1.4	20.3 ± 1.1
Body weight (kgs)	61.8 ± 9.1	59.6 ± 10.5
Body fat (%)[a]	20.6 ± 5.6	25.8 ± 6.5
$VO_2 \cdot min^{-1}$ [a]	2.9 ± 0.5	2.1 ± 0.4

* Values represent means ± standard deviation
[a] Values significantly different between groups ($p<0.05$).

Table 8
Subjects' bone mineral density measurements (g/cm^2)*

	Athletes		Sedentary[a]	
Time**	**Boron (n=10)**	**Placebo (n=7)**	**Boron (n=6)**	**Placebo (n=5)**
1	1.27 ± 0.14	1.30 ± 0.08	1.25 ± 0.11	1.19 ± 0.11
2	1.30 ± 0.16	1.33 ± 0.10	1.26 ± 0.13	1.17 ± 0.11

* Values represent means ± standard deviation
** Time 1 = baseline, Time 2 = final
[a] Values significantly different between groups ($p<0.05$).

were 1.33 ± 0.10 and 1.17 ± 0.11 g/cm^2, respectively. The average bone mineral densities were not influenced by supplementation.

The medical history questionnaires completed by the subjects at the beginning of the present study indicated that 9 subjects, 32%, were experiencing menstrual dysfunction. However, hormone assays and monthly records revealed that all of the subjects, with the exception of two amenorrheic subjects, (12% of subjects studied) were eumenorrheic. One of the amenorrheic athletes had normal hormonal patterns without menses (Volpe-Snyder, 1991).

The dietary intake results obtained with laboratory analysis did not differ between the two groups in relation to total calories consumed per day (Table 9). A comparison of the dietary results obtained with laboratory analyses and Nutritionist III analyses is summarized in Table 10. Statistical differences were found between methods in total calories and dietary magnesium ingested per day ($p<0.05$). Total energy intake determined by Nutritionist III was greater, by 23%, than that calculated based on proximate analyses ($p<0.05$). Calcium and phosphorous did not differ in intake levels between the two methods of dietary analysis. The level of magnesium consumed was 2.6 times greater with Nutritionist III assessment, as compared to atomic absorption assessment ($p<0.05$).

Baseline and final dietary assessments, using Nutritionist III computer analysis, showed changes in carbohydrate and fat intakes over time (Table 11). Among all subjects, the average percentage of total calories consumed as

Table 9
Subjects' daily dietary intakes assessed by laboratory analyses*

	Athletes (n=17)	Sedentary (n=11)
Energy (Kcal)	1468 ± 503	1417 ± 584
Protein (%)	14.1 ± 5.1	14.9 ± 5.2
Fat (%)	28.7 ± 7.7	30.8 ± 6.3
Carbohydrate (%)	57.3 ± 9.8	54.3± 6.3
Calcium (mg)	650 ± 558	714 ± 442
Phosphorous (mg)	915 ± 616	840 ± 330
Magnesium (mg)	103 ± 107	73 ± 30

* Values represent means ± standard deviation

Table 10
Comparison of laboratory analyses and Nutritionist III assessments of subjects' baseline daily dietary intakes*

	Athletes		Sedentary	
	LA** (N=17)	N III (n=17)	LA (n=11)	N III (N=11)
Energy (kcal)[a]	1468 ± 503	1810 ± 696	1417 ± 584	1927 ± 595
Protein (%)	14.1 ± 5.1	14.3 ± 3.1	14.8 ± 5.1	14.7 ± 2.5
Fat (%)	28.8 ± 7.8	30.9 ± 9.8	30.8 ± 6.5	35.9 ± 8.3
Carbohydrate (%)	57.4 ± 9.7	54.9 ± 10.9	54.6 ± 6.3	48.6 ± 6.9
Calcium (mg)	650 ± 558	698 ± 342	714 ± 441	963 ± 378
Phosphorous (mg)	915 ± 616	1030 ± 423	840 ± 330	1169 ± 251
Magnesium (mg)[a]	103 ± 107	224 ± 106	73 ± 30	233 ± 69

* Values represent means ± standard deviation
** LA = Laboratory Analysis, N III = Nutritionist III
[a] Values significantly different between methods ($p<0.05$).

Table 11
Baseline and final comparison of subjects' daily dietary energy, protein, fat, carbohydrate, and minerals assessed by Nutritionist III*

		Athletes		Sedentary	
	Time**	Boron (n=10)	Placebo (n=7)	Boron (n=6)	Placebo (n=5)
Energy (kcal)	1	1946 ± 814	1615 ± 472	1764±349	2121±804
	2	2070 ± 521	1786 ± 674	1700±398	1537±365
Protein (%)	1	14.6 ± 3.6	13.9 ± 2.5	15.0 ± 2.0	14.4 ± 3.1
	2	13.7 ± 3.6	12.9 ± 2.8	15.3 ± 3.1	14.8 ± 2.3
Fat (%)[a]	1	29.5 ± 9.1	32.9 ± 11.1	37.2 ± 9.3	34.4 ± 7.7
	2	27.3 ± 8.9	29.7 ± 10.4	27.2 ± 6.7	27.2 ± 7.8
Carbohydrates (%)[a]	1	55.6 ± 10.7	53.9 ± 11.8	46.2 ± 6.7	51.4 ± 6.7
	2	58.5 ± 10.1	57.1 ± 12.6	57.2 ± 4.4	58.0 ± 9.0
Dietary Fiber (g)	1	14.2 ± 7.7	12.1 ± 5.2	10.8 ± 1.9	12.7 ± 4.2
	2	12.7 ± 7.9	17.1 ± 13.7	12.6 ± 8.5	12.9 ± 9.5
Calcium (mg)[bc]	1	715 ± 339	673 ± 372	761 ± 133	1206±448
	2	771 ± 241	668 ± 371	752 ± 235	975 ± 397
Phosphorous (mg)	1	1054 ± 450	996 ± 414	1058±130	1303±308
	2	1022 ± 296	1059 ± 464	1050±256	1132±466
Magnesium (mg)	1	238 ± 127	202 ± 69	224 ± 48	243 ± 93
	2	242 ± 107	278 ± 156	264 ± 168	247 ± 128

* Values represent means ± standard deviation
** Time, 1 = baseline, 2 = final
[a] Values significantly different between times ($p<0.05$).
[b] Values significantly different between groups ($p<0.05$).
[c] Significant group-supplement interaction ($p<0.05$).

carbohydrate for the baseline period was 52%. This was lower than the average percentage of total calories consumed as carbohydrate during the final period, 58% ($p<0.05$). Conversely, fat intakes decreased over time among all subjects ($p<0.05$); the baseline average percentage of total calories consumed as fat was 33% and the final average of total calories consumed as fat was 28%.

Although laboratory chemical analysis showed that there was no difference in calcium consumption between treatment groups, computer analysis of baseline and final dietary records revealed an activity-supplement interaction in relation to calcium consumption. There was a significantly higher calcium intake among the sedentary placebo group than among the athletes, both pre- and final ($p<0.05$).

The serum calcium levels, determined using atomic absorption, differed over time within activity-supplement groups; baseline values were lower than final values ($p<0.05$) (Table 12). Serum phosphorous levels, determined colorimetrically, also differed over time within groups: baseline values were higher than final values ($p<0.05$). Supplementation was found to influence serum phosphorous levels; boron supplementation was associated with lower serum phosphorous than that observed with placebo supplementation ($p<0.05$). A significant activity-supplement interaction was also observed ($p<0.05$). The sedentary group on boron supplementation, at both testing times, had the lowest serum phosphorous levels; the sedentary placebo group had the highest serum phosphorous levels, at both testing times ($p<0.05$). Activity depressed the effect of boron supplementation observed in sedentary controls ($p<0.05$).

Table 12
Baseline and final comparison of subjects' blood calcium, phosphorous, and magnesium assessed by atomic absorption (mg/dl)*

		Athletes		Sedentary	
	Time**	Boron (n=10)	Placebo (n=7)	Boron (n=6)	Placebo (n=5)
Calcium[a] (AA)[1]	1	8.9 ± 0.4	8.8 ± 0.2	9.1 ± 0.2	9.0 ± 0.5
	2	9.6 ± 0.3	9.8 ± 0.3	9.8 ± 0.2	9.7 ± 0.4
Phosphorous[a,b,c] (SD)[2]	1	4.7 ± 0.4	4.7 ± 0.6	4.3 ± 0.5	5.1 ± 0.3
	2	3.9 ± 0.2	4.0 ± 0.2	3.6 ± 1.0	4.4 ± 0.3
Magnesium[a,b,c] (AA)	1	1.5 ± 0.3	1.7 ± 0.2	1.9 ± 0.3	1.7 ± 0.2
	2	2.0 ± 0.0	2.0 ± 0.1	2.4 ± 0.4	2.0 ± 0.1

* Values represent means ± standard deviation
** Time, 1 = baseline, 2 = final
[1] AA = Atomic Absorption
[2] SD = Sigma Diagnostics
[a] Values significantly different between times ($p<0.05$).
[b] Values significantly different between supplements ($p<0.05$).
[c] Significant group-supplement interaction ($p<0.05$).
[d] Values significantly different between groups ($p<0.05$).

Final serum magnesium values, determined using atomic absorption, were higher than baseline values within activity groups ($p<0.05$). An activity-supplement interaction was identified; boron supplementation lowered serum magnesium levels in athletes and elevated serum magnesium levels in sedentary controls ($p<0.05$).

Blood calcium was also determined using an electrolyte analyzer, the NOVA 7 (NOVA Biomedical, Waltham, MA) (Table 13). A change over time in blood calcium, like that observed with atomic absorption analysis, was observed only for plasma ionized calcium; baseline plasma ionized values, combined over all subjects, were lower than final values ($p<0.05$). However, this change was not reflected in plasma normalized or total calcium determinations using the NOVA 7. When the total blood calcium values determined by the two procedures (AA and NOVA 7) were compared, a difference in total blood calcium values was found only during the baseline analysis (Table 14). The baseline serum total calcium values, combined over groups and determined by atomic absorption, were lower than the plasma total calcium values obtained by electrolyte analysis ($p<0.05$).

Daily excretion values for calcium, phosphorous, magnesium and boron are reported in Table 15. Urinary calcium values differed over time within groups; final calcium excretion levels were higher than baseline excretion levels ($p<0.05$). Urinary phosphorous and magnesium levels did not differ with time, activity level or supplementation.

Table 13
Baseline and final comparison of subjects' plasma calcium, assessed by the NOVA 7 (mg/dl)*

		Athletes		Sedentary	
	Time**	Boron (n=10)	Placebo (n=7)	Boron (n=6)	Placebo (n=5)
Total Calcium	1	9.4 ± 0.5	9.7 ± 0.6	9.4 ± 0.4	9.7 ± 0.2
	2	9.6 ± 0.2	9.7 ± 0.3	9.5 ± 0.2	9.4 ± 0.1
Normalized Calcium	1	4.7 ± 0.3	4.9 ± 0.2	4.8 ± 0.1	4.8 ± 0.0
	2	4.8 ± 0.2	4.8 ± 0.1	4.8 ± 0.1	4.7 ± 0.2
Ionized Calcium[a]	1	4.5 ± 0.3	4.7 ± 0.2	4.6 ± 0.1	4.5 ± 0.0
	2	4.7 ± 0.2	4.7 ± 0.2	4.7 ± 0.1	4.7 ± 0.2

* Values represent means ± standard deviation
** Time, 1 = baseline, 2 = final
[a] Values significantly different between times ($p<0.05$).

Table 14
Comparison of subjects' blood calcium, assessed by the NOVA 7 and AA (mg/dl)*

		Athletes		Sedentary	
	Time**	Boron (n=10)	Placebo (n=7)	Boron (n=6)	Placebo (n=5)
Serum Calcium[a,b] (AA)[1]	1	8.9 ± 0.4	8.8 ± 0.2	9.1 ± 0.2	9.0 ± 0.5
	2	9.6 ± 0.3	9.8 ± 0.3	9.8 ± 0.2	9.7 ± 0.4
Plasma Calcium (NOVA 7)[2]	1	9.4 ± 0.5	9.7 ± 0.6		
	2	9.6 ± 0.2	9.7 ± 0.3	9.4 ± 0.4	9.7 ± 0.2
				9.5 ± 0.2	9.4 ± 0.1

* Values represent means ± standard deviation
** Time, 1 = baseline, 2 = final
[1] AA = Atomic Absorption
[2] NOVA 7 = NOVA 7 Electrolyte Analyzer, NOVA Biomedical, Waltham, MA
[a] Values significantly different between times ($p<0.05$).
[b] Values significantly different between methods at time 1 only ($p<0.05$).

Table 15
Baseline and final comparison of subjects' daily urinary calcium, phosphorous, magnesium and boron*

		Athletes		Sedentary	
	Time**	Boron (n=10)	Placebo (n=7)	Boron (n=6)	Placebo (n=5)
Calcium (AA) (mg)[1,a]	1	50 ± 45	72 ± 50	62 ± 37	47 ± 31
	2	106 ± 40	89 ± 27	74 ± 41	53 ± 16
Phosphorous (SD) (mg)[2]	1	760 ± 798	719 ± 394	755 ± 364	480 ± 316
	2	601 ± 280	606 ± 178	767 ± 312	672 ± 240
Magnesium (AA) (mg)	1	53 ± 30	67 ± 44	60 ± 23	53 ± 33
	2	118 ± 98	72 ± 34	70 ± 36	52 ± 33
Boron (ICP) (ug)[3,a-d]	1	0.7 ± 0.6	0.7 ± 0.3	0.7 ± 0.3	0.5 ± 0.3
	2	2.8 ± 1.6	0.8 ± 0.8	1.1 ± 1.1	0.7 ± 0.6

* Values represent means ± standard deviation
** Time, 1 = baseline, 2 = final
[1] AA = Atomic Absorption
[2] SD = Sigma Diagnostics
[3] ICP = Inductively Coupled Plasma Spectroscopy
[a] Values significantly different between times ($p<0.05$).
[b] Values significantly different between groups ($p<0.05$).
[c] Values significantly different between supplements ($p<0.05$).
[d] Significant time-drug interaction ($p<0.05$).

Boron excretion, summed over subjects, was affected by time, activity, and supplementation ($p<0.05$). Boron supplementation increased the final urinary excretion of boron 4 times over baseline urinary excretion in athletes and 1.6 times over baseline values in sedentary controls ($p<0.05$).

When urinary minerals were assessed using ICP the values obtained were not different from those obtained using AA for calcium (Table 16). However, the statistical analysis revealed a significant difference between groups when the data from the two methods (AA and ICP) were being compared. Athletes had higher calcium excretion rates than did sedentary subjects ($p<0.05$). This difference was not seen when the statistical analysis was performed on the data obtained using only AA. Although the urinary values for phosphorous were above the recommended analytical range for ICP analysis the values obtained by this method were not different from those obtained using AA. However, a difference between values obtained using ICP and AA was observed with urinary magnesium. Urinary magnesium values were higher using AA than when determined using ICP.

Data collected but not reported here would include: information from the health and fitness questionnaires, menstrual records, subject height, subject body fats and weights at the midpoint and final data collection periods, plasma calcium analyses at the midpoint, and additional bone mineral density information.

Table 16
Comparison of subjects' daily urinary calcium, phosphorous and magnesium, assessed using AA, SD and ICP* (mg)

		Athletes		Sedentary	
	Time**	Boron (n=10)	Placebo (n=7)	Boron (n=6)	Placebo (n=5)
Calcium (AA) [1,a,b]	1	50 ± 45	72 ± 50	62 ± 37	47 ± 31
	2	106 ± 40	89 ± 27	74 ± 41	53 ± 16
Calcium (ICP)[2]	1	56 ± 35	74 ± 42	82 ± 35	70 ± 40
	2	102 ± 60	119 ± 126	50 ± 24	52 ± 26
Phosphorous (SD)[3]	1	760 ± 798	719 ± 394	755 ± 364	480 ± 316
	2	601 ± 280	606 ± 178	767 ± 312	672 ± 240
Phosphorous (ICP)	1	476 ± 317	701 ± 347	601 ± 363	564 ± 400
	2	610 ± 316	567 ± 306	598 ± 498	668 ± 457
Magnesium (AA)[c]	1	53 ± 30	67 ± 44	60 ± 23	53 ± 33
	2	118 ± 98	72 ± 34	70 ± 36	52 ± 33
Magnesium (ICP)	1	31 ± 21	40 ± 28	45 ± 19	38 ± 24
	2	52 ± 31	46 ± 28	25 ± 12	40 ± 26

* Values represent means ± standard deviation
** Time, 1 = baseline, 2 = final
[1] AA = Atomic Absorption
[2] ICP = Inductively Coupled Plasma Spectroscopy
[3] SD = Sigma Diagnostics
[a] Values significantly different between times ($p<0.05$).
[b] Values significantly different between groups ($p<0.05$).
[c] Values significantly different between methods ($p<0.05$).

CHAPTER V

DISCUSSION

Physical Characteristics

Menstrual status. Initially, college-age female athletes had been targeted for this study with the expectation that many of these subjects would be experiencing menstrual dysfunction. The incidences of amenorrhea, 12%, (2 out of 28), and oligomenorrhea, (0%), in this study were not as high as had been anticipated. Previous investigations (Speroff, 1982; Boyden, et al., 1983; Lloyd et al., 1986; Boyden et al.,1983) have reported that exercise increases the rates of oligomenorrhea and amenorrhea. Some reports state that the rates increase from about 5% of the sedentary population to approximately 20% of women who exercise regularly and vigorously (Lloyd et al., 1986). Glass et al. (1987) reported amenorrhea occurring in olympic marathon runners at a rate of 19% and the incidence of all menstrual dysfunctions at 34%.

Hormonal changes, specifically lower estrogen levels, are characteristic of athletic amenorrhea and menstrual dysfunction. A number of studies have shown that regular vigorous exercise is associated with decreased levels of estrogen (Boyden et al., 1983; Drinkwater et al., 1984). Other researchers have stated that circulating estrogens have an effect on the maintenance of bone composition because of their effects on calcium metabolism (Lloyd et al., 1987). This same effect has been observed in a previous study where a 20-30% decrease in spinal trabecular bone mass was associated with premenopausal amenorrhea in women

engaged in vigorous exercise. In postmenopausal women, low serum estrogen levels have been reported to lead to an increase in osteoporosis (Cann et al., 1984).

Although the athletic subjects in this study were participating in intercollegiate sports and/or other competitions they did not portray a low serum estrogen profile (Volpe-Snyder, 1991). Only 2 of the 17 athletes were amenorrheic; the other 15 were eumenorrheic. The athletes had a higher mean bone mineral density than did sedentary subjects. Thus, the estrogen levels observed would suggest that the female college athletes in this study are not at an increased risk for osteoporosis later in life.

It is possible that the subjects in the present study were not as competitive, or not exercising at as high an intensity level as the subjects in previous studies reporting higher incidences of menstrual dysfunction. It is likely that other factors, i.e., improper diet, stress, and/or low body fat, may be associated with the higher reported incidences of menstrual dysfunction found in the literature. In the present study the method of recruiting subjects may have influenced the characteristic profiles of the subjects completing the study. Individuals on oral contraceptives were not permitted to participate or complete the study. Some potential athletic subjects were already on oral contraceptives to manage irregular menstrual patterns.

Method of determining menstrual status. In the present study there was a discrepancy between the athletes' perceived menstrual dysfunction and that revealed by monthly records and hormone assays. In baseline medical history questionnaires, 32% of subjects (9) in the present study indicated that they were experiencing menstrual dysfunction. However, hormone assays and monthly records revealed that all of the subjects, with the exception of two amenorrheic subjects, (12% of subjects studied) were eumenorrheic. Defining and identifying menstrual dysfunction and amenorrhea has been a chronic concern among researchers working in this area. Previous studies have not always indicated what methods (questionnaires, monthly records, hormone assays) were used to determine menstrual status.

The two amenorrheic athletes in this study differed from the other athletes in certain parameters measured. One amenorrheic athlete was in the low end of the range of body weights and had low bone mineral density measurements compared to the other athletes. Body fat was also particularly low in this individual, in comparison to the body fat measures of the other athletes. Both amenorrheic athletes had maximal oxygen consumption measures of 3.3 l O_2*min^{-1}. The average for the athletic group was 2.8 l O_2*min^{-1}, ranging from 2.0 to 3.6 l O_2*min^{-1}. Thus, the two amenorrheic athletes' levels of physical fitness, assessed by PWC^{170}, were not extreme measures within their group.

Bone mineral density. The higher bone mineral densities found in the athletic group, support the theory that moderate exercise may afford females some protection against osteoporosis (Dalsky, 1987, 1990; Marcus and Carter, 1988). The reported values in this study, averaging 1.30 ± 0.1 and 1.22 ± 0.1 g/cm^2 for athletes and sedentary groups, respectively, are expected levels for this age group. Drinkwater et al. (1984), using dual photon absorptiometery, reported mean (± S.E.M.) bone mineral densities of the lumbar spine to be 1.12 ± 0.04 g/cm^2 for amenorrheic athletes and 1.30 ± 0.03 g/cm^2 for eumenorrheic athletic controls ($p<0.05$).

The fact that significant differences in bone mineral density could be detected is indicative of the fact that the methods of assessment are adequate to identify changes in this metabolically slow tissue. Although we do not know for how long the athletic subjects had been physically active nor the intensity of the exercise during these years, it can be assumed that if they are competing in college sports they most likely competed in high school sports also. Thus, in order to observe changes in bone mineral density due to boron supplementation a longer treatment period, such as that seen with differences in physical activity, could be beneficial in detecting differences in bone mineral content as a result of boron supplementation.

Dietary Intake Analysis

When assessing dietary intakes, no differences were found between activity groups. It is interesting to note that daily calories consumed, by all subjects, are two-thirds of the 2,200 kcal expected for this age group. Other studies have reported the daily nutrient intake of college athletes. Marcus et al. (1985) observed mean daily caloric intakes of 1715 calories in eumenorrheic subjects and 1272 kcal in amenorrheic subjects. Drinkwater et al. (1984) observed mean values of 1965 kcal and 1623 kcal in eumenorrheic and oligomenorrheic female athletes, respectively. Lloyd et al. (1987), using the Nutritionist II computer program, reported 1585 ± 109 kcal intakes in sedentary control subjects, 2000 ± 99 kcal in eumenorrheic athletes, and 2131 ± 144 kcal in oligomenorrheic athletes, all 18-19 years of age (values for athletes were significantly different from those for controls).

A recent report investigating the incidence of eating disorders and calorie intake levels in Swiss female athletes found that the total energy intake per day was 1849 ± 391 for the control group, 1892 ± 446 kcal in the swimmers and 1544 ± 398 kcals in the gymnasts (Benson et al., 1990). These subjects were 12-13 years of age. Thus, the reported intakes among the subjects in the present study appear to be consistent with the literature, although there is considerable individual variation among intakes reported.

Dietary intakes, particularly of the minerals, also varied considerably, as reflected in the large standard deviations reported. When assessing dietary

minerals there were no differences found among subjects for average intakes of calcium, phosphorous, and magnesium. Calcium intakes of 650 ± 558 and 714±442 mg/day for athletes and sedentary controls, respectively, are approximately one half of the RDA of 1200 mg/day for this age group. Similar calcium intakes have been found by other researchers analyzing young female athletes' diets. For example, Lloyd et al. (1987) reported calcium intakes of 595, 786, and 973 mg/day for sedentary controls, eumenorrheic athletes and oligomenorrheic athletes, respectively (values for athletes were significantly different from those for controls ($p<0.05$)).

In the present study, slightly higher levels of phosphorous than calcium were consumed. These intakes, 915 ± 616 and 840 ± 330 mg/day for athletes and sedentary controls, respectively, were also below the 1200 mg/day RDA. Again, Lloyd et al. (1987) reported similar phosphorous intakes of 967, 1183, and 1359 mg/day for sedentary controls, eumenorrheic athletes and oligomenorrheic athletes, respectively. (Values for athletes were significantly different from those for controls ($p<0.05$)).

The calcium to phosphorous ratios calculated from the intakes in this study are 0.71 for the athletes and 0.85 for the sedentary group. Ratios that are 1:1 to 2:1 are preferred (McDonald, 1981). Long-term dietary intakes of calcium and phosphorous at levels reported here could pose a possible health risk, particularly with regard to bone metabolism. Previous studies have suggested that high phosphorous, low calcium diets can induce osteoporosis and alter

parathyroid function (Saville and Krook, 1969; Calvo et al., 1990). However, the changes in serum parathyroid secretion reported were induced by a diet containing 1700 mg/d phosphorous and only 400 mg/d calcium (Calvo et al., 1990). The subjects in our study did not approach these extreme levels of intake. Thus, their diets, although not properly balanced with respect to calcium and phosphorous, are not likely to induce such hormonal changes. Furthermore, these calcium to phosphorous ratios fall within the range of those calculated from intakes reported by Lloyd et al. (1987); 0.61, 0.74, and 0.72 for sedentary controls, eumenorrheic and oligomenorrheic athletes, respectively and those reported by Drinkwater et al. (1984), 0.92 ± 0.17 and 0.88 ± 0.09 in amenorrheic and eumenorrheic athletes, respectively.

The dietary intakes of magnesium in the subjects in the present study are low also. Intakes of 103 ± 107 and 73 ± 30 mg/day for athletes and sedentary controls, respectively, were markedly below the 280 mg/day RDA for their age group. The current recommendation for magnesium intake is lower than the 1979 version of the RDAs in which recommended magnesium intakes for this group were set at 300 mg/day. The newer recommendations are being contested. Shils (1990) has commented that the 1990 RDAs for magnesium are too low. Abraham and Grewal (1990) agree, stating that the 1990 RDAs for magnesium are the minimum to which the body can adapt and at the cost of increased susceptibility to stress and probably primary postmenopausal osteoporosis. There are also reports that much greater intakes of magnesium,

up to 1,000 mg/day, may be needed to maintain a positive balance under stressful conditions (1981). In other countries, such as the Soviet Union, 500-1250 mg/day is the recommended magnesium intake for women (Lederer, 1984). Although there appears to be considerable controversy over what the recommended intake level of magnesium should be, the intake observed in our study of female college athletes is only one third the present recommended level.

The consequences of low magnesium intakes in young females are of concern. As early as 1960, magnesium deficiencies were linked to osteopathies (Dalderup, 1960). In 1982, Abraham postulated that premenopausal osteoporosis is a skeletal manifestation of magnesium deficiency; postmenopausal trabecular bone loss would not occur, even without estrogen therapy, if the magnesium intake were sufficient to maintain an adequate bone magnesium reserve. Recently, Abraham and Grewal (1990) proposed a total dietary program emphasizing magnesium instead of calcium for the management of postmenopausal osteoporosis.

In another instance, Nielsen et al (1988b) demonstrated, in rats, that magnesium deprivation affected the response of boron deprivation. A series of experiments, conducted to study the effects of dietary boron and magnesium and their interactions with various calcium, phosphorous, and magnesium parameters, confirmed that the deprivation of either boron or magnesium made the deficiency signs of the other more marked (Nielsen et al., 1988b).

Methods of dietary intake analysis. The subjects' diets were assessed using laboratory analyses to analyze duplicate plate collections and computerized data banks to analyze diet records. The only differences between the two methods of analysis were found when comparing total calories consumed and the dietary magnesium intakes. The percent of total calories consumed as protein, fat and carbohydrate did not differ. Neither did calcium and phosphorous intakes.

The difference in calculation of total calories consumed could be due to any of several factors. The laboratory analyses were dependent on accurate collection procedures on the part of subjects. In addition, in homogenized and freeze dried samples, the fat was visibly separating from the rest of the mixture. The difficulty in maintaining a consistent food mixture because of the fat content could have been a complication affecting the accuracy of sampling procedures. However, it is interesting to note that the fat content in the food determined by

Soxhlet procedure and Nutritionist III were not statistically different. The significant difference, between the two methods used, in total calories consumed per day could be attributed to several factors. Total calories consumed per day determined by proximate analyses were calculated values. After determining the percentages of protein, fat and acid detergent fiber, the actual grams of protein, fat and acid detergent fiber were calculated from the total dry matter, determined by freeze drying. Grams of carbohydrates consumed were determined by difference. Then the respective caloric content of each of these macronutrients

was used to determine the total calories provided by the macronutrient components, their sum being the total calories consumed per subject per day. The percentages of total calories consumed as protein, fat and carbohydrate were then calculated from this total energy intake. (Possibly because protein, fat and carbohydrate are percentages rather than actual intake values the determination did not differ with the methods of analyses used, proximate analyses and Nutritionist III.)

It is also possible that the Nutritionist III computerized method of analyzing total calories consumed provided values that were too high as compared to the proximate analyses method of assessing total energy intake. The Nutritionist III computerized system is, like proximate analyses, subject to some inherent problems including accurate recording of the foods consumed, amounts of foods consumed and methods of food preparation. Also, the computer database uses an average value and does not take into account regional differences, seasonal differences, bioavailability, etc.

These same factors may explain the differences found in magnesium determinations. However, for other minerals, calcium and phosphorous, intake did not differ with the methods of analyses. Again, very large individual differences in intake of all nutrients measured, as reflected by the large standard deviations, may explain why there where not more differences detected between methods.

Mineral analyses were performed on freeze-dried, wet ashed samples of food composites. Gorsuch (1975) has discussed in detail the inherent problems associated with wet and dry ashing samples for analyses such as those used for mineral determinations in this study.

Baseline and Final Dietary Analysis. The only changes over time in nutrient intake were observed in the percentages of total calories consumed as fat and carbohydrate. In all subjects there was a decrease in total calories consumed as fat and an increase in the total calories consumed as carbohydrate. There is no apparent explanation for this other than a change in dietary habits among the subjects. A change in the reporting of their intakes is unlikely since the other nutrients did not change. It is possible that, because the participants in the study were aware of the fact that they were human subjects in a nutrition study, they may have altered their eating patterns, or the reporting of their eating patterns, during the time of diet analysis. It is also possible that it is simply coincidental.

It is interesting to see that when both baseline and final calcium intakes, determined via Nutritionist III, were statistically analyzed a group difference was detected. Also a significant group-supplement interaction was identified. Sedentary controls, taking the placebo, had the highest dietary intakes and the athletes taking the placebo had the lowest dietary calcium intakes. Overall, the athletes consumed a lower level of calcium than did the sedentary controls. Again, no readily apparent explanation is available, other than differences in

personal dietary habits, or small sample sizes, or recording errors. This difference was not observed when calcium intakes were assessed by atomic absorption of food composites nor was it apparent when baseline diet records alone were assessed using Nutritionist III.

Blood Mineral Analysis

In general, the blood values for calcium, phosphorous and magnesium fell within the expected blood concentration ranges. However, magnesium concentrations, ranging in this study between 1.5 ± 0.3 and 2.4 ± 0.4 mg/dl, were at the low end of the expected 1.7 - 2.8 mg/dl range. Low serum magnesium levels have been reported by others in relation to bone mineral metabolism. Stiedl et al. (1990) reported serum and red cell magnesium and calcium concentrations in 60 osteoporotic patients (33 with senile osteoporosis, 18 with postmenopausal osteoporosis, and 9 with osteoporosis due to corticoids). In the former two groups there were signs of chronic magnesium deficiency; in the third group, there was a trend toward low serum calcium levels. The authors stated that magnesium plays an important role in the development of bone disorders.

The effects of boron supplementation on blood minerals has also been the focus of other investigations (Nielsen et al., 1987a,b; Nielsen et al., 1988a). In one instance, boron supplementation for 4 months, in postmenopausal women, was coordinated with magnesium and aluminum deficient and adequate diets.

The effects of boron supplementation on blood minerals were most marked in the 13 postmenopausal subjects when they were consuming the magnesium-deficient diets (Nielsen et al., 1987a,b; Nielsen 1988a).

In the present study, a similar situation existed; daily supplementation with 3 mg of boron for 10 months affected blood mineral concentrations. Like the postmenopausal subjects on a magnesium deficient diet, the female athletes consumed a low magnesium diet, although voluntarily. However, in the present study, serum magnesium levels were highest among sedentary individuals supplemented with boron ($p<0.05$); whereas, in the previous study, serum magnesium was depressed through an interaction with aluminum.

Of the three major minerals implicated in bone metabolism, phosphorous has, thus far, received little attention other than concern over phosphorous intakes that are too high (Calvo et al., 1990; Wyshak et al., 1989; Heaney and Recker, 1982). In the present study there were changes in serum phosphorous associated with supplementation; sedentary individuals supplemented with boron, had the lowest serum phosphorous levels ($p<0.05$); whereas, in the previous study, serum phosphorous was elevated in magnesium-low postmenopausal women, possibly through an interaction with high dietary aluminum.

Plasma normalized calcium, in the present study, did not differ with time, supplementation or activity. However, Nielsen has reported the effects of boron supplementation on blood calcium in a number of communications. In one study, Nielsen et al. (1988a) state that serum ionized calcium was elevated with

boron supplementation. In a recent review, Nielsen (1990c) stated that boron deprivation depressed plasma ionized calcium and calcitonin and elevated plasma total calcium. In one human study, magnesium deprivation depressed plasma ionized calcium. Because boron and/or magnesium deprivation causes changes similar to those seen in women with postmenopausal osteoporosis, Nielsen (1990c) contends that these elements are needed for optimal calcium metabolism and are, thus, needed to prevent the excessive bone loss which often occurs in postmenopausal women and older men.

Boden and Kaplan (1990) recently stated that normal serum calcium balance could be upset by dietary calcium, phosphorous, and/or magnesium deficiencies. The significance of an association between lower serum phosphorous, lower dietary phosphorous and lower bone mineral density should be investigated further. Also, an association with seasonal variations should be examined further since changes over time were noted with all serum minerals.

Methods of Blood Calcium Analysis. The fact that the plasma and serum calcium values found in this study were within the normal ranges reported for clinical controls was indicative that the methods used to assess blood levels were valid. SeraChem (1991) publishes clinical control data sheets for commercially supplied serum standards. Reported values for the clinical chemistry serum pool used at the time of analysis were in the range of 9.5 $\pm$ 1.5 for AA determinations.

In the present study a difference over time was observed in serum total calcium, determined by AA, and plasma ionized calcium, determined by NOVA 7; while the plasma total or normalized calcium values determined by NOVA 7 did not differ over time. Plasma ionized calcium is considered to be a more valuable indicator of calcium status than is blood calcium. Plasma ionized calcium is more readily available to respond to homeostatic mechanisms maintaining normal blood calcium levels.

Ballantine and Herbein (1990) have reported differences between AA and NOVA 7 methodology in serum calcium concentrations in Holstein cattle. Concentrations of plasma total calcium determined potentiometrically, using the NOVA 7 were lower than those determined by AA. However, in general, the total calcium concentrations, determined by either method, appeared to be similar to those determined by AA in other studies.

Urine Mineral Analysis

In general, the urine calcium values reported in this study, ranging from 47 ± 31 to 106 ± 40 mg/d, are high in relation to those reported as normal by Free and Free (1975), 23 mg/d. When compared to those reported by Nielsen et al., (1988a) who reported urinary excretion values of 65 to 132 mg/d, the current values appear comparable. The excretion of phosphorous, magnesium and boron can be considered within normal ranges.

A difference over time was noted with urinary calcium determinations, with calcium excretion consistently being higher during the final collection period than the baseline collections ($p < 0.05$). As would be expected, boron excretion rates were significantly influenced by boron supplementation. This would explain the significantly different boron excretion rates reported over time and between supplement groups. However, activity also appears to influence boron excretion. The higher boron excretion levels in the athletes can not be explained by supplementation alone, since an increase of the same magnitude was not seen in the sedentary controls. The final urine analysis was done on urine collected on one day. A composite collected over several days would have been preferred to represent average daily excretions.

Diet should not be discounted. At this time the boron content of the diets is not known and this could possibly be a factor in the difference in excretion between the two activity groups. Phosphorous and magnesium did not display this same effect over time.

These observations do not coincide with those of Nielsen et al. (1987a,b) who reported that dietary boron had a marked effect on major mineral metabolism in postmenopausal women. These authors stated that a supplement of 3.0 mg of boron/day, added to a low boron diet for four months (167 days), markedly depressed the urinary excretion of calcium and magnesium in postmenopausal women. They concluded that supplementing a low-boron diet with an amount of boron, commonly found in diets high in fruits and vegetables,

induces changes in postmenopausal women consistent with the prevention of calcium loss and bone demineralization.

The inability to confirm the findings of Nielsen et al. (1987a,b, 1988a) in the present study may be attributed to differences in age, hormonal status, dietary management, duration of supplementation and/or activity levels. In the present study the females ranged in age from 18 to 25 years, had normal estrogen levels, consumed their normal diets, and were free living (either sedentary or athletic). The boron supplementation period lasted 10 months. The boron content of diets in the present study was unknown. The postmenopausal women were 48 to 82 years, hypoestrogenic, were housed in a metabolic unit, fed a 3-day menu rotation diet, low in boron, with supplements added to ensure adequacy of the non-variable nutrients and had normal energy expenditures for daily living. Boron supplementation lasted for 4 months.

After further experimentation in animals and another human study, Nielsen et al. (1990c) contend that boron has a role in calcium metabolism, probably at the kidney level. Moreover, this role seems more apparent with magnesium deprivation or under conditions in which increased calcium loss from bone is likely, i.e., osteoporosis. The data also suggest that magnesium deprivation alone may be detrimental to bone health.

Methods of urine mineral analysis. Urine minerals were assessed using several different methods, including atomic absorption, colorimetric procedures, and inductively coupled argon plasma spectroscopy. Differences in sample

preparation and sample analysis did not influence the determinations of urinary calcium and phosphorous, even though, with the determination of phosphorous, the levels obtained using ICP were higher than the detection limits recommended for this instrument. Atomic absorption values for urinary magnesium were consistently higher than values obtained using ICP. The samples prepared for atomic absorption were wet ashed; whereas samples prepared for ICP were digested using the WALTTT procedure described by Schuler and Hunt (1989). Thus, the difference in urinary magnesium determined using two different methods may be inherent to the methods of analysis and/or the method of sample preparation.

To conclude: 1) Boron supplementation lowered serum phosphorous and elevated serum magnesium in the sedentary subjects; 2) Dietary patterns do not appear to differ between athletic and sedentary individuals, both groups having low intakes of calcium, phosphorous, magnesium and energy. Effects of low intakes needs further investigation. 3) Urinary boron increased with boron supplementation and this increase was greater in athletic subjects.

CHAPTER VI

RECOMMENDATIONS FOR FUTURE RESEARCH

The effects of boron supplementation and activity on bone and bone mineral metabolism, from the findings in this study, indicate that more information on the effects of various levels of the treatments and the controlling mechanism are of interest. A few recommendations would include:

1. Studying the effects of various boron supplementation levels on female athletes at various physical work capacities, measuring;
   (a) dietary, serum, and urinary calcium, phosphorous and magnesium
   (b) calcitonin and parathyroid hormone.
2. Studying the effects of these same treatments on the same parameters in college males, and males and females of various ages, physical work capacities, and hormone conditions.
3. Comparisons of the various methods of analysis for calcium, phosphorous, magnesium, and boron for food, blood and urine.
4. Studying the effects of diets high in boron containing foods in these same populations measuring bone mineral indices.

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JOURNAL ARTICLE

BORON SUPPLEMENTATION EFFECTS ON BLOOD AND URINARY MINERALS IN COLLEGE FEMALE ATHLETES[1]

ABSTRACT

The effects of boron supplementation on blood and urinary minerals were studied in 17 female college athletes and 11 sedentary college control subjects. The subjects were similar in age and weight. The athletic subjects had lower percent body fat (20.6 ± 5.6 and 25.8 ± 6.5 %, respectively, ($p<0.05$)) and higher aerobic capacities (2.9 ± 0.5 and 2.1 ± 0.4 l O_2*min_{-1}, respectively, ($p<0.05$)) than did sedentary controls. No differences in dietary intake were observed. Serum phosphorous levels were lower in boron supplemented subjects than in subjects receiving placebos ($p<0.05$) and were lower during final analysis than during baseline analysis ($p<0.05$). Activity depressed changes in serum phosphorous due to boron supplementation. Serum magnesium was greatest in the sedentary controls supplemented with boron and increased with time in all subjects ($p<0.05$). Again, a group supplement interaction was observed with serum magnesium; exercise in boron supplemented subjects lowered serum magnesium ($p<0.05$). In all subjects, calcium excretion increased over time ($p<0.05$); in boron supplemented subjects, boron excretion

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increased over time ($p<0.05$). In conclusion, boron supplementation affected serum phosphorous and magnesium and the excretion of boron in the urine. The significance of these findings in relation to overall mineral status, bone mineral density, and exercise in college female athletes needs further investigation. It is possible that increasing the intake of foods high in boron may be found to be beneficial in the treatment and/or prevention of metabolic bone disorders such as those related to excessive exercise. (To be submitted to the Am J Clin Nutr)

(Key words: boron supplementation, female athletes, serum minerals, urinary mineral excretion, calcium, phosphorous, magnesium)

INTRODUCTION

It has been shown that weight bearing exercise increases bone hypertrophy and bone mineral density (1-3). However, female athletes may be at risk for developing exercise-induced menstrual dysfunction (2,4,5). The consequences of the hypoestrogenic status of these individuals can include the loss of bone mineral density, an increased risk of osteoporosis and increased musculoskeletal injuries (2,4,6-8).

The underlying physiologic mechanisms relating exercise to changes in bone mineral density have not been elucidated (2). Numerous studies have selectively characterized the effects of various nutritional patterns in estrogen deficient females, all of whom are at risk of osteoporosis. The effects of various nutritional supplements on bone and mineral status in exercising and sedentary, eumenorrheic and amenorrheic, females have been reported (6,9-12).

Several reports indicate that estrogen replacement and calcium supplementation have a positive influence on bone mineral density in exercising individuals. However, the effectiveness of nutrient supplementation is conflicting (13). High calcium supplementation levels have been supported by some and deemed by others as inappropriate, potentially causing metabolic disturbances in relation to other nutrients (14). Recently, Nielsen et al. (14) found that boron supplementation in postmenopausal women induced changes consistent with the prevention of calcium loss and bone demineralization in these subjects.

Nutritional approaches to the prevention and/or treatment of osteoporosis would be preferable to hormone therapy, particularly in young female populations.

The present study was designed to study the effect of boron supplementation on mineral metabolism in college female athletes. The effects of boron supplementation on bone mineral density and other nutritional indices in these same subjects will be presented elsewhere.

METHODOLOGY

Subjects and Study Design

The protocol for the recruitment and treatment of human subjects was reviewed and monitored by the Institutional Review Board for Research Involving Human Subjects at the University. Twenty-eight college females, ages 18 - 25 years, were selected to participate and completed the 10 month study. The subjects were classified, as either athletes or sedentary, depending on the level of activity in which they engaged. Athletic subjects (n=17) were recruited from basketball, volleyball, tennis, and track and field programs; one was a triathlete. Sedentary controls (n=11) were selected from among their contemporaries. Subjects were excluded if they had a history of smoking, previous pregnancies, eating disorders, orthopedic problems, use of recreational drugs, oral contraceptives or anabolic steroids within the previous six months, or a cumulative oral contraceptive use of greater than six months. Menstrual status

was classified as eumenorrheic if menses occurred every 25-35 days, or 10-13 menses per year.

All subjects received an oral and written explanation of the purpose of the study and procedures to be followed. All gave written consent. Each subject completed a medical history and exercise questionnaire which included information regarding their exercise regimen and menstrual history. Subjects also completed monthly menstrual history records in order to accurately assess menstrual status. They were given calendars in which to record the dates and symptoms of their periods, i.e., headaches, bloating, back or breast aches, and daily gauge menstrual flow as heavy, medium or light. An additional questionnaire was completed at the end of the study to ascertain that their health, exercise and dietary habits had been consistent throughout the year.

The experimental design and actual parameters measured are shown in Table 1. In a single blind, random assignment, the 28 college students were given either daily boron supplementation (Tri-Boron, Twin Laboratories, Inc., Ronkonkoma, NY) or placebos consisting of cornstarch in gelatin capsules (Revco Pharmacy, Blacksburg, VA). Tri-Boron is a combination of three highly utilizable, 100% natural, chelated sources of the trace mineral boron. Each capsule contains 3 mg of pure elemental boron derived from boron citrate, aspartate and glycinate chelates. The subjects took one capsule per day. No other dietary restrictions were imposed on either the athletes or the sedentary controls during the study.

Table 1
Summary of the experimental design

Parameter	Athletes		Sedentary	
Supplement	Boron	Placebo	Boron	Placebo
Time[1]	1 2 (n=10)	1 2 (n=7)	1 2 (n=6)	1 2 (n=5)
Bone mineral density	* *	* *	* *	* *
Percent body fat	* *	* *	* *	* *
Aerobic capacity	*	*	* *	* *
Weight	* *	* *	* *	* *
Nutrient Intake:				
(food analysis)				
•Protein	*	*	*	*
•Fat	*	*	*	*
•Carbohydrate	*	*	*	*
•Acid Detergent Fiber	*	*	*	*
•Minerals:				
•Calcium	*	*	*	*
•Phosphorous	*	*	*	*
•Magnesium	*	*	*	*
(3 day dietary record)				
•Total Kilocalories	* *	* *	* *	* *
•Protein	* *	* *	* *	* *
•Fat	* *	* *	* *	* *
•Carbohydrate	* *	* *	* *	* *
•Dietary Fiber	* *	* *	* *	* *
•Minerals:				
•Calcium	* *	* *	* *	* *
•Phosphorous	* *	* *	* *	* *
•Magnesium	* *	* *	* *	* *
Blood Minerals:				
•plasma				
•Ionized Calcium	* *	* *	* *	* *
•Total Calcium	* *	* *	* *	* *
•Normalized Calcium	* *	* *	* *	* *
•serum				
•Calcium	* *	* *	* *	* *
•Phosphorous	* *	* *	* *	* *
•Magnesium	* *	* *	* *	* *

[1] 1 = baseline, 2 = final

This study is part of a larger study, whereby, the blood samples collected were also analyzed for various hormones, alkaline phosphatase, and vitamin D (12).

Procedures

Aerobic Work Capacity. Aerobic work capacity was assessed to verify that the athletes differed from the sedentary controls in physical condition. Subjects with higher aerobic capacities can exercise longer and obtain greater workloads than subjects with lower aerobic capacities. A Physical Work Capacity 170 Test (PWC_{170}) was performed (15). The PWC_{170}, a submaximal exercise test, is designed to predict subjects' maximal oxygen consumption (VO_2max) by assessing their aerobic work capacity when a submaximal heart rate of 170 beats per minute is obtained. A Monark bicycle ergometer (Monark-Cresent AB, Vargerg, Sweden) was calibrated and the seat height adjusted before each subject's testing. Subjects were instructed to keep a constant cadence, 50 revolutions per minute (rpm) by watching the cycle speedometer.

Percent Body Fat. Percent body fat was determined using skinfold thicknesses obtained with Lange calipers (10/mm, constant pressure) (Cambridge Scientific Industries, Inc., Cambridge, MD). The average of five measurements was used as the representative score for each site tested, the triceps, suprailiac, and mid-thigh. The equation developed by (16) was used to calculate the percent fat for each subject and is as follows:

$$\text{Percent Fat} = \left(\frac{4.92}{BD} - 4.5\right) \times 100$$

Body Density (BD) = 1.0994921 - 0.0009929(x) + 0.0000023(x)2 - 0.001392(y)

x = sum of triceps, suprailiac, and thigh skinfolds (mm)

y = age, in years

Height and Weight. All subjects were weighed on a physicians' scale in light clothing without shoes. Weight was recorded to the nearest 0.5 kg; height was recorded to the nearest cm.

Sample Collection. Prior to supplementation, the subjects collected duplicate plates of all food and beverages consumed for three days, including one weekend day. The subjects were provided with gallon plastic containers, triple lined with plastic bags to collect all food and beverages. The total intake was homogenized and 5% composites were made in duplicate and frozen until later nutrient analysis.

Twelve to 14-hour fasting blood samples (30 ml), taken from the subjects' antecubital vein, were drawn by qualified personnel at baseline and at 10 months (final or treatment effect). Samples were taken on the same day of the week for four weeks during each collection period. The samples for ionized calcium determinations were collected in sodium heparinized vacutainers, centrifuged and analyzed within one hour of being drawn. Mineral free vacutainers were used for serum collections for the determination of calcium, phosphorous, and magnesium. Duplicate aliquots of serum were frozen at -20° C for later analysis.

Baseline urine samples were collected during the same three-day period during which food collections were made and food records kept; a single day collection was made final on a day coinciding with food collections and dietary recall. Urine was collected in polyethylene containers for each 24-hr period. The collection period was defined as beginning with the second voiding of one day and including the first voiding of the following day. Duplicate aliquots of urine composites were frozen at -20° C and stored until later analysis.

Sample Analysis. Duplicate samples were analyzed for moisture (freeze drying 5-7 days, followed by overnight drying in a 90° C oven), protein (17), fat (18) and acid detergent fiber (17). Food samples were digested using a wet ash procedure and analyzed for calcium and magnesium content using a Model 2100 Atomic Absorption Spectrometer (Perkin-Elmer Corp., Norwalk, CT). Phosphorous was determined using a colorimetric procedure (Sigma Diagnostics, #670-C, St. Louis, MO).

Blood analyses were performed on samples drawn from one week during the baseline month and one week during the final analysis month. Plasma normalized calcium was determined on the day of collection using a NOVA 7 Electrolyte Analyzer (NOVA Biomedical, Waltham, MA). The samples were diluted with 0.3% (w/v) lanthanum diluent, as chloride. Magnesium was determined in stored serum samples, collected and frozen as reported above. The thawed samples were diluted with deionized water and analyzed using a Model 2100 Atomic Absorption Spectrometer (Perkin-Elmer Corp., Norwalk, CT). Serum

phosphorous was determined using the procedures and materials obtained from Sigma Diagnostics (Sigma Diagnostics, St. Louis, MO) for the quantitative, colorimetric analysis of inorganic phosphorous on a spectrophotometer at 660 nm.

Urinary calcium and magnesium were determined using a Model 2100 Atomic Absorption Spectrometer (Perkin-Elmer Corp., Norwalk, CT) and an appropriate lamp for each element. The urine samples were diluted with 0.3% lanthanum as chloride for urinary calcium determinations; urinary magnesium was determined on samples diluted with deionized water. Inorganic phosphorous was determined following the quantitative colorimetric technique described by Sigma Diagnostics (Sigma Diagnostics, St. Louis, MO). For urinary boron determinations, samples were digested using the wet-ash, low-temperature, Teflon tube procedure developed by Hunt and Shuler (19) and analyzed using inductively coupled argon plasma spectroscopy.

Statistical analysis. Factorial analysis of variance procedures were used to test for between treatment differences and within treatment changes over time in the various experimental parameters (20). Student's t-test was employed whenever significant interactions were present. The level of significance was set, a priori, at $p = 0.05$.

RESULTS

Physical Characteristics

Twenty-eight subjects, ages 18-25 years, completed the 10 month supplementation period and pre- and final protocols. It was assumed that subjects complied with the daily supplementation regime. The athletic subjects (n=17) did not differ from the sedentary group (n=11) in age and body weight (Table 2). The athletic subjects differed from the sedentary group in body fat as assessed by skinfold thickness measurements. The baseline percent body fat for athletes was lower than that for the sedentary group, 20 ± 5% and 25 ± 6%, respectively ($p<0.05$). Physical fitness, assessed by the PWC_{170} test, differed between subject groups. The athletic subjects had greater estimated work capacities. Baseline estimates of maximal oxygen consumption were VO_2max = 2.9 ± 0.5 L O_2 per minute for athletes and VO_2max = 2.1 ± 0.4 L O_2 per minute for sedentary subjects ($p<0.05$).

Dual photon absorptiometry measurements of bone mineral densities in the lumbar vertebrae were higher for athletes than for sedentary subjects ($p<0.05$) (Table 3). The greatest difference was observed between athletes and sedentary subjects receiving placebos at ten months; bone mineral densities were 1.33 ± 0.10 and 1.17 ± 0.11 g/cm^2, respectively. Bone mineral densities were not influenced by supplementation.

Table 2
Baseline age, body weight, body fat, and VO_2max. of subjects in this study*

	Athletes (n=17)	Sedentary (n=11)
Age (yrs)	19.8 ± 1.4	20.3 ± 1.1
Body weight (kgs)	61.8 ± 9.1	59.6 ± 10.5
Body fat (%)[a]	20.6 ± 5.6	25.8 ± 6.5
VO_2max, (1 0_2*min^{-1})[a]	2.9 ± 0.5	2.1 ± 0.4

* Values represent means ± standard deviation
[a] significant difference between groups ($p<0.05$).

Table 3
Average bone mineral density measurements of subjects in this study (g/cm^2)*

	Athletes		Sedentary[a]	
Time**	**Boron (n=10)**	**Placebo (n=7)**	**Boron (n=6)**	**Placebo (n=5)**
1	1.27 ± 0.14	1.30 ± 0.08	1.25 ± 0.11	1.19 ± 0.11
2	1.30 ± 0.16	1.33 ± 0.10	1.26 ± 0.13	1.17 ± 0.11

* Values represent means ± standard deviation
** Time, 1 = baseline, 2 = final
[a] significant difference between groups ($p<0.05$).

Dietary Intake Analysis

The dietary habits between the two groups, did not differ in relation to total calories consumed per day. Daily percentages of calories consumed as protein, fat and carbohydrate also did not differ between groups. In addition, intakes of calcium, phosphorous, magnesium and dietary fiber did not differ between activity groups (Table 4).

Blood Mineral Analysis

Plasma calcium and serum phosphorous and magnesium levels were determined pre- and final (Table 5). Plasma normalized calcium levels did not differ with time, activity or supplementation. However, final serum phosphorous levels, determined colorimetrically and summed over all groups, were lower then baseline values ($p<0.05$). Supplementation was also found to influence serum phosphorous levels ($p<0.05$); additional boron was associated with lower serum phosphorous. A group-supplement interaction was also identified in relation to serum phosphorous values ($p<0.05$). Activity significantly depressed the changes seen in serum phosphorous in sedentary controls ($p<0.05$).

Final magnesium values, determined using atomic absorption, were significantly higher then baseline values ($p<0.05$). A difference in serum magnesium levels was also found between activity groups ($p<0.05$). The athletes on boron supplementation had lower serum magnesium levels than did the sedentary controls. In addition, a significant group-supplement interaction

Table 4
Baseline daily dietary intakes of subjects in this study*

	Athletes (n=17)	Sedentary (n=11)
Energy (Kcal)	1468 ± 503	1417 ± 584
Protein (%)	14.1 ± 5.1	14.9 ± 5.2
Fat (%)**	28.7 ± 7.7	30.8 ± 6.3
Carbohydrate (%)**	57.3 ± 9.8	54.3± 6.3
Calcium (mg)	650 ± 558	714 ± 442
Phosphorous (mg)	915 ± 616	840 ± 330
Magnesium (mg)	103 ± 107	73 ± 30

* Values represent means ± standard deviation
** Percentage of total kcal consumed per day

Table 5
Baseline and ost-test comparison of subjects' blood calcium, phosphorous, and magnesium (mg/dl)*

		Athletes		Sedentary	
	Time**	Boron (n=10)	Placebo (n=7)	Boron (n=6)	Placebo (n=5)
Normalized Calcium	1	4.7 ± 0.3	4.9 ± 0.2	4.8 ± 0.1	4.8 ± 0.0
	2	4.8 ± 0.2	4.8 ± 0.1	4.8 ± 0.1	4.7 ± 0.2
Phosphorous[a,b,c]	1	4.7 ± 0.4	4.7 ± 0.6	4.3 ± 0.5	5.1 ± 0.3
	2	3.9 ± 0.2	4.0 ± 0.2	3.6 ± 1.0	4.4 ± 0.3
Magnesium[a,c,d]	1	1.5 ± 0.3	1.7 ±0.2	1.9 ± 0.3	1.7 ±0.2
	2	2.0 ± 0.0	2.0 ± 0.1	2.4 ± 0.4	2.0 ±0.1

* Values represent means ± standard deviation
** Time, 1 = baseline, 2 = final
[a] significant difference between times ($p<0.05$).
[b] significant difference between supplements ($p<0.05$).
[c] significant group-supplement interaction ($p<0.05$).
[d] significant difference between groups ($p<0.05$).

was identified; boron supplementation in sedentary controls significantly increased serum magnesium levels as compared to the levels found in athletes receiving boron supplements ($p<0.05$). No difference was identified between activity groups receiving placebos.

Urinary Mineral Analysis

Daily excretion values for calcium, phosphorous, magnesium, and boron are reported in Table 6. Calcium excretion differed over time; final calcium excretion levels were higher than baseline excretion levels. The athletes also had higher calcium excretion rates than did sedentary controls. The phosphorous and magnesium levels in the urine did not differ with time, activity level, or supplementation. In regard to boron excretion, a significant time-supplement interaction was observed; urinary excretion of boron increased after 10 months of supplementation ($p<0.05$) (Figure 4). There was no significant difference over time in the urinary excretion of boron in subjects receiving placebos. Boron supplementation increased the urinary excretion rate of boron over time ($p<0.05$). The final excretion of boron increased, 4 fold, over baselineurinary excretion rates in athletes. This same increase was seen among sedentary controls; however, the increase was not as dramatic, (1.6 fold).

Table 6
Subjects' urinary calcium, phosphorous, magnesium and boron*

		Athletes		Sedentary	
	Time**	Boron (n=10)	Placebo (n=7)	Boron (n=6)	Placebo (n=5)
Calcium[a] (mg/d)	1	50 ± 45	72 ± 50	62 ± 37	47 ± 31
	2	106 ± 40	89 ± 27	74 ± 41	53 ± 16
Phosphorous (mg/d)	1	760 ± 798	719 ± 394	755 ± 364	480 ± 316
	2	601 ± 280	606 ± 178	767 ± 312	672 ± 240
Magnesium (mg/d)	1	53 ± 30	67 ± 44	60 ± 23	53 ± 33
	2	118 ± 98	72 ± 34	70 ± 36	52 ± 33
Boron[a,b,c,d] (ug/d)	1	0.7 ± 0.6	0.7 ± 0.3	0.7 ± 0.3	0.5 ± 0.3
	2	2.8 ± 1.6	0.8 ± 0.8	1.1 ± 1.1	0.7 ± 0.6

* Values represent means ± standard deviation
** Time, 1 = baseline, 2 = final
[a] significant difference between times ($p<0.05$).
[b] significant difference between groups ($p<0.05$).
[c] significant difference between supplements ($p<0.05$).
[d] significant time-supplement interaction ($p<0.05$).

DISCUSSION

Physical Characteristics

Initially, college-age female athletes had been targeted for this study with the expectation that many of these subjects would be experiencing menstrual dysfunction. The incidences of amenorrhea, 12%, (2 out of 28), and oligomenorrhea, (0%), in this study were not as high as had been anticipated. Previous investigations (21-23) have reported that exercise increases the rates of oligomenorrhea and amenorrhea. Some reports state that the rates increase from about 5% of the sedentary population to approximately 20% of women who exercise regularly and vigorously (21). Glass et al. (22) reported amenorrhea occurring in olympic marathon runners at a rate of 19% and the incidence of all menstrual dysfunctions at 34%.

Hormonal changes, specifically lower estrogen levels, are characteristic of athletic amenorrhea and menstrual dysfunction. A number of studies have shown that regular vigorous exercise is associated with decreased levels of estrogen (4,23). Other researchers have stated that circulating estrogens have an effect on the maintenance of bone composition because of their effects on calcium metabolism (2). This same effect has been observed in a previous study where a 20-30% decrease in spinal trabecular bone mass was associated with premenopausal amenorrhea in women engaged in vigorous exercise. In postmenopausal women, low serum estrogen levels have been reported to lead to an increase in osteoporosis (24).

Although the athletic subjects in this study were participating in intercollegiate sports and/or other competitions they did not portray a low serum estrogen profile (12). Only 2 of the 17 athletes were amenorrheic; the other 15 were eumenorrheic. The athletes had a higher mean bone mineral density than did sedentary subjects. Thus, the estrogen levels observed would suggest that the female college athletes in this study are not at an increased risk for osteoporosis later in life.

It is possible that the subjects in the present study were not as competitive, or not exercising at as high an intensity level as the subjects in previous studies reporting higher incidences of menstrual dysfunction. It is likely that other factors, i.e., improper diet, stress, and/or low body fat, may be associated with the higher reported incidences of menstrual dysfunction found in the literature.

In the present study there was a discrepancy between the athletes' perceived menstrual dysfunction and that revealed by monthly records and hormone assays. In baselinemedical history questionnaires, 32% of subjects (9) in the present study indicated that they were experiencing menstrual dysfunction. However, hormone assays and monthly records revealed that all of the subjects, with the exception of two amenorrheic subjects, (12% of subjects studied) were eumenorrheic.

The problem with defining and identifying menstrual dysfunction and amenorrhea has been a chronic concern among researchers working in this area.

Previous studies have not always indicated what methods (questionnaires, monthly records, hormone assays) were used to determine menstrual status.

The two amenorrheic athletes in this study differed from the other athletes in certain parameters measured. One amenorrheic athlete was in the low end of the range of body weights and had low bone mineral density measurements compared to the other athletes. Body fat was also particularly low in this individual, in comparison to the body fat measures of the other athletes. Both amenorrheic athletes had maximal oxygen consumption measures of 3.3 l O_2*min^{-1}. The average for the athletic group was 2.8 l O_2*min^{-1}, ranging from 2.0 to 3.6 l O_2*min^{-1}. Thus, the two amenorrheic athletes' levels of physical fitness, assessed by PWC^{170}, were not extreme measures within their group.

Dietary Intake Analysis

When assessing dietary intakes, no differences were found between activity groups. It is interesting to note that daily calories consumed, by all subjects, are two-thirds of the 2,200 kcal expected for this age group. Other studies have reported the daily nutrient intake of college athletes. Marcus et al. (8) observed mean daily caloric intakes of 1715 calories in eumenorrheic subjects and 1272 kcal in amenorrheic subjects. Drinkwater et al. (4) observed mean values of 1965 kcal and 1623 kcal in eumenorrheic and oligomenorrheic female athletes, respectively. Lloyd et al. (2), using the Nutritionist II computer program, reported 1585 ± 109 kcal intakes in sedentary control subjects, 2000 ± 99 kcal

in eumenorrheic athletes, and 2131 ± 144 kcal in oligomenorrheic athletes, all 18-19 years of age (values for athletes were significantly different from those for controls).

A recent report investigating the incidence of eating disorders and calorie intake levels in Swiss female athletes found that the total energy intake per day was 1849 ± 391 for the control group, 1892 ± 446 in the swimmers and 1544 ± 398 in the gymnasts (25). These subjects were 12-13 years of age. Thus, the reported intakes among the subjects in the present study appear to be consistent with the literature, although there is considerable individual variation among intakes reported.

Dietary intakes, particularly of the minerals, also varied considerably, as reflected in the large standard deviations reported. When assessing dietary minerals there were no differences found among subjects for average intakes of calcium, phosphorous, and magnesium. Calcium intakes of 650 ± 558 and 714 ± 442 mg/day for athletes and sedentary controls, respectively, are approximately one half of the RDA of 1200 mg/day for this age group. Similar calcium intakes have been found by other researchers analyzing young female athletes' diets. For example, Lloyd et al. (2) reported calcium intakes of 595, 786, and 973 mg/day for sedentary controls, eumenorrheic athletes and oligomenorrheic athletes, respectively (values for athletes were significantly different from those for controls ($p<0.05$).

In the present study, slightly higher levels of phosphorous than calcium were consumed. These intakes, 915 ± 616 and 840 ± 330 mg/day for athletes and sedentary controls, respectively, were also below the 1200 mg/day RDA. Again, Lloyd et al (2) reported similar phosphorous intakes of 967, 1183, and 1359 mg/day for sedentary controls, eumenorrheic athletes and oligomenorrheic athletes, respectively. (Values for athletes were significantly different from those for controls ($p<0.05$).

The calcium to phosphorous ratios calculated from the intakes in this study are 0.71 for the athletes and 0.85 for the sedentary group. Ratios that are 1:1 to 2:1 are preferred (26). Long term dietary intakes of calcium and phosphorous at levels reported here could pose a possible health risk, particularly with regard to bone metabolism. Previous studies have suggested that high phosphorous, low calcium diets can induce osteoporosis and alter parathyroid function (27-28). However, the changes in serum parathyroid secretion reported were induced by a diet containing 1700 mg/d phosphorous and only 400 mg/d calcium (28). The subjects in our study did not approaching these extreme levels of intake. Thus, their diets, although not properly balanced with respect to calcium and phosphorous, are not likely to induce such hormonal changes. Furthermore, these calcium to phosphorous ratios fall within the range of those calculated from intakes reported by Lloyd et al. (2); .61, .74, and 0.72 for sedentary controls, eumenorrheic and oligomenorrheic athletes, respectively and those reported by

Drinkwater et al. (4), 0.92 ± 0.17 and 0.88 ± 0.09 in amenorrheic and eumenorrheic athletes, respectively.

The dietary intakes of magnesium in the subjects in the present study are low also. Intakes of 103 ± 107 and 73 ± 30 mg/day for athletes and sedentary controls, respectively, were markedly below the 280 mg/day RDA for their age group. The current recommendation for magnesium intake is lower than the 1979 version of the RDAs in which recommended magnesium intakes for this group were set at 300 mg/day. The newer recommendations are being contested. Shils (29) has commented that the 1990 RDAs for magnesium are too low. Abraham and Grewal (30) agree, stating that the 1990 RDAs for magnesium are the minimum to which the body can adapt and at the cost of increased susceptibility to stress and probably primary postmenopausal osteoporosis. There are also reports that much greater intakes of magnesium, up to 1,000 mg/day, may be needed to maintain a positive balance under stressful conditions (31). In other countries, such as the Soviet Union, 500-1250 mg/day is the recommended magnesium intake for women (32). Although there appears to be considerable controversy over what the recommended intake level of magnesium should be, the intake observed in our study of female college athletes is only one third the present recommended level.

The consequences of low magnesium intakes in young females are of concern. As early as 1960, magnesium deficiencies were linked to osteopathies (33). In 1982 Abraham (34) postulated that premenopausal osteoporosis is a

skeletal manifestation of magnesium deficiency; postmenopausal trabecular bone loss would not occur, even without estrogen therapy, if the magnesium intake were sufficient to maintain an adequate bone magnesium reserve. Recently, Abraham and Grewal (30) proposed a total dietary program emphasizing magnesium instead of calcium for the management of postmenopausal osteoporosis.

In another instance, Nielsen et al. (1988b) demonstrated, in rats, that magnesium deprivation affected the response of boron deprivation. A series of experiments, conducted to study the effects of dietary boron and magnesium and their interactions with various calcium, phosphorous. and magnesium parameters, confirmed that the deprivation of either boron or magnesium made the deficiency signs of the other more marked.

Blood Mineral Analysis

In general, the blood values for calcium, phosphorous and magnesium fell within the expected blood concentration ranges. However, magnesium concentrations, ranging in this study between 1.5 ± 0.3 and 2.4 ± 0.4 mg/dl, were at the low end of the expected 1.7 - 2.8 mg/dl range. Low serum magnesium levels have been reported by others in relation to bone mineral metabolism. Stiedl et al. (37) reported serum and red cell magnesium and calcium concentrations in 60 osteoporotic patients (33 with senile osteoporosis, 18 with postmenopausal osteoporosis, and 9 with osteoporosis due to

corticoids). In the former two groups there were signs of chronic magnesium deficiency; in the third group, there was a trend toward low serum calcium levels. The authors stated that magnesium plays an important role in the development of bone disorders.

The effects of boron supplementation on blood minerals has also been the focus of other investigations (14,35). In one instance boron supplementation for 4 months, in postmenopausal women, was coordinated with magnesium and aluminum deficient and adequate diets. The effects of boron supplementation on blood minerals were most marked in the 13 postmenopausal subjects when they were consuming the magnesium-deficient diets (14,35).

In the present study, a similar situation existed; daily supplementation with 3 mg of boron for 10 months affected blood mineral concentrations. Like the postmenopausal subjects on a magnesium deficient diet, the female athletes consumed a low magnesium diet, although voluntarily. However, in the present study, serum magnesium levels were highest among sedentary individuals supplemented with boron ($p<0.05$); whereas, in the previous study, serum magnesium was depressed through an interaction with aluminum.

Of the three major minerals implicated in bone metabolism, phosphorous has, thus far, received little attention other than concern over phosphorous intakes that are too high (28, 39-40). In the present study there were changes in serum phosphorous associated with supplementation; sedentary individuals supplemented with boron, had the lowest serum phosphorous levels ($p<0.05$);

whereas, in the previous study, serum phosphorous was elevated in magnesium-low postmenopausal women, possibly through an interaction with high dietary aluminum.

Plasma normalized calcium, in the present study, did not differ with time, supplementation or activity. However, Nielsen has reported the effects of boron supplementation on blood calcium in a number of communications. In one study, Nielsen et al. (35) stated that serum ionized calcium was elevated with boron supplementation. In a recent review, Nielsen (41) stated that boron deprivation depressed plasma ionized calcium and calcitonin and elevated plasma total calcium. In one human study, magnesium deprivation depressed plasma ionized calcium. Because boron and/or magnesium deprivation causes changes similar to those seen in women with postmenopausal osteoporosis, Nielsen contends that these elements are needed for optimal calcium metabolism and are, thus, needed to prevent the excessive bone loss which often occurs in postmenopausal women and older men.

Boden and Kaplan (38) recently stated that normal serum calcium balance could be upset by dietary calcium, phosphorous, and/or magnesium deficiencies. The significance of an association between lower serum phosphorous, lower dietary phosphorous and lower bone mineral density should be investigated further.

Urine Mineral Analysis

In general, the urine calcium values reported in this study, ranging from 47 ± 31 to 106 ± 40 mg/d, are high in relation to those reported as normal. When compared to those reported by Nielsen et al.(35), who reported urinary excretion values of 65 to 132 mg/d, the current values appear comparable. The excretion of phosphorous, magnesium and boron can be considered within normal ranges.

A difference over time was noted with urinary calcium determinations, with calcium excretion consistently being higher during the final collection period than the baseline collections ($p<0.05$). As would be expected, boron excretion rates were significantly influenced by boron supplementation. This would explain the significantly different boron excretion rates reported over time and between supplement groups. However, activity also appears to influence boron excretion. The higher boron excretion levels in the athletes can not be explained by supplementation alone, since an increase of the same magnitude was not seen in the sedentary controls. Diet should not be discounted. At this time the boron content of the diets is not known. Phosphorous and magnesium did not display this same effect over time.

These observations do not coincide with those of Nielsen et al (14) who reported that dietary boron had a marked effect on major mineral metabolism in postmenopausal women. These authors stated that a supplement of 3.0 mg of boron/day, added to a low boron diet for four months (167 days), markedly

depressed the urinary excretion of calcium and magnesium in postmenopausal women. They concluded that supplementing a low-boron diet with an amount of boron, commonly found in diets high in fruits and vegetables, induces changes in postmenopausal women consistent with the prevention of calcium loss and bone demineralization.

The inability to confirm the findings of Nielsen et al (14) in the present study may be attributed to differences in age, hormonal status, dietary management, duration of supplementation and/or activity levels. In the present study the females ranged in age from 18 to 25 years, had normal estrogen levels, consumed their normal diets, and were free living (either sedentary or athletic). The boron supplementation period lasted 10 months. The boron content of diets in the present study was unknown. The postmenopausal women were 48 to 82 years, hypoestrogenic, were housed in a metabolic unit, fed a 3-day menu rotation diet, low in boron, with supplements added to ensure adequacy of the non-variable nutrients and had normal energy expenditures for daily living. Boron supplementation lasted for 4 months.

After further experimentation in animals and another human study, Nielsen et al. (41) contend that boron has a role in calcium metabolism, probably at the kidney level. Moreover, this role seems more apparent with magnesium deprivation or under conditions in which increased calcium loss from bone is likely, i.e., osteoporosis. The data also suggest that magnesium deprivation alone may be detrimental to bone health.

To conclude: 1) boron supplementation affected serum phosphorous and magnesium and the effect was modified by exercise 2) dietary patterns do not appear to differ between athletic and sedentary individuals, both groups having low intakes of calcium, phosphorous, magnesium and energy. Effects of low intakes needs further investigation.

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APPENDIX A

INSTITUTIONAL REVIEW BOARD

Principal Investigator(s) L. Janette Taper Department Human Nutrition and Foods

Project Title Changes in Bone Mineral Density in Eumenorrheic and Oligomenorrheic Athletes

Source of Support: Departmental Research ☐ Sponsored Research ☐ Proposal No. _____

1. The criteria for "expedited review" by the Institutional Review Board for a project involving the use of human subjects and with minimal risk* is one or more of the following. Please initial all applicable conditions and provide a substantiating statement of protocol.

☐ a. Collection of:
   a) hair or nail clipping in a non-disfiguring manner;
   b) deciduous teeth;
   c) permanent teeth if patient care indicates need of extraction.

☑ b. Collection of excreta and external secretions: sweat, uncanulated saliva, placenta removed at delivery, amniotic fluid obtained at time of rupture of the membrane.

☑ c. Recording of data from subjects 18 years or older, using noninvasive procedures routinely employed in clinical practice. Exemption does not include exposure to electromagnetic radiation outside the visible range.

☑ d. Collection of blood samples by venipuncture (not exceeding 450 ml 8 week period, and no more than twice a week) from subjects 18 years or older, in good health and not pregnant.

☐ e. Collection of supra- and subgingival dental plaque and calculus, provided the procedure is no more invasive than routine scaling of the teeth.

☐ f. Voice recordings.

☑ g. Moderate exercise by healthy volunteers.

☐ h. Study of existing data, documents, records, pathological specimens or diagnostic specimens.

☐ i. Research on drugs or devices for which an investigational exemption is not required.

2. If the project involves human subjects who are exposed to "more than minimal risk" and are not covered by the criteria above (a to i), the IRB review must involve the full IRB board. Please check if the research involves more than minimal risk** ☐ and provide a substantiating statement of protocol.

3. Human subjects would be involved in the proposed activity as either:
Minors and/or Children* ☐, Fetuses ☐, Abortuses ☐, Pregnant Women ☐, Prisoners ☐, Mentally Retarded ☐, Mentally Disabled ☐.

Note that if children are involved in the research as human subjects, they may have to provide consent as well as their parents.

Whether or not the project may undergo "expedited review" or must be reviewed by the full Institutional Review Board, it is necessary that the required informed consent forms also be reviewed. These should be submitted with the proposal. However, if there is insufficient time to meet the sponsor's deadline, submittal can be delayed up to thirty days after submittal of the proposal without jeopardizing the IRB certification to the prospective sponsor.

* Minimal risk means that the risks of harm anticipated in the proposed research are not greater, considering the probability and magnitude, than those encountered in daily life or during performance of routine physical or psychological examinations or tests.

**Subject at risk is an individual who may be exposed to the possibility of injury as a consequence of participation as a subject in any research, development or related activity which departs from the application of those established and accepted methods necessary to meet his needs, or which increases the ordinary risks of daily life, including the recognized risks inherent in a chosen occupation or field of science.

This is to certify that the project identified above will be carried out as approved by the Human Subject Review Board, and will neither be modified nor carried out beyond the period approved below without express review and approval by the Board.

L. J. Taper 3/31/[illegible] _____ 3/7/[illegible]
Signature, Principal Investigator, Date Signature, Departmental Reviewer, Date

The Human Subjects Review Board has reviewed the protocol identified above, as it involves human subjects, and hereby approves the conduct of the project for ___ months, at which time the protocol must be resubmitted for approval to continue.

_____
Signature, Board Chairman, Authorized Reviewer, Date

APPENDIX B

WRITTEN INFORMATION AND INFORMED CONSENT

Athletic Menstrual Dysfunction and Boron Supplementation

Studies have shown that female athletes who have disturbed menstrual function may also have reduced bone density which may predispose them to osteoporosis, a condition more frequently found in postmenopausal women. Normal bone accretion peaks between 20 and 30 years of age, thereafter declining gradually until menopause, when the loss of bone mineral accelerates.

Athletes with reduced bone density are at risk for increased stress fractures and musculoskeletal injuries much like the older women. Calcium and vitamin D supplements and estrogen therapy have all been recommended as possible treatment or prevention measures. The results are conflicting and nonconclusive. Recently, boron supplementation has been recommended and shown to have positive effects on bone mineralization and serum hormone levels in postmenopausal women. This supplementation has not been tested in young female athletes.

The follcwing study is designed to test the effects of boron supplementation on normal female college students, eumenorrheic (normal) college athletes, and oligomenorrheic (irregular) college athletes. The effects of supplementation will be measured in serum hormones, minerals and vitamins, urinary vitamins and minerals, bone density, body fat and muscle development. Dietary intakes of several other nutrients will also be assessed.

During the first, sixth and final months of a year long study, participants will be asked to provide the following:

- an initial health and dietary survey
- initial duplicate food intake collections for 3 days concomitantly with total daily urine collections
- blood collections (approx. 20 ml each day) for one day a week for 4 weeks; first, sixth and final months
- bone density determinations by dual photon absorptiometry, first and final months
- fitness testing on the bicycle ergometer and anthropometric measurements, first and final months

The following will be determined in relation to bone density: serum hormone levels, serum calcium, serum boron, urinary boron, dietary calcium and boron.

Consent of Participation
In Nutrition Study

Department of Human Nutrition and Foods
Virginia Polytechnic Institute and State University

I have received an oral and written explanation of the study. I understand the following:

All subjects will maintain routine activities throughout the study.

All subjects will consume their usual diet. Boron will be provided at a level of 3 mg per day as sodium borate. The usual consumption of boron is approximately 1.5-2 mg per day. A level of 3 mg would be obtained from a diet high in fruits and vegetables. Subjects will keep a complete 3 day dietary record at the beginning of the study at a ten month follow-up. Subjects will also collect duplicate plates of their typical food consumption for 3 days while, simultaneously collecting urine for 3 days, at the beginning of the study.

Venous samples of blood, approximately 30 ml, will be taken by a qualified medical technician at the beginning of the study and at six and ten month follow-ups. The subjects will donate 30 ml of blood, 4 times per month.

Bone density of the lumber spine will be determined using dual photon absorptiometry. This procedure will be done by qualified personnel at Montgomery Regional Hospital, Blacksburg, Virginia. The test takes about one hour. The dose of radiation is approximately 1% of the radiation obtained from an ordinary forearm x-ray. This will be measured at the beginning and end (10 months) of the study. Percent body fat will be assessed based on skinfold measures and will be measured at the beginning, six months, and ten months. Maximal oxygen uptake will be measured by a submaximal bicycle ergometry test. Because this is not a dependent measure, maximal oxygen uptake will only be measured at the beginning of the study.

If at anytime a participant or the investigators believe that the health of the subject may be impaired or jeopardized, the subject may drop from the study.

The following people may be contacted if subjects have any concerns or questions about their participation in this study.

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Stella Snyder, M.S.
Graduate Student
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Department of Human Nutrition and Foods
Virginia Polytechnic Institute and State University
Blacksburg, VA 24061-0430

I understand the above and agree to participate in the human nutrition study to be conducted at Virginia Polytechnic Institute and State University from April, 1990 to April 1991.

_____
(Name)

_____
(Date)

APPENDIX C

PRESTUDY QUESTIONNAIRE

Health and Fitness Appraisal

MEDICAL HISTORY QUESTIONNAIRE

Last Name First Name Middle Initial

Date of Birth Sex Home Phone

Address City, State Zip

SS Number Work Phone Family Physician

In order to assess cardiovascular function, body composition, and other physical fitness components, the undersigned hereby voluntarily consents to engage in one or more of the following tests (check the appropriate boxes):

- ☐ Graded exercise stress test
- ☐ Underwater weighing
- ☐ Muscle fitness tests
- ☐ Flexibility tests

EXPLANATION OF THE TESTS

The graded exercise stress test is performed on a bicycle ergometer or motor-driven treadmill. The work load is increased every few minutes until exhaustion or until other symptoms dictate termination of the test. We may stop the test at any time because of fatigue or discomfort.

The underwater weighing procedure involves being completely submerged in a tank or tub while breathing through respiratory equipment. This test provides an accurate assessment of your body composition.

For muscle fitness testing, you lift weights for a number of repetitions using barbells or exercise machines. These tests assess the strength and endurance of the major muscle groups in the body.

For evaluation of flexibility, you perform a number of calisthenic-type exercises. During these exercises, we measure the range of motion in your joints.

RISKS AND DISCOMFORTS

During the graded exercise stress test, certain changes may occur. These changes include abnormal blood pressure responses, fainting, irregularities in heartbeat, and heart attack. Every effort is made to minimize these occurrences. Emergency equipment and trained personnel are available to deal with these situations if they occur.

You may experience some discomfort during the underwater weighing, especially if you have a fear of being submerged. Breathing through respiratory equipment while underwater should minimize this discomfort. If necessary, alternative procedures (e.g., skinfold techniques) are used to estimate body composition.

There is a slight possibility of pulling a muscle or spraining a ligament during the muscle fitness and flexibility testing. In addition, you may experience muscle soreness 24–48 hr after testing. These risks can be minimized by performing warm-up exercises prior to taking the tests. If muscle soreness occurs, appropriate stretching exercises to relieve this soreness will be demonstrated.

EXPECTED BENEFITS FROM TESTING

These tests allow us to assess your physical working capacity scientifically and to appraise your physical fitness status clinically. The results are used to prescribe a safe, sound exercise program for you. Records are kept strictly confidential unless you consent to release this information.

INQUIRIES

Questions about the procedures used inthe physical fitness tests are encouraged. If you have any questions or need additional information, please ask us to explain further.

FREEDOM OF CONSENT

Your permission to perform these physical fitness tests is strictly voluntary. You are free to deny consent if you so desire.

I have read this form carefully and I fully understand the test procedures. I consent to participate in these tests.

_____
Signature of patient

_____ _____
Date Witness

QUESTIONS: _____

_____

RESPONSE: _____

4. Give the following information pertaining to the last three times you have been hospitalized. (Women: do not list normal pregnancies.)

	Hospitalization Number 1	Hospitalization Number 2	Hospitalization Number 3
Type of operation			
Month and year hospitalized			
Name of hospital			
City and state			

During the past twelve months...

1.	Has a physician prescribed any form of medication for you?	Yes☐	No☐
2.	Has your weight fluctuated more than a few pounds?	Yes☐	No☐
3.	Did you attempt to bring about this weight change through diet and/or exercise?	Yes☐	No☐
4.	Have you experienced any faintness, lightheadedness, blackouts?	Yes☐	No☐
5.	Have you occasionally had trouble sleeping?	Yes☐	No☐
6.	Have you experienced any blurred vision?	Yes☐	No☐
7.	Have you had any severe headaches?	Yes☐	No☐
8.	Have you experienced chronic morning cough?	Yes☐	No☐
9.	Have you experienced any temporary change in your speech pattern such as slurring or loss of speech?	Yes☐	No☐
10.	Have you felt unusually nervous or anxious for no apparent reason?	Yes☐	No☐
11.	Have you experienced unusual heartbeats such as skipped beats or palpitations?	Yes☐	No☐
12.	Have you experienced periods in which your heart felt as though it were racing for no apparent reason?	Yes☐	No☐

At present...

1.	Do you experience shortness of breath or loss of breath while walking with others your own age?	Yes☐	No☐
2.	Do you experience sudden tingling, numbness, or loss of feeling in your arms, hands, legs, feet, or face?	Yes☐	No☐
3.	Have you ever noticed that your hands or feet sometimes feel cooler than other parts of your body?	Yes☐	No☐
4.	Do you experience swelling of your feet and ankles?	Yes☐	No☐
5.	Do you get pains or cramps in your legs?	Yes☐	No☐
6.	Do you experience any pain or discomfort in your chest?	Yes☐	No☐
7.	Do you experience any pressure or heaviness in your chest?	Yes☐	No☐

8. Have you ever been told that your blood pressure was abnormal? Yes☐ No☐
9. Have you ever been told that your serum cholesterol or triglyceride level was high? Yes☐ No☐
10. Do you have diabetes? Yes☐ No☐
    If yes, how is it controlled?
    ☐ Dietary means ☐ Insulin injection
    ☐ Oral medication ☐ Uncontrolled
11. How often would you characterize your stress level as being high?
    ☐ Occasionally ☐ Frequently ☐ Constantly
12. Have you ever been told that you have any of the following illnesses?

☐ Myocardial infarction	☐ Arteriosclerosis	☐ Heart disease	☐ Heart block
☐ Coronary thrombosis	☐ Rheumatic heart	☐ Heart attack	☐ Aneurysm
☐ Coronary occlusion	☐ Heart failure	☐ Heart murmur	☐ Angina

Has any member of your immediate family been treated for or suspected to have had any of these conditions? Please identify their relationship to you (father, mother, sister, brother, etc.).

A. Diabetes
B. Heart disease
C. Stroke
D. High blood pressure

SMOKING HABITS

1. Have you ever smoked cigarettes, cigars, or a pipe? Yes☐ No☐
2. Do you smoke presently? Yes☐ No☐

Cigarettes_____per day

Cigars _____per day

Pipefuls _____per day

3. At what age did you start smoking? _____years
4. If you have quit smoking, when did you quit? _____

DRINKING HABITS

1. During the past month, how many days did you drink alcoholic beverages?

_____days

2. During the past month, how many times did you have five or more drinks per occasion?

_____times

3. On the average, how many glasses of beer, wine, or highballs do you consume per week?

Beer _____ glasses or cans

Wine _____ glasses

Highballs _____ glasses

Other _____ glasses

EXERCISE HABITS

1. Do you exercise vigorously on a regular basis? Yes☐ No☐
2. What activities do you engage in on a regular basis?

3. If you walk, run, or jog, what is the average number of miles you cover per workout?

   _____ miles

4. How many minutes on the average is each of your exercise workouts?

   _____ minutes

5. How many workouts per week do you participate in on the average?

   _____ workouts

6. Is your occupation:

   _____ Inactive (e.g., desk job)

   _____ Light work (e.g., housework, light carpentry)

   _____ Heavy work (e.g., heavy carpentry, lifting)

7. Check those activities that you would prefer in a regular exercise program for yourself:

   _____ Walking/running/jogging
   _____ Stationary running
   _____ Jumping rope
   _____ Bicycling
   _____ Stationary cycling
   _____ Handball/raquetball/squash
   _____ Basketball
   _____ Swimming
   _____ Tennis
   _____ Aerobic dance
   _____ Others (specify)

DIETARY HABITS

1. What is your current weight? _____lb height? _____in.
2. What would you like to weigh? _____lb
3. What is the most you ever weighed as an adult? _____lb
4. What is the least you ever weighed as an adult? _____lb

Psychological Stress Level Assessment and Relaxation Techniques

APPENDIX E.1. TYPE A BEHAVIOR INVENTORY

Respond to the following questions with yes or no answers:

Yes	No		
___	___	1.	I have an intense sustained drive to get ahead.
___	___	2.	I'm anxious to reach my goals, but I'm uncertain what those goals are.
___	___	3.	I feel a need to compete and win.
___	___	4.	I have a persistent desire for recognition.
___	___	5.	I always seem to be involved in too many things at once.
___	___	6.	I'm always racing the clock, constantly on edge, have deadlines.
___	___	7.	I have a need to speed things up, get things done faster.
___	___	8.	I'm extraordinarily alert mentally and physically.

From Falls, H. B., Baylor, A. M., and Dishman, R. K. *Essentials of Fitness*. Philadelphia: Saunders College Publishing, 1980. Reprinted with permission of the publisher.

5. What weight loss methods have you tried?

6. Which do you eat regularly?
   - ☐ Breakfast
   - ☐ Midmorning snack
   - ☐ Lunch
   - ☐ Midafternoon snack
   - ☐ Dinner
   - ☐ After-dinner snack

7. How often do you eat out per week? _____times

8. What size portions do you normally have?
   ☐ Small ☐ Moderate ☐ Large ☐ Extra large
   ☐ Uncertain

9. How often do you eat more than one serving?
   ☐ Always ☐ Usually ☐ Sometimes ☐ Never

10. How long does it usually take you to eat a meal? _____minutes

11. Do you eat while doing other activities (e.g., watching TV, reading, working)?

12. When you snack, how many times per week do you eat the following?

    Cookies, cake, pie_____ Candy_____ Diet soda_____

    Soft drinks_____ Doughnuts_____ Fruit_____

    Milk or milk beverage_____ Potato chips, pretzels, etc._____

    Peanuts or other nuts_____ Ice cream_____

    Cheese and crackers_____ Other_____

13. How often do you eat dessert? _____times per day _____times per week

14. What dessert do you eat most often?_____

Answer yes or no to each of the following questions:

Yes	No		
___	___	1.	Do you often experience headaches or backaches?
___	___	2.	When sitting in a chair and talking to someone, do you continually move in the chair to seek a comfortable position?
___	___	3.	When retiring for the night, are you unable to fall asleep immediately?
___	___	4.	Do you often grind your teeth when you are confronted with an unpleasant experience?
___	___	5.	Do you easily become angry or frustrated when you are faced with a problem for which there is no immediate solution?
___	___	6.	Do you often complain of being tired?
___	___	7.	Does your face often hold expressions of intense concentration?
___	___	8.	Do you often drum your fingers aimlessly or forcibly to express irritation?
___	___	9.	Does your posture appear stiff when you sit or walk?
___	___	10.	Are you unable to concentrate on one problem at a time?
___	___	11.	Are you unable to relax voluntarily?
___	___	12.	Do you often experience nervousness and uneasy feelings?
___	___	13.	Do you become upset when your plans are interrupted or must be changed?
___	___	14.	Are you highly competitive in sports, in your test grades, in your daily responsibilities?
___	___	15.	Are you time-conscious?
___	___	16.	Do you experience extreme dissatisfaction and anxiety when you fail to achieve success in your endeavors?
___	___	17.	Are you an aggressive person?
___	___	18.	Are you often too busy to allow time for physical activity?
___	___	19.	Do you plan your day's activities and often budget your time?
___	___	20.	Are you critical of yourself when you make a mistake?

____ ____ 21. Do you feel "uptight" at the end of the day?

____ ____ 22. Are you impatient when others are late for an appointment with you?

____ ____ 23. Do you often set high goals or levels of achievement for yourself?

____ ____ 24. Do you experience bad moods often?

____ ____ 25. Are you unyielding when others disagree with your beliefs or convictions?

From Miller, D. K., and Allen, T. E. *Fitness: A Lifetime Commitment* (ed. 2). Minneapolis, MN: Burgess Publishing Co., 1982. Reprinted with permission of the publisher.

Are you taking any medications on a regular basis?

no _____ yes _____

If yes, please list any and all medications you are taking (both prescription and non-prescription drugs).

Are you currently taking a vitamin and/or mineral supplement? _____ If yes, please list;

brand name of supplement(s) _____

dosage _____ frequency _____

Have you ever suffered a;

broken bone _____

bone fracture _____

sprain _____

if yes, was the incident exercise related?

_____

Have you ever suffered a training related injury ?

_____

if yes, please elaborate.

Age of menarche _____ No. of menses per year

_____

Does your menstrual pattern change during training ?

if yes, please elaborate:

Are you currently:	Yes	No	Since
amenorrheic (0 menses/year)	____	____	_____
oligomenorrheic (0-6 menses/year)	____	____	_____
eumenorrheic (normal menses pattern)	____	____	____

APPENDIX D

MENSTRUAL RECORDS

MONTHLY MENSTRUAL RECORD

1. Record day menses begins

2. Record day menses ends

3. Record daily heavy (H), Moderate (M), or light (L) rates of menstrual discharge

4. Note any signs of bloating (fluid retention), cramps, weight gain, headaches, medication used, etc.

5. Note any other causes of menses cessation or abnormalities

MONTH _____ YEAR _____

SUNDAY	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SATURDAY

APPENDIX E

POSTSTUDY QUESTIONNAIRE

FINAL QUESTIONNAIRE

NAME:_____ DATE:_____

(*If your need more space, use back of paper)

1. Have your exercise patterns changed drastically over the past year? Please describe. (If they haven't changed, please describe an average daily workout).

2. Describe the difference between a workout in your competitive season versus a workout out of your competitive season.

3. Does your menstrual cycle change with the intensity/duration of your workouts? Please describe.

4. Has your menstrual cycle remained the same (for you) during the duration of this study? Please describe YOUR normal cycle.

5. Have your eating patterns remained approximately the same during the duration of this study? Please describe even slight changes in your diet.

6. Do you take vitamin/mineral supplements? If so, please list the supplement and the amount. Also, please note when you began taking the supplement(s).

7. Were you placed on any medications over the past year? If so, please list, describe them, and explain the reason you had to take them.

8. Were you under any emotional stress this past year? If so, please elaborate.

9. EXTRA CREDIT: What do you think you were taking during this study: the boron or the placebo?

APPENDIX F

INDIVIDUAL DATA

INDIVIDUAL DATA FOR BORON SUPPLEMENTATION STUDY

SUBJECT NAME:_____

SUBJECT NUMBER:_____

THE SUPPLEMENT YOU WERE ON WAS:___(boron/placebo)___

CIRCLE ONE: BASELINE MIDPOINT FINAL

AEROBIC CAPACITY:

Maximal Oxygen Uptake (VO_2max):_____ml/kg/min

ANTHROPOMETRIC VALUES:

Percent Body Fat:_____%

Midpoint Leg Girth:_____ cm

3" Above Knee:_____ cm Midpoint Arm:_____ cm
Height:_____ inches Weight:_____ lb

HAND DYNAMOMETER:

Grip Strength:_____ kg

DUAL PHOTON ABSORPTIOMETRY:

Bone Density:_____g/cm^2

BLOOD VALUES:

Ionized Calcium:_____ Normalized Calcium:_____

Total Calcium:_____ Blood pH:_____

BLOOD VALUES (continued):

Minerals:

Calcium:_____ Boron:_____

Magnesium:_____ Phosphorous:_____

Zinc:_____ Aluminum:_____

Alkaline Phosphatase:_____ U/L
(normal range = 39 - 117 U/L)

Vitamin D:_____

Hormones:

Estrogen:_____ pg/ml Progesterone:_____ ng/ml

Testosterone:_____ ng/ml Calcitonin:_____ pg/ml

Parathyroid Hormone:_____ ng/ml

Luteinizing Hormone:_____ mIU/ml

Follicle Stimulating Hormone:_____ mIU/ml

Cholesterol Results:

Total Cholesterol:_____ mg/dl
(goal = less than 200 mg/dl)

High Density Lipoprotein (HDL) Cholesterol:_____ mg/dl
(goal = greater than 45 mg/dl)

Total Cholesterol/HDL Cholesterol Ratio:_____
(goal = less than 5.0)

URINE MINERAL VALUES:

Calcium:_____ Boron:_____

Magnesium:_____ Phosphorous:_____

Zinc:_____ Aluminum:_____

FOOD VALUES:

Your diet was comprised of:

_____% Carbohydrate _____% Protein

_____% Fat _____% Fiber

APPENDIX G

UNDERGRADUATE RESEARCH CREDITS FORM

DEPARTMENT OF HUMAN NUTRITION AND FOODS
COLLEGE OF HUMAN RESOURCES APPROVAL FORM FOR
INDEPENDENT STUDY, SPECIAL STUDY, OR UNDERGRADUATE RESEARCH
(2974,4974,4984,4994)

DEPARTMENT_____ DATE_____

COURSE NO._____ INDEX NO._____

COURSE TITLE _____

_____

HOURS OF CREDIT_____ SEMESTER_____

I. Objectives:

II. Plan of Study:

III. Methods of Evaluation:

NAME OF STUDENT
ENROLLED _____

SOCIAL SECURITY NUMBER _____

_____APPROVED:_____ DATE_____
INSTRUCTOR DEPARTMENT HEAD

APPROVED:_____
DEAN, COLLEGE OF HUMAN RESOURCES

_____
STUDENT'S CURRENT QCA

APPROVED:_____
DEAN, GRADUATE SCHOOL

INDEPENDENT STUDY

2974 Undergraduate Credit Only
4974 Undergraduate Credit Only

Independent Studies are designed for a particular student and are not intended to accommodate low enrollment sections of formal courses. A separate request is necessary for each student.

SPECIAL STUDY

4984 Undergraduate and/or Graduate Credit

If for undergraduate credit please mark Approval Form "Not for Graduate Credit," otherwise, it will be handled as a graduate course and must be submitted to the Graduate Committee 10 days prior to the beginning of the semester.

Special Studies are considered as "Experimental Courses" and are not to be offered on a recurring basis. (These are subject matter courses and require syllabi for approval.) Minimum enrollments are required (15 for undergraduate, 5 for graduate; 12 and 4 for summer terms).

STUDENT

The student should summarize the plans developed in cooperation with the instructor who will supervise your independent study. Final approval from the Department Head and the Dean must be obtained prior to the first day of classes in the applicable semester.

INSTRUCTOR

1. Prepare one copy for each student enrolled and submit to Department Head for approval.

2. Department Head will forward original plus four copies to the Dean for approval.

3. The Dean will retain one signed copy for the files, forward one signed copy to the Registrar for the student's records, and return three copies to the Department Head. Two copies will be returned to the instructor; one for instructor's file and one for student.

APPENDIX H

SUMMARY OF COMPLETE STUDY ANALYSES

Summary of the Experimental Design			
Parameter Measured (units)	**0 months**	**6 months**	**10 months**
Bone mineral density (g/cm^2)	*		*
Percent body fat (%)	*	*	*
Aerobic capacity (L 0_2/min)	*		
Height (cm)	*		
Weight (kg)	*	*	*
Nutrient Intake:			
(Food analysis)			
•Energy (kcal)	*		
•Protein (%[2])	*		
•Fat (%[2])	*		
•Carbohydrate (%[1])	*		
•Acid Detergent Fiber (g/day)	*		
•Minerals:			
•Calcium (mg/day)	*		
•Phosphorous (mg/day)	*		
•Magnesium (mg/day)	*		
(3 day dietary record)[1]			
•Energy (kcal)	*		*
•Protein (%[2])	*		*
•Fat (%[2])	*		*
•Carbohydrate (%[2])	*		*
•Dietary Fiber (g)	*		*
•Minerals			
•Calcium (mg/day)	*		*
•Phosphorous (mg/day)	*		*
•Magnesium (mg/day)	*		*
Blood hormones:[1]			
•17-B Estradiol (pg/ml)	*	*	*
•Progesterone (ng/ml)	*	*	*
•Testosterone (ng/ml)	*	*	*
•Calcitonin (pg/ml thyroid)	*		*
•Hormone (ng/ml)	*		*
•Follicle Stimulating Hormone (mIU/ml)	*		*
•Luteinizing Hormone (MIU/ml)	*		*

continued

Parameter	0 months	6 months	10 months
Blood minerals (mg/dl):			
• Plasma			
• Ionized Calcium	*	*	*
• Total Calcium	*	*	*
• Normalized Calcium	*	*	*
• Serum			
• Calcium	*	*	*
• Phosphorous	*	*	*
• Magnesium	*	*	*
Blood vitamins[1]			
• Plasma Vitamin D	*		*
Blood enzymes[1]			
• Plasma Alkaline Phosphatase	*		*
Blood Cholesterol[3]			
• Total Cholesterol	*	*	*
• HDL Cholesterol	*	*	*
• LDL Cholesterol	*	*	*
Urine minerals (mg/day)			
• Calcium	*		*
• Phosphorous	*		*
• Magnesium	*		*
• Boron	*		*

[1] published by Volpe-Snyder, 1991
[2] percentage of total calories consumed per day
[3] unpublished data

APPENDIX I

PWC_{170} AND ANTHROPOMETRIC DATA SHEETS

PWC_{170} TEST

NAME:_____

DATE:_____

AGE:_____ GROUP:_____
(active or inactive)

PREDICTED MAX HEART RATE (220 - age): _____

WEIGHT: _____ lbs. _____kg.

RESTING HEART RATE: _____

	RPM	KPN	WATTS	HR	RPE
STAGE 1	___	___	___		
Minute:					
1				_____	
2				_____	
3				_____	
4				_____	
STAGE 2	___	___	___		
5				_____	
6				_____	
7				_____	
8				_____	

	RPM	KPN	WATTS	HR	RPE
STAGE 3	___	___	___		
9				_____	
10				_____	
11				_____	
12				_____	
STAGE 4	___	___	___		
13				_____	
14				_____	
15				_____	
16				_____	
STAGE 5	___	___	___		
17				_____	
18				_____	
19				_____	
20				_____	

STAGE 6 ___ ___ ___

21 _____

22 _____

23 _____

24 _____

RPM KPN WATTS HR RPE

STAGE 7 ___ ___ ___

25 _____

26 _____

27 _____

28 _____

RECOVERY (IPE) HR RPE

1 _____

2 _____

3 _____

4 _____

SKINFOLD DATA: HEIGHT AND WEIGHT

CIRCLE ONE: BASELINE MIDPOINT FINAL

SUBJECT NAME:_____

DATE:_____

AGE:_____ TEST ADMINISTRATOR:_____

	TRICEP	SUPRAILIAC	THIGH
TRIAL 1	_____	_____	_____
TRIAL 2	_____	_____	_____
TRIAL 3	_____	_____	_____
TRIAL 4	_____	_____	_____
TRIAL 5	_____	_____	_____
AVERAGE:	_____	_____	_____

SUM OF 3 SITES (average in mm):_____ mm

PERCENT BODY FAT:_____%

HEIGHT:_____ inches _____ cm

WEIGHT:_____ pounds _____ kg

GIRTH MEASUREMENTS

CIRCLE ONE: BASELINE MIDPOINT FINAL

SUBJECT NAME:_____

TEST ADMINISTRATOR:_____

DATE:_____

MIDPOINT THIGH:

TRIAL 1	_____	inches	_____	cm
TRIAL 2	_____	inches	_____	cm
TRIAL 3	_____	inches	_____	cm
AVERAGE:	_____	inches	_____	cm

3" ABOVE PATELLA:

TRIAL 1	_____	inches	_____	cm
TRIAL 2	_____	inches	_____	cm
TRIAL 3	_____	inches	_____	cm
AVERAGE:	_____	inches	_____	cm

MIDPOINT ARM:

TRIAL 1	_____	inches	_____	cm
TRIAL 2	_____	inches	_____	cm
TRIAL 3	_____	inches	_____	cm
AVERAGE:	_____	inches	_____	cm

HAND DYNAMOMETER (GRIP STRENGTH)

CIRCLE ONE: BASELINE MIDPOINT FINAL

SUBJECT NAME:_____

TEST ADMINISTRATOR:_____

DATE:_____

GRIP STRENGTH (RIGHT ARM):

TRIAL 1	_____	kg
TRIAL 2	_____	kg
TRIAL 3	_____	kg
TAKE HIGHEST VALUE:	_____	kg

APPENDIX J

BORG'S RATING OF PERCEIVED EXERTION

BORG'S RATING OF PERCEIVED EXERTION 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
20	

From: Pollock, M. L., Wilmore, J. L., & Fox, S. M. (1984) Exercise in health and disease: evaluation and prescription for prevention and rehabilitation. Philadelphia: W.B. Saunders Company.

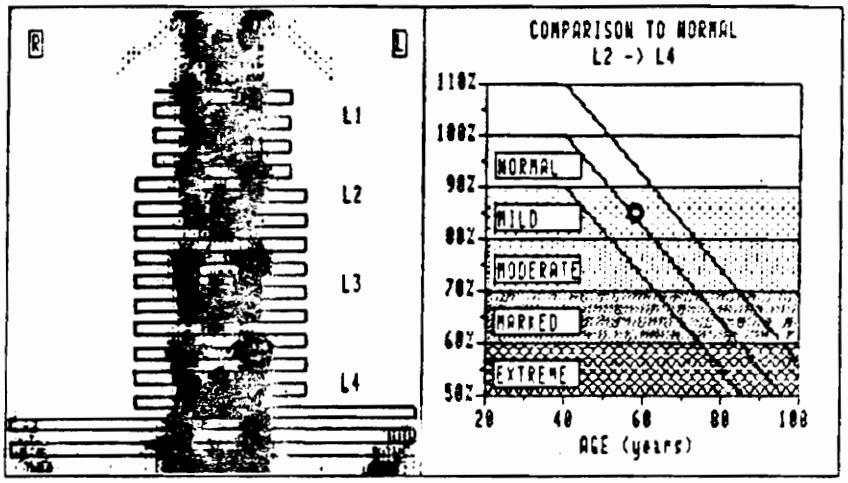
APPENDIX K

SAMPLE DATA SHEET FOR DUAL PHOTON ABSORPTIOMETRY

Graphic Display of Spine

UNIVERSITY HOSPITAL
Bone Diagnostic Center
Department of Nuclear Medicine

ID#: SAMPLE **FILENAME: SPINE-00** **DATE: 01-01-85**



CALIBRATED SPINE RESULTS VERSION-0.0

Age (years).....	58	Large Standard.....	18.00	Scan Speed (mm/s)...	5.0
Sex	Female	Medium Standard.....	13.50	Step Distance (mm)..	4.5
Weight (Kg).....	50.4	Small Standard.....	9.00	Collimation (mm)....	13
Height (cm).....	165	44 KeV Air Value....	98125	Corrected R Value...	1.43
Ethnic	White	100 KeV Air Value...	65740		

REGION	BMD g/cm²	% Young Normal	% Age Matched	Fracture Risk
L1	.939	78.8	91.2	MODERATE
L2	1.002	84.0	97.2	MILD
L3	1.030	86.4	99.9	MILD
L4	1.002	84.0	97.2	MILD
L1 -> L2	.970	81.4	94.2	MILD
L1 -> L3	.993	83.2	96.3	MILD
L1 -> L4	.996	83.5	96.6	MILD
L2 -> L3	1.017	85.3	98.7	MILD
L2 -> L4	**1.011**	**84.8**	**98.1**	**MILD**
L3 -> L4	1.014	85.1	98.4	MILD

APPENDIX L

FOOD COLLECTION PROCEDURES

Food Collections:

1. Provide subjects with an extensive information session on how to collect all food and beverage consumed and why.

2. If collections are to be in a cafeteria they may have a special room for weighing and storing.

3. Record all amounts and how prepared. No apple cores, popsicle sticks, grapefruit rinds, candy bar wrappers, or vitamin or other nutrient supplements in jugs please!

4. Do report any supplements consumed and keep them in a separate bag.

5. Collect food and fluid in large plastic 1 gallon jugs lined with small plastic trash bags (triple layered) and twist wires used to secure closing. After homogenizing, store aliqots in ziplock bags in duplicate for each day.

APPENDIX M

DIETARY FAT ANALYSIS

PROCEDURES FOR FAT EXTRACTION IN FOOD COMPOSITES USING ETHER EXTRACT

1. Weigh out 0.5 g of freeze dried food composite.
2. Dry sample overnight (with top off) in 90°C oven. The next day remove from oven and store in a desiccator with the top on until cool. On Soxhlet instrument turn on heat switch and water valve.
3. Dry cups and thimbles in the oven for 30 min. Place in desiccator to cool to room temperature.
4. Tare cups on balance. Weigh sample into thimbles.
5. Put cups and thimbles in holders and thimbles on instrument.
6. Measure some petroleum ether into each cup. Place on instrument.
7. Slowly lower thimble in petroleum ether - open stop cock.
8. Let "boil" for 30 min.
9. Raise thimbles to "rinse" for 60 mins.
10. Close condenser valves.
11. Collect solvent for 15 mins.
12. Press air button and open evaporation valve for 15 mins.
13. Turn off air and evaporation.
14. Remove cups and thimbles.
15. Put cups and thimbles in oven for 10 mins.
16. Place in desiccator and weigh back.
17. If doing a second set, check level of petroleum ether.

18. Add more, pouring in from the top, to the 50 ml mark on the holding section.

19. Repeat above procedure.

20. After 30 min remove ether and put in waste container.

21. Turn off water.

Notes: If sample is low weight use only 40 mls of ether/sample. If sample weight is high in fat use 0.25 - 0.50 g. Clean cups with EtOH (80%) and dump thimbles and blow out with air hose.

APPENDIX N

ATOMIC ABSORPTION PROCEDURES

INSTRUCTIONS FOR PERKIN-ELMER AA

1. Turn on the gases, both the air pressure (60 psi) and the acetylene (<100 psi).
2. Turn on the AA power button.
3. Turn on the computer, printer, and monitor.
4. Wait for logo screen.
5. Press the '2100' key on the key pad.
6. Wait for the system diagnostics test to run.

   Downloading 'm2100'
   Burner
   Wavedrive
   Wave calib

7. Select element to run.

   Example: (1) enter lamp position at curser, then hit (F8)
   (2) enter element number or symbol 'Cu', hit (F6)
   (3) enter lamp current '18', hit (F7)

8. Press 'Program Element'. Enter 'Element File Name'.
9. Enter 'element' to be run. Example 'Cu' (F2). If the file name is not certain press 'Data Manage' to list existing files. A message will appear (No coded lamp found for this element). This message is ok and appears because we do not have coded lamps.
10. Press 'F2', recall file. Wait for the red light to go out on recall file. To see parameter press 'Program Element' again.
11. Press 'Run Element'. Wait.

12. Press 'Flame On'. The flame on the AA should now light. Make sure the tubing is in the water now.

13. Aspirate a blank. (* Hit 'Cont Graph'). Press 'F1' for autozero. ('Cont Graph' is optional. It will give you the burner head horizontal and vertical positions.

    Then return to 'Run Element'.

14. Press the 'Print' key and then 'F1' for printer on.

15. Press 'Run Element' to return to program.

16. Press 'F2' for 1-4 standards. Aspirate first standard, press 'F2'. Aspirate second standard, press 'F3'. Aspirate third standard, press 'F4'.

17. Aspirate some deionized water for a few minutes before doing any samples.

18. Aspirate samples and press 'Read'. The printer will print out the results.

Notes: When the flame is lit check to make sure there are no 'dancing ladies' (uneven spots on the burner head flame); if so the burnerhead may need cleaning.

SHUTDOWN PROCEDURE

1. Aspirate deionized water for 5-10 minutes.
2. Remove tubing from water.
3. Turn off flame by pressing the 'Flame On/Off' button.
4. Turn off the gases, both air and acetylene.
5. Press 'Atom Contr'.
6. Press the 'F7' button. Check gases.
7. Press 'F1' several times to bleed the lines. When lines are completely bled the red light will go out on the screen.
8. Press 'Element Select'. Press 'F1' for yes.
9. Press 'F4' for return to DOS. Press 'F1' for yes.
10. Enter C>cd\ and hit 'Enter'
11. Enter C>parkhead and hit 'Enter'
12. Turn off the AA, computer, printer, and monitor.

APPENDIX O

PHOSPHOROUS ANALYSIS

Inorganic Phosphorous Assay

Sigma, Diagnostics Phosphorous, Inorganic Assay Kit (#661-11)

Method of Fiske and Subbarow (1925)

Serum collection:

1. Serum was collected in mineral free tubes, separated from cells by centrifugation and frozen at -20° C until later analysis.

Analysis procedure:

1. Serum was thawed and shaken, 0.5 ml or 0.25 ml was added to 15 ml polypropylene Sarstedt tubes, in duplicate, depending upon the amount available. Samples were also used to determine calcium and magnesium. Several additional tubes from other weeks were needed in order to obtain enough serum.
2. 2.5 ml or 1.26 ml of water and 2.0 ml or 1.0 ml of trichloroacetic acid, 20% (w/v) were added, shaken and allowed to stand 5 minutes for protein precipitation.
3. Centrifuged for 5 minutes in the clinical centrifuge on the highest setting.

4. The following was pipetted into 7 or 15 ml polypropylene Sarstedt tubes: 2.0 ml of filtrate, 3.0 ml of water and 1.0 ml of acid molybdate solution, and contents shaken. (amounts halved if serum volume low)
5. Fiske and Subbarow reducer, 0.25 ml (or 1.26 ml) was added, mixed by inversion and allowed to stand 10 min for color development.
6. Solutions were transferred to cuvets and read and absorbance recorded using a blank (2 ml of TCA, 20%, 3.0 ml water, and 1.0 ml acid molybdate solution) as reference at 660 nm on the spectrophotometer.
7. Standard concentrations of 0, 2.5, 5.0, 7.5, 10.0, 12.5 ml, and respective absorbance readings were entered to obtain a standard curve on a Texas Instruments Calculator and inorganic phosphorous determined on all samples.

Standard Curve:

1. The following reagents were pipetted into test tubes:

Tube	Phos. Std.	Water	Concentration
1	0.00 ml	5.00 ml	0 mg/dL
2	0.25	4.75	2.5
3	0.50	4.50	5.0
4	0.75	4.25	7.5
5	1.00	4.00	10.0
6	1.25	3.75	12.5

2. Acid Molybdate solution, 1 ml, was added and contents mixed by swirling.
3. Fiske and Subbarow solution was added, 0.25 ml, mixed by inversion and allowed to stand 10 min for color development. Standard clinical control serum was used to verify results each day.

Urine:

1. The same procedure was followed with the exception of using 0.05 ml of urine and 2.95 ml of water, a 10-fold dilution, during the precipitation of proteins. The precipitate was not as visible as with serum when urine was not precipitated. The preliminary trials with unprecipitated urine values did not duplicate.

2. Calculations:
   a) obtain concentration from calibration curve
   b) multiply by 10 to correct for 10-fold-dilution
   c) convert mg/dL to g/24 hr by using the formula:

$$\text{urine Pi (g/24)} = \frac{\text{Pi (mg/dL)} \times \text{24 hr urine (ml)}}{100 \times 1000}$$

Food Composites:

1. The precipitation step was omitted on the wet ashed diets and the sample and reagent amounts altered adding 0.1 ml of wet ashed diet (50% of serum concentration used), 4.90 ml of water and 1 ml of acid molybdate solution to the initial test tube.

2. Calculations:

a) obtain concentration from calibration curve

b) then calculate:

$$\frac{2(12.5 \text{ ml wet ash sample})[\text{Pi mg/dl}](0.1)}{0.5 \text{ grams freeze dried diet}} =$$

c) Pi mg/gram freeze dried diet x total grams freeze dried diet per day = Pi mg consumed per day

Comments:

Directions suggest running Pi on fasting blood since glucose ingestion lowers Pi.

"It is imperative that the serum be separated as soon as possible from the red cells. However, once the serum or plasma is separated, phosphorous is stable at refrigerator temperature for 1 week. The maximum increase standing at room temperature for 6 hours is only a few tenths of a milligram per dL. Plasma obtained with heparin (0.2 mg/mL) yielded results identical to serum. Oxalate, citrate and EDTA have been reported to yield lower results due to possible changes in water balance." (Sigma Chemical Company, 1985).

Our samples were frozen for 14 and 4 months, and at refrigerator temperatures for extended periods during months 13 and 14 during analysis.

Suggestion for next time: Prepare pools of urine, food and serum for internal controls in large batches to run each time samples are analyzed. Analyze for phosphorous at time of collection and do not store. If frozen, run all samples at one time.

APPENDIX P

NUTRITIONIST III PROCEDURES

DIRECTIONS FOR NUTRITIONIST III

1. Type cd\NUT3
2. Type N3
3. Select '< 1 > RUN NUTRITIONIST III'
4. Select '< F > RENAME CURRENT DIET' to name your diet
5. Select '< P > Select/Print RDA TYPES'
6. Enter desired selection
7. Select '< A > ENTER FOODS AND SERVINGS'
8. Entries are to be based on '< 2 > DIET ANALYSIS'
9. Enter name of food (optional meal codes needs to be added after ITEM #)
10. Enter servings, unit (gram, pound, item, etc. shows on the bottom of the screen select your choice using scroll)
11. Push 'ENTER' for position (position refers to the number, order, given to the foods listed
12. Repeat from step 8 until all foods have been entered
13. Return (go back to selection list; A - Z, 1 - 5)

14. Select '< M > LIST/PRINT DIET ANALYSIS'

    < V > if you want a printout

    < N > for screen display only

    Press 'SPACEBAR' for continue

    Select < 1 >/ < 2 > for your choice

    'ESC' to get back to program selection menu

15. Select '< H > ANALYZE DIET/RECIPE'

    Choose your selection from the bottom of the screen

    < A > to add food items

    < B > to delete items

    < L > list of food entered

    < C > composition of protein, carbohydrates, fat, alcohol in the diet

    < R > ratio of Ca/P, PUFA/SFA, PUFA/MUFA

    < F > fat and fatty acid content of the diet

    < P > to print the screen display

16. To end - 'ESC'

17. Select < 8 > to quit NUTRITIONIST III

APPENDIX Q

URINE COLLECTION PROCEDURES

Urine collections:

1. Give subjects adequate instructions on how and why collections are done: Void first urination in the morning and start collecting with the next one for the rest of the day including the first urination the next morning.
2. Give subjects 3-4 acid washed/rinsed, one liter bottles/person, rinse in ddH20 3X, Give bags to carry the bottles in and paper bags too. Preservative (HCl) is not used if urine is processed rapidly the day it is brought in. (Please turn in the day the morning collection is made). Please use our plastic bottles, do not substitute with other containers, especially glass.
3. Record total volume. One volumetric was used and acid rinsed between each measurement. Saved 200-250 ml in white plastic and brown plastic aliquot bottles to freeze until analysis.
4. Creatinine determination to check for complete 24 hr collection is possible but a 2% body weight value needs to be checked to establish each subject's creatinine value 3X before actual urine check.

Note: May need to replace collection and storage bottles; they are beginning to crack.

APPENDIX R

WET ASH DIGESTION PROCEDURES

I. Boron Digestion and Analysis

Digestion: Boron analysis was performed following the procedures established by Hunt and Shuler (1989) An open-vessel, wet-ash, low-temperature, Teflon tube (WALTTT) protocol was used to digest the samples. Inductively Coupled Argon Plasma spectroscopy (ICP) was utilized for the determination of boron and other minerals.

Urine samples:

1. Thawed samples were shaken and allowed to settle for 30 seconds.
2. Five mls of urine from the top of the container were pipetted into Teflon vials and samples prepared in duplicate. Duplicate blanks were also prepared using ultrapure distilled water. (Tuf-Tainer Vials; 30 ml; translucent; vial without cap, height and vial diameter, 76 x 29 mm)(Pierce, P.O. Box 117, Rockford, IL 61105).
3. One ml of redistilled 16.1 N HNO^3 was added and the vial loosely capped with a screw cap to allow release of generated gas. Samples were allowed to digest at room temperature for 24 hr before being heated.
4. The next morning the uncapped vials were placed in the preheated (1-2 hrs) sandbath, 15-20 mm deep, under a laboratory hood. The sandbath was filled with fine grain sand and heated to <140° C. An ice bath was available in case samples started to rapidly boil. Remove to cool. Internal

vial temperature was kept at a maximum of 120.5° C (the constant boiling point of nitric acid) by removing the vials before evaporating to dryness to prevent loosing boron compounds which are volatile above 180° C.

5. The vials were removed from the sandbath, cooled, and 1 ml of 16.1 N HNO^3 and then 3 mls of 30% H^2O^2 were added, and heated until almost dry, on a low setting. CAUTION: Always add nitric acid first and then the hydrogen peroxide. Never make into a solution! Explosive! Have ice bath ready in case samples start to rapidly boil. Remove to cool. Cap.

6. Reconstitute with 1 ml 6N HCl and heat in the sandbath, <140° C, for about five mins.

7. Labeled 15 ml polyethylene tubes were tared on a Sartoris balance. The samples and blanks were transferred from the Teflon vessels to the tared vessels using an Eppendorf pipet, rinsing three times with 2-3 mls of 0.1 N HCL, bringing the final sample weight to 10 g (approximately a 1:10 dilution). The samples were taken to the Soil Science Testing Laboratory for analysis of boron using ICP spectroscopy. Citrus leaves and bovine liver were used to validate the method.

Before use, vials and polypropylene tubes were soaked overnight in a 5% HCl solution and rinsed eight times in distilled H_20, dried in a glassware-drying oven (50°C). Repeated analysis of blanks confirmed the lack of boron contamination in the vials.

Human samples can be approved for analysis in other laboratories on campus when deactivated by heat following the recommendations of Dr. Harvey Klein as described in Morbidity and Mortality Weekly Report, Vol. 37/No. S-4, April 1, 1988.

"...heating small volumes of serum for 30 minutes at 56° C before serologic testing reduces residual infectivity to below detectable levels" (p. 4).

Handling and disposal of infectious waste was managed within the Department of Human Nutrition and Foods according to the procedures described by Dr. Barbara Chrisley, included with blood collection procedures.

Notes:

Where:- Litton Reaves Poultry Science Laboratory under the hood, Nancy Frank's sandbath for 30 Teflon vials/run.

-Approximately 5-7 hours/run

-Label all tubes and caps

-Gloves and goggles used and small hand mirror to observe when the evaporation has just begun to expose the bottom of the Teflon vials. Once this is observed remove the vials, cool, and if complete dilute as described above or add more liquid and heat to almost dryness again.

-Be particularly cautious when moving Teflon vessels in and out of the sandbath to prevent dropping sand in to uncapped sample vials.

Analysis:

Standards were prepared and samples run by Nancy Phillips, Soil Testing Laboratory, Agronomy Department, Virginia Tech.

II. NITRIC-PERCHLORIC WET ASH PROCEDURE

1. Weigh out 100 mg sample in duplicate into acid washed test tubes and add 3.0 ml redistilled nitric acid. Let sit over night.

2. The next morning turn on heating block, use the lowest setting.

3. Under the hood, add 1.0 ml redistilled $HCLO_4$ to the blanks and then to the samples and place the test tubes on the heating block, also under a hood.

4. Allow samples to heat. A brown smoke will appear in the test tubes.

5. After they have heated on low for about 30 minutes to 1 hour and brown smoke has stabilized, swirl the samples by lifting them from the block and gently agitating the contents. This can be done every 15 minutes until the brown smoke disappears.

6. The samples should now be yellow. Turn up the heat to the middle (2nd) setting.

7. The samples will smoke again, eventually the smoke will be white. Continue heating until almost dry. The sample remaining should turn clear after swirling without bubbling.

8. Remove tubes from the heating block to cool. Place then in a test tube rack under a hood. Turn off heating block and wash down.

9. Once samples have cooled, add 10% HCl, bringing the volume to the 12.5 mark on the tube.

10. Cap samples with parafilm and allow to sit for two hours.

11. Transfer to labeled disposable polypropylene tubes.

APPENDIX S

ICP USER INFORMATION

Updated 12/90
SJD, DWA

SPECTRAL ANALYSIS OF ELEMENTS WITH

INDUCTIVELY COUPLED PLASMA SPECTROMETRY (ICP)

SOIL TESTING AND PLANT ANALYSIS LABORATORY

145 Smyth Hall, VPI & SU, Blacksburg, VA 24061-0404
Phone: (703) 231-6893

Introduction

An Inductively Coupled Plasma (ICP) spectrometer system is available for analyzing extracts of soil, plant and other materials for over 60 different elements. The laboratory is equipped to process samples submitted by Virginia Tech researchers for a nominal fee. The ICP system consists of a **simultaneous** spectrometer (Jarrell-Ash ICAP 9000) and a **sequential** (scanning) spectrometer (Jarrell-Ash Atomscan 2400). The simultaneous spectrometer records the concentration of pre-selected elements simultaneously, and is limited to 13 elements (Table 1). The sequential spectrometer scans the spectrum from 190 to 535 nm, measuring specified wavelengths corresponding to over 60 individual elements (Table 2).

The following sections outline procedures and costs for submitting samples for analysis.

Scheduling of Work

All ICP analytical work must be scheduled in advance. Samples will not be accepted without prior scheduling. Contact Mrs. Nancy Phillips in Room 145 Smyth Hall (231-9806) to set up a time for sample processing. Analyses will be scheduled so as to minimize sample storage.

Sample Preparation

Samples to be analyzed must be extracted and filtered by the researcher and delivered in solution form, along with information of sample type and matrix used. Suspensions and radioactive materials are not acceptable. The Laboratory retains the right to refuse samples which are deemed unacceptably hazardous. For up to 4 elements, at least 25 ml of solution should be submitted per sample; an extra 5 ml per sample is required for each additional element. Sufficient volume of blank solution is also required for standard preparation. Please contact Mrs. Nancy Phillips for the appropriate volume needed.

Sample Charges

A nominal fee will be charged for all analytical work done. A completed Interdepartmental Service Request (ISR) and an estimate of the number of samples to be analyzed are required before any work is done. The cost of analysis depends upon which instrument is required, number of elements requested per sample, and total number of samples. Additional charges may be required.

Pricing Structure

Cost per sample on the simultaneous ICP (see Table 1):

	Within University / Federal Govt.	Outside the University
1 - 6 elements per sample:	$3.50	$4.00
Each additional element / sample:	$0.30	$0.50

Cost per element per sample on the sequential ICP (see Table 2):

	Within University / Federal Govt.	Outside the University
Each element per sample	$3.00	$8.35

NOTE: There is a minimum charge of $30.00 for any work done to cover start up costs.

Additional Charges

Additional charges may be necessary under certain conditions to defray the cost of analysis. Specifically, any unfamiliar matrix (acid, base, etc.) must be evaluated to isolate and minimize spectral interferences. Wavelength patterns for many matrices have already been determined. An additional $75 is required for matrices not yet evaluated for the ICP. Also, elements which have not been previously analyzed require additional setup time and, therefore, an extra $25 per new element is required.

Analysis of samples containing hydrofluoric acid necessitate installation of a special torch on the sequential ICP. An additional $50 is required to cover installation and setup of the hydrofluoric torch.

Please contact Mrs. Nancy Phillips (231-9809) for details on which additional charges may apply.

Table 1. Detection limits, Simultaneous Spectrometer (Jarrell Ash ICAP 9000).

Element		Analytical Range*	Element		Analytical Range
		— ppm —			— ppm —
Al	Aluminum	0.025 - 5000	B	Boron	0.006 - 150
Ca	Calcium	0.100 - 1500	Cu	Copper	0.002 - 150
Fe	Iron	0.005 - 150	K	Potassium	0.300 - 1000
Li	Lithium	0.006 - 150	Mg	Magnesium	0.010 - 350
Mn	Manganese	0.001 - 150	Na	Sodium	0.010 - 200
P	Phosphorus	0.060 - 250	S	Sulfur	0.100 - 500
Zn	Zinc	0.004 - 150			

* Analytical ranges were compiled from published values and may not reflect actual ICP performance. Detection limits will vary somewhat depending on sample matrix.

Table 2. Detection limits, Sequential Spectrometer (Jarrell Ash Atomscan 2400).

Element	Analytical Range*	Element	Analytical Range
	— ppm —		— ppm —
Ag Silver	0.007 - 150	Nd Neodymium	0.010 - 1500
Al Aluminum	0.025 - 3800	Ni Nickel	0.100 - 2500
As Arsenic	0.100 - 10000	Os Osmium	0.050 - 5000
Au Gold	0.010 - 7500	P Phosphorus	0.100 - 10000
B Boron	0.006 - 600	Pb Lead	0.100 - 10000
Ba Barium	0.001 - 8000	Pd Palladium	0.070 - 10000
Be Beryllium	0.001 - 2100	Pr Praseodymium	0.030 - 3500
Bi Bismuth	0.040 - 10000	Pt Platinum	0.030 - 10000
Ca Calcium	0.001 - 10000	Rb Rubidium	37.50 - 10000
Cd Cadmium	0.002 - 200	Re Rhenium	0.015 - 6300
Ce Cerium	0.030 - 3000	Rh Rhodium	0.020 - 6300
Co Cobalt	0.003 - 300	Ru Ruthenium	0.030 - 7500
Cr Chromium	0.005 - 7500	Sb Antimony	0.050 - 10000
Cu Copper	0.002 - 4000	Sc Scandium	0.001 - 100
Dy Dysprosium	0.003 - 1500	Se Selenium	0.080 - 10000
Er Erbium	0.003 - 1000	Si Silicon	0.010 - 3000
Eu Europium	0.001 - 200	Sm Samarium	0.010 - 2000
Fe Iron	0.005 - 10000	Sn Tin	0.050 - 8000
Ga Gallium	0.020 - 5000	Sr Strontium	0.001 - 6300
Gd Gadolinium	0.010 - 1000	Ta Tantalum	0.020 - 5000
Ge Germanium	0.100 - 5000	Tb Terbium	0.010 - 2000
Hf Hafnium	0.010 - 5000	Te Tellurium	0.060 - 10000
Hg Mercury	0.100 - 10000	Th Thorium	0.030 - 1500
Ho Holmium	0.003 - 400	Ti Titanium	0.002 - 4000
In Indium	0.040 - 4000	Tl Thallium	0.100 - 10000
Ir Iridium	0.020 - 2500	Tm Thulium	0.080 - 400
La Lanthanum	0.002 - 300	U Uranium	0.200 - 10000
Li Lithium	1.000 - 10000	V Vanadium	0.001 - 1000
Lu Lutetium	0.001 - 250	W Tungsten	0.020 - 3000
Mg Magnesium	0.001 - 5000	Y Yttrium	0.002 - 2500
Mn Manganese	0.001 - 5000	Yb Ytterbium	0.001 - 1050
Mo Molybdenum	0.005 - 1000	Zn Zinc	0.004 - 10000
Na Sodium	1.000 - 1000	Zr Zirconium	0.003 - 900
Nb Niobium	0.010 - 4000		

* Analytical ranges were compiled from published values and may not reflect actual ICP performance. Detection limits will vary somewhat depending on sample matrix.

CHAPTER 6

THE ICAP SOURCE

I. Formation of a Plasma

A. A plasma is defined as a gas in which a significant fraction of its atoms are ionized.

B. It can be formed by the interaction of a strong magnetic field on charged particles within a stream of argon.

1. The high frequency AC current in the coil which surrounds the quartz tube containing argon produces a pulsating magnetic field.

2. The magnetic lines of force are axially oriented inside the tube and follow closed elliptical paths outside the tube.

3. The magnetic field does not interact with the un-ionized argon until a few "seed" electrons are introduced with a Tesla Coil.

4. Under the influence of the alternating magnetic field, these electrons form eddy currents which flow in annular paths within the argon.

5. The eddy currents heat the argon gas and produce more ionization.

C. A plasma formed in this way attains a gas temperature of 9000 - 10,000 K.

1. With this sort of temperature, it is necessary to shield the quartz tube from the plasma by use of a tangental flow of coolant argon.

2. The coolant argon, cools the inside walls of the outermost quartz tube and centers the plasma radially in the tube.

D. The plasma assumes a donut or toroidal shape.

1. The sample is injected into the narrow channel up through the toroidal hole.

2. The temperature in the channel is about 7000 K--twice as high as any flame used in AA.

3. The plasma core which resides inside and extends a few millimeters above the coil emits an intense continuum and is of little analytical usefulness.

4. The next zone extends 1 to 3 cms over the coil.

    1. The continuum is much reduced.

    2. Background structure: OH bands between 2600 - 3250A, Ar lines and weak emission from NH, NO, CH, etc.

    3. Highest signal to noise ratio for analyte species are observed in the mid-to-upper region of this zone.

5. The tail flame or third zone is barely visible when water is aspirated, but assumes typical flame colors when analytes are added to plasma.

E. Advantages of a plasma as a source.

1. The combination of high temperature and relatively long sample resident time (5 msec.) lead to complete solute vaporization and high atomization within the plasma core.

2. Free atoms are generated in a chemically inert enviornment.

3. Free atoms emit light in a narrow cylindrical channel. The optical aperture or viewing field of most spectrometers can be easily filled by this sort of source.

4. At the normal observation height in the plasma, the central axial channel has a very uniform temperature.

5. High density of emitting atoms in a central channel and few in surrounding hotter sheath.

6. Atoms tend to form optically thin emitting source with little self-absorption--wide dynamic range up to 5 orders of magnitude.

II. <u>The RF Generator</u>

A. Converts the power obtained from the powerline to RF energy required to create a plasma.

Comparison of Detection Limits for AA, GFAA, ICP				
Element	**Detection Limit (ug/L)**			
	AA	**GFAA**	**ICP***	**ICP-ST**
Calcium	1	0.05	5	.0001
Phosphorous	50,000	30	20	.00006
Magnesium	0.1	0.004	0.1	.00001
Boron	700	20	0.1	.000006
* ICP detection limits for multielement analysis				

APPENDIX T

BLOOD COLLECTION PROCEDURES

Blood collection:

1. Subjects- call and remind the night before about 12 hour fast and appointed time to appear in the morning. They may have only water, tea, coffee during the 10-13 hour fast. Scheduled at 5 minute intervals, this is hard to maintain, especially if any problems arise.
2. Each subject must sign a release form each day blood is drawn (see appendix with consent forms).
3. Materials needed:

consent forms
labeled tubes
needles
test tube racks
vacutainer holder
ice and wash tub
styrofoam light protecting tub
tourniquet
ammonium slats
cotton balls
alcohol swabs
bandages
lab coats
paper towels
gloves
biohazard bags and bucket
kimwipes
baby powder
pencil, pen, paper
data sheet
labeling tape
parafilm
Sharpie
food
phone numbers

4. Vacutainers are prelabeled, name and date

   1-sodium heparin, 7 ml (Mineral free)
   2-mineral free, 7 ml@
   1-15 ml EDTA for B6 vitamers, cholesterol, HDL-C, Hct, Hb

5. Escort subjects to 310 Wallace Hall and make sure they eat something, or drink something, and are there for at least 10 minutes.

Blood preparation following collection:

1. The green labeled 7 ml vacutainers (Beckton-Dickenson) (Na Heparin for plasma) are spun on the clinical centrifuge on setting 3 for 10 min (bring up slowly to "3" on dial and then start timer).
2. The plasma is then removed with a disposable pipette to a plastic tube for freezing. If being used for ionized calcium determinations these tubes are carried over to (Dairy Science, Litton-Reaves) for analysis on the Nova 7 and the remaining plasma frozen and the cells discarded.
3. The sera from the red labeled vacutainers (7 ml) (additive free/mineral free) are for mineral analyses. The blood is allowed to sit, refrigerated, several hours or over night to allow clotting to occur.
4. The samples are spun down and the sera drawn off of the cells and clot with a disposable transfer pipette and transferred just like the plasma samples to plastic Sarsted tubes (7 ml, 65x15ml) for storage (-20°C). These tubes are collected in duplicate, labeled with name, date, and duplicate number, 1 or 2. Use different colors of tape for all 3 sets.

Notes:

Biohazard precautions: To decontaminate waste (transfer pipettes, gloves, vacutainers with cells) dispose of waste in sharps containers or biohazard bags in red buckets. See attached department memo on proper disposal procedures.

Use gloves and 10% chlorox to wipe up spills and clean desk top.

-use biohazard bag ("glad bag in beaker"), for disposal of glass tubes, plastic pipettes, gloves, etc.

-use Sarstedt tubes, water fast labeling pen, wrap tape all the way around tube and overlap slightly to prevent peeling off when freezing or thawing tubes.

-follow top of sera/plasma down with tip of pipette, use different pipette for each subject (Manual advised with calcium samples to take sample from top of red cell area to prevent aeration of plasma and alteration of pH.

-set out samples, labeled storage tubes, and counted pipettes before starting, cap after each subject's sample is transferred.

-to obtain permission to carry tubes with human blood components into other labs on campus be sure to inform them that these are human samples and that

we have obtained the proper protocol to decontaminate them for HIV and Hepatitis. See attached letter and MMWR information.

-size and cost of vacutainers, transfer tubes, transfer pipettes

-see procedures for ionized calcium and blood collection

APPENDIX U

HUMAN WASTE DISPOSAL PROCEDURES

INFECTIOUS WASTE MANAGEMENT

Protocol for Waste Disposal for Department of Human Nutrition and Foods

Categories of waste include:

a. Biological/"infectious" waste

b. Glass waste and "sharps"

c. Chemical waste and toxic waste

d. General lab waste, i.e., trash

e. Radioactive waste

Definition of each category of waste:

a. Biological/"infectious" waste:

INFECTIOUS - biomedical waste which is infectious to humans (not necessarily infectious but is treated as though capable of producing disease in humans or is perceived as "infectious")

Infectious waste:

1. Cultures and biologicals
2. Blood and blood products, including anything that comes in contact with blood, i.e., gloves, paper towels, Kimwipes, pipet tips, tubes (plastic)
3. Pathological waste
4. Sharps
5. Animal blood, carcasses, tissues, etc. presently human and animal waste treated the same

b. Glass waste and "sharps":

"Sharps" (includes infectious and noninfectious)

- needles
- syringes
- vacutainers
- glass slides
- pipet tips

- razor blades, scalpels
- Pasteur pipets
- all other pipets
- all blood or serum tubes for disposal
- all other glass tubes (broken)
- broken plastic
- small disposable filter units
- disposable loops
- swabs
- capillary tubes
- droppers
- glass vials
- ANY GLASS, PLASTIC, OR METAL WHICH CAN PUNCTURE A BIOHAZARD BA

c. Chemical waste and toxic waste:

- reagents and solvents used in lab procedures

d. General lab waste:

- regular trash includes everything from candy to flowers

e. Radioactive waste:

- never handle unless authorized

III. DISPOSAL PROCEDURE

a. Biological/"infectious waste":

- All "infectious waste" MUST BE AUTOCLAVED.* Therefore dispose all non-sharp materials in ORANGE BIOHAZARD BAGS, i.e., gloves, paper towels, Kimwipes, plastic tips.
- Liquids (solvents, etc.) that have been exposed to human or ani blood must be saved in a separate container. It must have a chemical label on it with the approximate amount of blood, plas serum, etc. that is in the container.

- Tell Barbara Chrisley or Kathy Reynolds so they can Autoclave the bag.
- Have information ready for log (record-keeping) i.e., kinds of materials including reagents added, how much, who generated, and Principal Investigator.
- After Autoclaving, "non-infectious" tag will be put on bag.
  *Some solvents exposed to blood cannot be autoclaved, i.e., cyanmethemoglobin reagent has toxic cyanide in it.
- Autoclaved bags of material will be kept in walk-in freezer until final packaging in RED BAG for contracted waste people.

b. Glass waste and "sharps":

- Dispose of "sharps" in orange biohazard bag which is in a labeled/red hard plastic container.
- Follow procedure as listed under a "infectious" waste.

c. Chemical waste and toxic waste:

- Before beginning any chemical analysis, fill out laboratory safety check list.
- Review MSDS (material safety data sheet) supplied from company from which the reagent or solvent was purchased.
- Use chemical labels on solvents or reagents that are stored in containers other than original one.
- Fill out waste label.
- TA's and students involved with research check with Carolyn Harris, Kathy Reynolds or Barbara Chrisley who call Health & Safety: the Hazardous Waste department to pick-up.

d. General lab waste:

- Keep trash segregated from "infectious" waste because we are charged by the pound for disposal of "infectious" waste.

e. Radioactive waste:

- Special guidelines

- Barbara Chrisley is the licensed and responsible person for radioisotopes in Human Nutrition and Foods.

GENERAL SAFETY GUIDELINES

Wear gloves, goggles and lab coats at all times when doing lab work!

Assume that almost nothing goes down the sink!

When in doubt, ASK!

IV. VA TECH REGULATIONS

- Refer to:

  Commonwealth of Virginia "Infectious waste management regulations"

- June 12, 1990 memo from Keith Furr

  "Processing of Biological Research/Clinical Waste

- University Co-Chairman for Infectious Waste Disposal

  Dr. Don Cordes

  Dr. Keith Furr

- Research Resource Information from NIH

  301-984-2870 (answer questions)

THE UNIVERSITY MUST COMPLY WITH ESTABLISHED DISPOSAL PROCEDURES. FAILURE TO COMPLY WILL RESULT IN CENSURE AND FINES TO THIS DEPARTMENT AND LOSS OF YOUR LABORATORY PRIVILEGES.

Table 7 Universal Precautions to Prevent Transmission of HTV

Universal Precautions

Because a medical history and physical examination cannot reliably identify all patients infected with HIV or other blood-borne pathogens, blood and body-fluid precautions should be consistently used for all patients, especially those in emergency-care settings in which the risk of blood exposure is increased and the infection status of the patient is usually not known.

1. Use appropriate barrier precautions to prevent skin and mucous membrane exposure when exposure to blood, body fluids containing blood, or other body fluids to which universal precautions apply (see below) is anticipated. Wear gloves when touching blood or body fluids, mucous membranes, or nonintact skin of all patients; when handling items or surfaces soiled with blood or body fluids; and when performing venipuncture and other vascular access procedures. Change gloves after contact with each patient; do not wash or disinfect gloves for reuse. Wear masks and protective eye wear or face shields during procedures that are likely to generate droplets of blood or other body fluids to prevent exposure of mucous membranes of the mouth, nose, and eyes. Wear gowns or aprons during procedures that are likely to generate splashes of blood or other body fluids.
2. Wash hands and other skin surfaces immediately and thoroughly following contamination with blood, body fluids containing blood, or other body fluids to which universal precautions apply. Wash hands immediately after gloves are removed.
3. Take care to prevent injuries when using needles, scalpels, and other sharp instruments or devices; when handling sharp instruments after procedures; when cleaning used instruments; and when disposing of used needles. Do not recap used needles by hand, do not remove used needles from disposable syringes by hand, and do not bend, break, or otherwise manipulate used needles by hand. Place used disposable syringes and needles, scalpel blades, and other sharp items in puncture-resistant disposal containers, which should be located as close to the use area as is practical.
4. Although saliva has not been implicated in HIV transmission, the need for emergency mouth-to-mouth resuscitation should be minimized by making mouthpieces, resuscitation bags, or other ventilation devices available for use in areas in which the need for resuscitation is predictable.
5. Health-care workers with exudative lesions or weeping dermatitis should refrain from all direct patient care and from handling patient-care equipment until the condition resolves.

Universal precautions are intended to supplement rather than replace recommendations for routine infection control, such as hand washing and use of gloves to prevent cross-microbial contamination of hands. In addition, implementation of universal precautions does not eliminate the need for other category- or disease-specific isolation precautions, such as enteric precautions for infectious diarrhea or isolation for pulmonary tuberculosis. Universal precautions are not intended to change waste management programs undertaken in accordance with state and local regulations.

Body Fluids to Which Universal Precautions Apply

Universal precautions apply to blood and other body fluids containing visible blood. Blood is the single most important source of HIV, hepatitis B virus, and other blood-borne pathogens in the occupational setting. Universal precautions also apply to tissues, semen, vaginal secretions, and the following fluids: cerebrospinal, synovial, pleural, peritoneal, pericardial, and amniotic.

Universal precautions do not apply to feces, nasal secretions, sputum, sweat, tears, urine, and vomitus unless they contain visible blood. Universal precautions also do not apply to human breast milk, although gloves may be worn by health-care workers in situations in which exposure to breast milk is frequent. In addition, universal precautions do not apply to saliva. Gloves need not be worn when feeding patients or wiping saliva from skin, although special precautions are recommended for dentistry, in which contamination of saliva with blood is predictable. The risk of transmission of HTV, as well as hepatitis B virus, from these fluids and materials is extremely low or nonexistent.

Use of Gloves for Phlebotomy

Gloves should be effective in reducing the incidence of blood contamination of hands during phlebotomy (drawing of blood samples), but they cannot prevent penetrating injuries caused by needles or other sharp instruments. In universal precautions, all blood is assumed to be potentially infectious for blood-borne pathogens. Some institutions have relaxed recommendations for the use of gloves for phlebotomy by skilled health-care workers in settings in which the prevalence of blood-borne pathogens is known to be very low (e.g., volunteer blood-donation centers). Institutions that judge that routine use of gloves for all phlebotomies is not necessary should periodically reevaluate their policy. Gloves should always be available for those who wish to use them for phlebotomy. In addition, the following general guidelines apply:

1. Use gloves for performing phlebotomy if cuts, scratches, or other breaks in the skin are present.
2. Use gloves in situations in which contamination with blood may occur—for example, when performing phlebotomy on an uncooperative patient.
3. Use gloves for performing finger or heel sticks on infants and children.
4. Use gloves when training persons to do phlebotomies.

Precautions for Laboratories

Blood and other body fluids from all patients should be considered infective. To supplement the universal precautions listed above, the following precautions are recommended for workers in clinical laboratories:

1. Put all specimens of blood and body fluids in a well-constructed container with a secure lid to prevent leakage during transport. Take care when collecting each specimen to avoid contaminating the outside of the container or the laboratory form accompanying the specimen.
2. Wear gloves when processing blood and body-fluid specimens (e.g., when removing tops from vacuum tubes). Wear masks and protective eye wear if it is anticipated that mucous membranes will come in contact with blood or body fluids. Change gloves and wash hands after completion of specimen processing.
3. For routine procedures, such as histologic and pathologic studies or microbiologic culturing, a biologic safety cabinet (class I or II) is not necessary. However, use a biologic safety cabinet (class I or II) when procedures are conducted that have a high potential for generating droplets, such as pipetting, sonicating, and vigorous mixing.
4. Use a mechanical pipetting device for manipulating all liquids in the laboratory. Do not pipette by mouth.
5. Limit use of needles and syringes to situations in which there is no alternative.
6. Decontaminate laboratory work surfaces with an appropriate chemical germicide after a spill of blood or other body fluids and after work is completed.
7. Decontaminate materials contaminated during laboratory tests before reprocessing them. Place contaminated materials for disposal in bags and discard in accordance with institutional policies for disposal of infective waste.
8. Decontaminate and clean scientific equipment that has been contaminated by blood or body fluids if repair in the laboratory or transport to the manufacturer is necessary.
9. Wash hands after completing laboratory work and remove protective clothing before leaving the laboratory.

Implementation of universal precautions eliminates the need for warning labels on specimens because blood and body fluids from all patients should be considered infective.

7 INF DIS XG ACQUIRED IMMUNODEFICIENCY SYNDROME-13

10/88 Received from P. Barkley, University Health Services, 11/30/90

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

National Institutes of Health
Bethesda, Maryland 20892
Building : 10
Room : 1C711
(301) 496- 9702

September 5, 1990

Dr. Susan Darnton
207 Wallace Hall
Virginia Tech
Blacksburg, VA 24060

Dear Dr. Darnton:

I am responding to your question concerning sterilization of serum samples. As I indicated to you, heat treatment is generally considered the most reliable and reproducible method. As you can see from the enclosed citation from the Centers for Disease Control, relatively gentle heating inactivates HIV. The hepatitis viruses appear to be more stable and heating for 10 hours at 60°C is generally a minimum.

A variety of agents have been added to plasma and serum to inactivate viruses. Most of these disrupt lipid encapsulated agents and include beta propiolactone with and without ultraviolet irradiation, sodium cholate and even dilute formaldehyde. Viral inactivation in blood components has been reviewed by Prince and Horowitz at the New York Blood Center.

Regardless of the means of inactivation, we routinely recommend universal precautions which include wearing gloves, hand washing, and forbidding eating and smoking whenever blood samples are being handled.

I hope that this is helpful to you.

Sincerely,

Harvey G. Klein, M.D.
Chief, Department of Transfusion Medicine
Clinical Center

MMWR

Supplement

MORBIDITY AND MORTALITY WEEKLY REPORT

1988 Agent Summary Statement for Human Immunodeficiency Virus

and

Report on Laboratory-Acquired Infection with Human Immunodeficiency Virus

Deposited by U.S. Gov't

MAY 07 1990

V.P.I. & S.U. Library

U.S. Department of Health and Human Services
Public Health Service
Centers for Disease Control
Center for Infectious Diseases
Hospital Infections Program
AIDS Program
Atlanta, Georgia

APPENDIX V

IONIZED CALCIUM ANALYSIS

Calcium Analyses - Ionized Calcium

1. Blood is collected in 7 ml vacutainers with sodium heparin.
2. Spin down, 4,000 rpms, 15 mins on a clinical centrifuge and leave in vacutainer.
3. Carry plasma quickly over to Herbien's lab (Wendy Wark).
4. Also take: 3.5 ml Sarsted transfer tubes prelabeled, transfer pipettes, Swinney filters and filter paper and O-rings, 5 ml plastic syringes, small rack to hold tubes while filtering, gloves, data sheet, pencil, marking pen, 10% chlorox for spills and wipe-up after leaving.
5. Standardize or calibrate the machine:

   Use one standard, there are three different standard levels, (found in the cabinet above the machine).

   Check values on digital readout with those in the manual on page 10-2 (NOVA 7/7 + 7 Electrolyte Analyzer Instruction Manual, NOVA Biomedical, Waltham, MA, 1986). Manual is found in the drawer to the right of the machine. If they are not within range, try again or another standard and this will generally check out, otherwise, call technician. Sometimes pH will not be within the range by just a few 1/100ths.

   The machine is slow to operate. Do not be impatient and push buttons before the "ready" signal is given.

5. Put together Swinney filters (25 mm diam., 0.45 um) and O-rings. Remove plunger from a syringe before attaching it to a filter unit to prevent displacing filter paper inside.

6. Attach the filter unit to a 2.5 B-D glass or 5 ml plastic syringe. Place the labeled transfer tube in a small test tube rack and position the filter unit-syringe apparatus over it (difficult to hold the tube and fill and push serum thru at the same time with just two hands). With disposable transfer pipette remove serum from vacutainer and transfer to syringe. Use plunger to gently push serum through filter. It should go through easily. However, if you have to apply forceful pressure it is most likely that something is wrong and you will loose the sample out the sides of the filter unit. (could be that the blue filter paper dividers are in the unit instead of the white filter papers or the filter clogged with large proteins).

Once filtered:

6. Push "analyze" on calibrated machine (see above) and wait for probe to appear (2 extensions). Place 5 ml tube with serum on probe and push "analyze" for serum to be drawn up, 200 ul, wait until "busy" light goes out and "test" light comes on and values will appear or push "test" button to display values. Values will "hold" until next sample is taken or after a given amount of time. Record all four values.

7. Push "purge" and "shift" at the same time to flush machine occasionally between samples and always twice when finished to clean machine.

8. Rinse syringes, filters and O-rings in a tub of water with some bleach (20 mls) a few hours or overnight, dry for next time and assemble carefully placing white filter paper and rubber O-ring.

Notes:

Use gloves

Dispose of pipettes, red cells in vacutainers, filter papers gloves and any papers used to wipe up spills in biohazard bag.

Contact Dr. Herbein and/or Wendy Wark a month or two in advance to ask for permission to use the machine and to schedule a time to use it.

If it is not in use they will have to start it up. The machine, once operable, will remain on and running for the month + that it will be used - do not turn it off.

Ionized calcium is very dependent upon pH and exposure to air. Even uncapping for analysis alters pH, that is why the normalized value is a calculated value to figure ionized calcium at pH = 7.4. Frozen samples can not be used! Even samples analyzed 2 hrs after collection will have shifted to high in pH to be dependable. Ionized calcium will be 48-52% of total. Calcium is very tightly and rapidly regulated in the body so differences in calcium status are hard to detect.

APPENDIX W

RAW DATA TABLES

Table of Subjects' Activity Group, Age and Supplement Assignment

SUBJECT#	GROUP*	AGE (yrs)	SUPPLEMENT+
1	S	21	B
2	S	19	P
3	S	21	B
4	S	23	P
5	S	20	B
6	S	20	P
7	S	20	B
8	S	20	P
9	S	20	B
10	S	19	P
11	S	20	B
12	A	19	B
13	A	19	B
14	A	18	P
15	A	21	B
16	A	19	P
17	A	19	B
18	A	20	P
19	A	19	B
20	A	19	B
21	A	24	P
22	A	21	B
23	A	20	P
24	A	19	B
25	A	19	P
26	A	18	B
27	A	21	P
28	A	21	B

* S = SEDENTARY EUMENORRHEIC; A = ATHLETIC EUMENORRHEIC/ AMENORRHEIC; THESE ABBREVIATIONS WILL BE USED THROUGHOUT THIS APPENDIX.

+ B = BORON SUPPLEMENT GROUP; P = PLACEBO GROUP

Table of Subjects' Maximal Oxygen Uptake Values (ml O_2/min)

SUBJECT#	GROUP	BASELINE*
1	S	1700
2	S	2700
3	S	2000
4	S	1700
5	S	2100
6	S	2200
7	S	1900
8	S	1700
9	S	2200
10	S	2700
11	S	1800
12	A	2600
13	A	3600
14	A	2900
15	A	2800
16	A	2900
17	A	2000
18	A	2900
19	A	2500
20	A	3300
21	A	3300
22	A	2700
23	A	2000
24	A	3400
25	A	3500
26	A	2600
27	A	2800
28	A	2700

* AEROBIC CAPACITY WAS ONLY MEASURED AT BASELINE BECAUSE IT WAS NOT A DEPENDENT VARIABLE.

Table of Subjects' Height (cm)

SUBJECT#	GROUP	BASELINE
1	S	166.4
2	S	167.6
3	S	161.3
4	S	162.6
5	S	157.5
6	S	160.0
7	S	156.2
8	S	156.8
9	S	167.6
10	S	165.1
11	S	180.3
12	A	171.5
13	A	165.1
14	A	175.3
15	A	154.7
16	A	177.2
17	A	166.4
18	A	162.6
19	A	160.0
20	A	165.1
21	A	175.3
22	A	165.1
23	A	166.4
24	A	177.8
25	A	164.5
26	A	177.8
27	A	170.2
28	A	172.7

Table of Subjects' Percent Body Fat

SUBJECT#	GROUP	BASELINE	MIDPOINT	FINAL
1	S	34.6	35.3	31.5
2	S	20.7	22.8	22.8
3	S	22.3	20.8	17.0
4	S	20.6	19.0	17.0
5	S	32.4	32.1	36.4
6	S	24.7	27.5	27.1
7	S	22.8	20.5	17.5
8	S	16.4	16.8	14.3
9	S	37.4	36.6	33.4
10	S	24.6	22.1	29.7
11	S	26.7	27.2	24.1
12	A	19.0	19.7	17.7
13	A	20.6	19.8	18.0
14	A	18.5	18.0	16.1
15	A	23.2	22.5	23.2
16	A	22.4	20.5	20.0
17	A	19.6	21.0	19.2
18	A	26.5	25.9	22.5
19	A	37.0	33.0	35.2
20	A	9.4	9.4	10.9
21	A	15.4	15.1	16.1
22	A	20.7	25.1	20.1
23	A	24.0	22.4	21.4
24	A	17.0	18.0	19.4
25	A	20.1	23.0	15.7
26	A	20.5	19.4	17.7
27	A	19.3	19.3	16.4
28	A	17.6	17.6	16.8

Table of Subjects' Body Weight (kg)

SUBJECT#	GROUP	BASELINE	MIDPOINT	FINAL
1	S	71.9	72.0	72.5
2	S	65.1	64.9	66.0
3	S	54.5	52.2	52.0
4	S	47.0	46.6	46.0
5	S	62.7	70.0	72.0
6	S	55.0	58.8	60.5
7	S	48.0	49.2	47.2
8	S	45.7	45.7	45.0
9	S	78.1	81.3	81.0
10	S	64.8	63.0	66.4
11	S	63.2	64.1	63.2
12	A	62.7	61.7	62.0
13	A	56.6	54.5	54.7
14	A	64.0	62.5	60.5
15	A	74.1	72.5	76.0
16	A	62.0	61.7	61.0
17	A	55.7	53.6	51.5
18	A	59.0	59.6	58.2
19	A	86.4	86.4	88.2
20	A	45.8	45.0	46.0
21	A	68.5	66.9	69.8
22	A	55.7	56.0	54.5
23	A	61.5	61.5	62.2
24	A	64.9	63.7	66.5
25	A	64.0	64.7	66.5
26	A	63.3	62.2	64.0
27	A	53.2	53.2	50.5
28	A	53.6	53.6	52.3

Table of Subjects' Bone Mineral Density (g/cm^2)*

SUBJECT#	GROUP	BASELINE	FINAL
1	S	1.32	1.38
2	S	1.22	1.23
3	S	1.22	1.24
4	S	1.26	1.20
5	S	1.21	1.14
6	S	1.31	1.28
7	S	1.07	1.08
8	S	1.03	0.99
9	S	1.34	1.35
10	S	1.15	1.15
11	S	1.35	1.39
12	A	1.47	1.52
13	A	1.30	1.36
14	A	1.29	1.28
15	A	1.47	1.54
16	A	1.43	1.47
17	A	1.13	1.16
18	A	1.35	1.43
19	A	1.21	1.28
20	A	1.13	1.10
21	A	1.27	1.29
22	A	1.07	1.11
23	A	1.16	1.24
24	A	1.35	1.40
25	A	1.34	1.39
26	A	1.32	1.29
27	A	1.28	1.27
28	A	1.28	1.21

* Analyzed by Gretchen Price, on the LUNAR DP III absorptiometer at Montgomery Regional Hospital, Blacksburg, VA.

Table of Subjects' Average Daily Kcals, and Percent Kcals as Protein, Fat, Carbohydrate, and Grams of Acid Detergent Fiber (Baseline)*

SUBJECT#	GROUP	TOTAL KCALS	% FAT	% PROT+	% CHO+	GRAMS ADF+
1	S	1165	31.2	15.8	53.0	31
2	S	886	30.1	18.4	51.5	28
3	S	1854	37.9	13.9	48.2	53
4	S	520	14.4	19.0	66.6	17
5	S	885	27.8	26.4	45.8	47
6	S	1845	32.2	9.3	58.6	57
7	S	1798	33.4	12.9	53.7	42
8	S	1921	37.3	10.1	52.5	33
9	S	874	33.6	16.2	50.2	15
10	S	2370	31.5	13.7	54.8	25
11	S	1470	28.8	8.2	63.1	19
12	A	1597	29.6	18.9	51.5	21
13	A	1519	26.7	16.5	56.8	44
14	A	633	31.7	11.2	57.1	13
15	A	892	29.2	18.1	52.7	26
16	A	2892	20.3	15.5	64.2	65
17	A	1253	32.6	15.2	52.2	36
18	A	1081	33.0	11.8	55.1	18
19	A	573	31.9	19.1	49.0	40
20	A	1212	10.9	16.4	72.7	26
21	A	1980	32.9	28.8	38.4	75
22	A	1486	26.1	11.6	62.4	26
23	A	1755	28.0	11.5	60.5	63
24	A	1961	20.8	14.4	64.8	78
25	A	1494	31.1	9.6	59.2	7
26	A	1369	24.8	12.6	62.7	47
27	A	1263	36.6	13.8	49.6	50
28	A	1351	31.8	16.5	51.7	12

* Determined via proximate analysis (baseline only): Protein by Kejldahl; Fat by Ether Extraction; Fiber by Acid Detergent Fiber; Carbohydrate by difference; Kcals by calculation

+ PROT = Protein; CHO = Carbohydrate; ADF = Acid Detergent Fiber

Table of Subjects' Average Daily Kcals, and Percent Kcals as Protein, Fat, Carbohydrate, and Grams of Dietary Fiber (Baseline)*

SUBJECT#	GROUP	TOTAL KCALS	% FAT	% PROT+	% CHO+	GRAMS D.F.+
1	S	1917	38.0	14.0	49.0	10.9
2	S	2637	36.0	15.0	50.0	15.6
3	S	1653	49.0	14.0	35.0	12.3
4	S	900	25.0	19.0	56.0	15.9
5	S	1183	22.0	17.0	54.0	8.7
6	S	2344	45.0	12.0	43.0	9.4
7	S	1780	34.0	18.0	47.0	13.4
8	S	2937	29.0	11.0	60.0	15.5
9	S	2249	44.0	13.0	42.0	10.9
10	S	1788	37.0	15.0	48.0	6.9
11	S	1804	36.0	14.0	50.0	8.5
12	A	2679	37.0	12.0	50.0	15.1
13	A	3448	33.0	13.0	53.0	23.5
14	A	1254	26.0	10.0	64.0	10.6
15	A	2246	40.0	17.0	42.0	9.0
16	A	2348	34.0	16.0	51.0	17.1
17	A	1840	33.0	13.0	54.0	8.4
18	A	1414	32.0	13.0	56.0	9.2
19	A	713	32.0	23.0	45.0	3.6
20	A	2240	17.0	16.0	67.0	29.2
21	A	1016	13.0	16.0	72.0	17.3
22	A	948	27.0	15.0	58.0	10.8
23	A	1884	35.0	11.0	54.0	13.8
24	A	2170	20.0	13.0	67.0	18.3
25	A	1391	46.0	16.0	38.0	2.5
26	A	1304	16.0	10.0	74.0	13.8
27	A	2000	44.0	15.0	42.0	14.3
28	A	1867	40.0	14.0	46.0	10.4

* Determined using Nutritionist III, Computer Software.
+ PROT = Protein; CHO = Carbohydrate; D.F. = Dietary Fiber

Table of Subjects' Average Daily Kcals, and Percent Kcals as Protein, Fat, Carbohydrate, and Grams of Dietary Fiber (Final)*

SUBJECT#	GROUP	TOTAL KCALS	% FAT	% PROT+	% CHO+	GRAMS D.F.+
1	S	1107	29.0	17.0	55.0	9.4
2	S	1126	26.0	12.0	62.0	7.3
3	S	1790	18.0	19.0	61.0	29.6
4	S	1509	21.0	13.0	66.0	25.8
5	S	1354	20.0	18.0	62.0	11.2
6	S	1278	40.0	17.0	43.0	4.1
7	S	1816	30.0	11.0	59.0	11.3
8	S	1726	21.0	17.0	62.0	20.1
9	S	1941	35.0	14.0	50.0	6.7
10	S	2047	28.0	15.0	57.0	7.0
11	S	2192	31.0	13.0	56.0	7.5
12	A	1978	26.0	13.0	61.0	10.8
13	A	2930	24.0	18.0	58.0	10.9
14	A	2399	26.0	9.0	64.0	16.3
15	A	2210	38.0	14.0	48.0	10.0
16	A	1433	25.0	14.0	60.0	7.5
17	A	2467	36.0	10.0	55.0	6.3
18	A	915	34.0	13.0	53.0	7.1
19	A	1727	35.0	20.0	45.0	6.5
20	A	1782	8.0	13.0	78.0	37.2
21	A	2765	13.0	9.0	78.0	44.3
22	A	1502	32.0	11.0	51.0	8.3
23	A	2211	34.0	16.0	51.0	13.0
24	A	2716	24.0	16.0	61.0	10.9
25	A	1469	47.0	15.0	37.0	12.1
26	A	2081	21.0	8.0	71.0	22.7
27	A	1311	29.0	14.0	57.0	9.8
28	A	1311	29.0	14.0	57.0	9.8

* Determined using Nutritionist III, Computer Software.
+ PROT = Protein; CHO = Carbohydrate; D.F. = Dietary Fiber

Table of Subjects' Average Daily Calcium Intake Determined By Laboratory Analysis (mg)*

SUBJECT#	GROUP	BASELINE
1	S	628.0
2	S	466.0
3	S	1037.0
4	S	288.0
5	S	664.0
6	S	597.0
7	S	627.0
8	S	1075.0
9	S	308.0
10	S	1790.0
11	S	378.0
12	A	883.0
13	A	541.0
14	A	284.0
15	A	431.0
16	A	1900.0
17	A	807.0
18	A	261.0
19	A	274.0
20	A	440.0
21	A	2108.0
22	A	408.0
23	A	156.0
24	A	230.0
25	A	249.0
26	A	626.0
27	A	603.0
28	A	846.0

* Determined using Atomic Absorption Spectroscopy

Table of Subjects' Average Daily Calcium Intake Determined by Nutritionist III (mg)*

SUBJECT#	GROUP	BASELINE	FINAL
1	S	891.5	494.9
2	S	790.7	623.8
3	S	912.5	767.4
4	S	796.7	877.6
5	S	707.6	518.4
6	S	1163.0	600.1
7	S	620.7	689.2
8	S	1846.0	1289.0
9	S	821.5	952.1
10	S	1433.0	1483.0
11	S	612.7	1085.0
12	A	882.8	763.1
13	A	785.7	728.9
14	A	586.7	757.7
15	A	1071.0	1230.0
16	A	1111.0	634.6
17	A	829.9	708.6
18	A	396.5	242.7
19	A	161.7	492.5
20	A	911.6	923.5
21	A	469.8	634.9
22	A	248.5	615.5
23	A	815.0	1422.0
24	A	939.3	1093.0
25	A	168.8	455.4
26	A	311.6	636.5
27	A	1163.0	528.5
28	A	1005.0	529.3

* Determined using Nutritionist III, Computer Software.

Table of Subjects' Average Daily Phosphorous Intake Determined By Laboratory Analysis (mg) *

SUBJECT#	GROUP	BASELINE
1	S	730.4
2	S	632.4
3	S	1178.9
4	S	427.5
5	S	953.9
6	S	796.0
7	S	1030.0
8	S	789.8
9	S	494.0
10	S	1570.5
11	S	633.0
12	A	1207.0
13	A	848.4
14	A	367.0
15	A	564.5
16	A	1397.2
17	A	567.6
18	A	472.2
19	A	404.8
20	A	733.0
21	A	2979.0
22	A	658.5
23	A	842.8
24	A	1180.0
25	A	513.0
26	A	706.0
27	A	879.0
28	A	1233.0

* Determined using Sigma Diagnostics procedures

Table of Subjects' Average Daily Phosphorous Intake Determined by Nutritionist III (mg)*

SUBJECT#	GROUP	BASELINE	FINAL
1	S	1148	702
2	S	1428	598
3	S	1102	1264
4	S	792	960
5	S	1071	874
6	S	1262	888
7	S	1190	930
8	S	1589	1461
9	S	1009	1158
10	S	1444	1752
11	S	826	1371
12	A	1121	786
13	A	1607	1558
14	A	747	1111
15	A	1388	1184
16	A	1605	776
17	A	748	893
18	A	608	530
19	A	437	988
20	A	1567	1438
21	A	1061	1532
22	A	546	691
23	A	1236	1820
24	A	1401	1095
25	A	442	907
26	A	535	847
27	A	1272	739
28	A	1190	739

* Determined using Nutritionist III, Computer Software.

Table of Subjects' Average Daily Magnesium Intake Determined by Laboratory Analysis (mg) *

SUBJECT#	GROUP	BASELINE
1	S	44
2	S	44
3	S	106
4	S	53
5	S	75
6	S	119
7	S	57
8	S	88
9	S	36
10	S	112
11	S	63
12	A	84
13	A	101
14	A	34
15	A	44
16	A	346
17	A	40
18	A	45
19	A	9
20	A	110
21	A	402
22	A	65
23	A	78
24	A	115
25	A	47
26	A	87
27	A	54
28	A	85

* Determined using Atomic Absorption Spectroscopy

Table of Subjects' Average Daily Magnesium Intake Determined by Nutritionist III (mg) *

SUBJECT#	GROUP	BASELINE	FINAL
1	S	212.2	134.9
2	S	359.4	148.3
3	S	179.0	326.3
4	S	140.7	220.2
5	S	274.3	575.1
6	S	178.9	107.1
7	S	289.8	206.1
8	S	320.3	379.3
9	S	175.5	127.5
10	S	216.5	382.4
11	S	213.1	213.3
12	A	256.8	208.0
13	A	488.1	321.2
14	A	161.6	296.9
15	A	217.1	211.8
16	A	253.8	175.5
17	A	138.8	198.3
18	A	145.5	103.7
19	A	77.4	159.3
20	A	420.6	516.6
21	A	245.0	582.6
22	A	158.0	144.4
23	A	289.1	346.6
24	A	262.7	218.1
25	A	97.4	228.6
26	A	161.3	227.6
27	A	223.8	212.8
28	A	216.1	213.2

* Determined using Nutritionist III, Computer Software.

Table of Subjects' Serum Normalized Calcium (NOVA 7) (mg/dl)*

SUBJECT#	GROUP	BASELINE	FINAL
1	S	4.65	4.73
2	S	4.79	4.60
3	S	4.86	4.59
4	S	4.75	4.86
5	S	4.96	4.88
6	S	4.80	4.81
7	S	4.71	4.79
8	S	4.82	4.45
9	S	4.83	4.66
10	S	4.84	4.70
11	S	4.76	4.86
12	A	4.96	4.80
13	A	4.93	5.08
14	A	4.87	4.79
15	A	4.70	4.63
16	A	4.91	4.85
17	A	4.43	4.75
18	A	4.75	4.92
19	A	4.32	4.51
20	A	4.81	4.79
21	A	5.10	4.63
22	A	4.83	4.95
23	A	5.11	4.95
24	A	4.99	4.62
25	A	4.74	4.66
26	A	4.91	4.76
27	A	4.78	4.69
28	A	4.32	4.74

* Determined using NOVA 7 Electrolyte Analyzer, NOVA Biomedical, Inc.

Table of Subjects' Serum Total Calcium (NOVA 7) (mg/dl)*

SUBJECT#	GROUP	BASELINE	FINAL
1	S	9.2	9.3
2	S	9.6	9.3
3	S	8.9	9.4
4	S	9.8	9.4
5	S	10.1	9.5
6	S	9.7	9.5
7	S	9.4	9.8
8	S	9.9	9.5
9	S	9.6	9.3
10	S	9.4	9.5
11	S	9.2	9.7
12	A	9.3	9.4
13	A	9.5	10.0
14	A	10.1	10.1
15	A	9.9	9.6
16	A	9.7	9.5
17	A	9.0	9.6
18	A	9.2	9.7
19	A	9.0	9.2
20	A	9.5	9.3
21	A	10.2	9.3
22	A	9.0	9.4
23	A	10.4	10.0
24	A	9.9	9.8
25	A	8.9	9.5
26	A	10.3	9.6
27	A	9.6	9.7
28	A	9.0	9.7

* Determined using NOVA 7 Electrolyte Analyzer, NOVA Biomedical, Inc.

Table of Subjects' Plasma Ionized Calcium (NOVA 7) (mg/dl)*

SUBJECT#	GROUP	BASELINE	FINAL
1	S	4.44	4.73
2	S	4.53	4.58
3	S	4.60	4.58
4	S	4.54	4.80
5	S	4.70	4.80
6	S	4.58	4.98
7	S	4.55	4.90
8	S	4.53	4.37
9	S	4.60	4.63
10	S	4.52	4.66
11	S	4.69	4.80
12	A	4.78	4.80
13	A	4.76	5.10
14	A	4.42	4.70
15	A	4.49	4.63
16	A	4.67	4.77
17	A	4.24	4.75
18	A	4.70	4.90
19	A	4.11	4.38
20	A	4.41	4.88
21	A	4.81	4.66
22	A	4.54	4.78
23	A	5.01	4.99
24	A	4.68	4.63
25	A	4.45	4.71
26	A	4.82	4.99
27	A	4.66	4.44
28	A	3.95	4.36

* Determined using NOVA 7, Electrolyte Analyzer, NOVA Biomedical, Inc.

Table of Subjects' Serum Total Calcium (AA) (mg/dl)*

SUBJECT#	GROUP	BASELINE	FINAL
1	S	8.75	9.63
2	S	9.00	10.00
3	S	9.00	9.88
4	S	9.25	9.13
5	S	9.00	9.75
6	S	8.75	9.38
7	S	9.25	10.00
8	S	9.50	9.63
9	S	9.25	10.00
10	S	8.25	10.13
11	S	9.25	9.63
12	A	8.75	9.38
13	A	8.25	10.13
14	A	8.75	10.13
15	A	9.25	9.75
16	A	8.75	9.88
17	A	8.75	10.00
18	A	8.50	10.00
19	A	8.50	9.25
20	A	9.00	9.38
21	A	9.00	9.50
22	A	9.25	9.75
23	A	8.75	9.50
24	A	9.00	9.75
25	A	8.50	10.13
26	A	9.50	9.33
27	A	9.00	9.33
28	A	9.00	9.50

* Determined using Atomic Absorption Spectroscopy

Table of Subjects' Serum Phosphorous (SD) (mg/dl)*

SUBJECT#	GROUP	BASELINE	FINAL
1	S	3.59	3.67
2	S	5.45	4.35
3	S	4.06	4.46
4	S	4.66	4.46
5	S	4.91	3.94
6	S	5.23	4.04
7	S	4.18	1.63
8	S	5.18	4.18
9	S	4.54	3.96
10	S	5.13	4.75
11	S	4.31	3.77
12	A	4.75	3.81
13	A	4.04	4.22
14	A	4.53	4.18
15	A	4.59	4.08
16	A	4.39	3.58
17	A	5.14	3.44
18	A	4.58	3.80
19	A	4.85	3.79
20	A	4.93	3.73
21	A	5.15	3.76
22	A	4.85	4.05
23	A	5.88	4.23
24	A	5.17	3.89
25	A	4.46	4.07
26	A	4.39	4.07
27	A	3.91	4.04
28	A	4.39	3.90

* Determined using Sigma Diagnostics

Table of Subjects' Serum Magnesium (AA) (mg/dl)*

SUBJECT#	GROUP	BASELINE	FINAL
1	S	1.5	2.5
2	S	1.5	2.0
3	S	1.5	2.0
4	S	2.0	2.0
5	S	2.0	2.5
6	S	1.7	2.0
7	S	2.0	2.0
8	S	1.7	2.0
9	S	2.3	3.0
10	S	1.5	2.3
11	S	1.8	2.3
12	A	1.5	2.0
13	A	1.5	2.0
14	A	1.5	2.0
15	A	1.5	2.0
16	A	2.0	2.3
17	A	1.5	2.0
18	A	2.0	2.0
19	A	1.0	2.0
20	A	1.8	2.0
21	A	1.8	2.0
22	A	2.0	2.0
23	A	1.5	2.0
24	A	1.5	2.0
25	A	1.5	2.0
26	A	1.5	2.0
27	A	1.5	2.0
28	A	1.5	2.0

* Determined using on Atomic Absorption Spectroscopy

Table of Subjects' Daily Urinary Calcium (AA) (mg)*

SUBJECT#	GROUP	BASELINE	FINAL
1	S	33.6	130.0
2	S	56.4	62.0
3	S	99.7	117.6
4	S	6.0	65.6
5	S	66.4	28.0
6	S	66.1	26.0
7	S	33.9	51.0
8	S	23.8	52.0
9	S	112.2	64.6
10	S	80.6	61.5
11	S	26.4	53.0
12	A	109.2	68.1
13	A	41.8	64.0
14	A	166.4	127.6
15	A	89.6	122.0
16	A	93.0	113.0
17	A	24.0	80.8
18	A	24.8	93.0
19	A	2.8	140.4
20	A	12.6	64.0
21	A	68.0	44.8
22	A	56.0	96.0
23	A	67.0	88.0
24	A	33.6	126.2
25	A	66.0	77.4
26	A	127.4	187.2
27	A	16.8	80.0
28	A	4.2	108.0

* Determined using Atomic Absorption Spectroscopy

Table of Subjects' Daily Urinary Calcium (ICP) (mg)*

SUBJECT#	GROUP	BASELINE	FINAL
1	S	49.8	56.9
2	S	90.6	27.8
3	S	136.1	56.5
4	S	12.9	67.8
5	S	74.1	15.3
6	S	80.6	20.2
7	S	62.2	75.3
8	S	48.5	74.2
9	S	114.9	71.2
10	S	116.9	70.1
11	S	56.4	25.9
12	A	84.0	32.7
13	A	46.3	127.1
14	A	135.5	137.9
15	A	108.4	51.9
16	A	95.6	391.5
17	A	23.7	36.4
18	A	22.8	90.4
19	A	24.2	194.5
20	A	18.9	82.7
21	A	100.7	58.1
22	A	77.2	130.1
23	A	73.3	84.4
24	A	33.0	132.4
25	A	73.0	33.0
26	A	119.7	184.4
27	A	19.3	36.7
28	A	21.7	45.8

* Determined using Inductively Coupled Plasma Spectroscopy

Table of Subjects' Daily Urinary Phosphorous (AA) (mg)*

SUBJECT#	GROUP	BASELINE	FINAL
1	S	260	920
2	S	390	570
3	S	820	480
4	S	60	570
5	S	950	730
6	S	440	580
7	S	480	580
8	S	580	540
9	S	1300	1320
10	S	930	1100
11	S	720	570
12	A	920	1160
13	A	245	250
14	A	1070	580
15	A	980	240
16	A	500	700
17	A	400	360
18	A	130	520
19	A	230	720
20	A	294	690
21	A	1260	570
22	A	2880	700
23	A	440	830
24	A	260	800
25	A	700	750
26	A	630	590
27	A	930	290
28	A	760	500

* Determined using Atomic Absorption Spectroscopy

Table of Subjects' Daily Urinary Phosphorous (ICP) (mg)*

SUBJECT#	GROUP	BASELINE	FINAL
1	S	271	418
2	S	547	267
3	S	1010	224
4	S	79	690
5	S	127	375
6	S	597	318
7	S	624	757
8	S	419	655
9	S	995	1540
10	S	1181	1411
11	S	581	272
12	A	948	393
13	A	273	420
14	A	649	773
15	A	1116	354
16	A	1186	307
17	A	430	204
18	A	159	633
19	A	268	956
20	A	146	778
21	A	846	621
22	A	316	920
23	A	760	1078
24	A	263	1076
25	A	938	368
26	A	528	722
27	A	371	189
28	A	474	273

* Determined using Inductively Coupled Plasma Spectroscopy

Table of Subjects' Daily Urinary Magnesium (AA)(mg)*

SUBJECT#	GROUP	BASELINE	FINAL
1	S	44.8	75.0
2	S	49.8	21.2
3	S	71.2	140.0
4	S	11.0	48.0
5	S	49.8	40.0
6	S	45.6	20.0
7	S	45.2	46.4
8	S	56.0	94.8
9	S	102.0	57.0
10	S	104.0	75.0
11	S	44.0	63.0
12	A	84.0	55.2
13	A	76.0	384.0
14	A	128.0	116.0
15	A	84.0	80.0
16	A	90.0	90.0
17	A	16.0	60.2
18	A	12.0	30.0
19	A	8.0	78.0
20	A	56.0	128.0
21	A	102.0	56.0
22	A	56.0	100.0
23	A	75.6	108.0
24	A	56.0	121.6
25	A	50.0	69.6
26	A	78.0	120.0
27	A	14.0	34.0
28	A	14.0	54.0

* Determined using Atomic Absorption Spectroscopy

Table of Subjects' Daily Urinary Magnesium (ICP)(mg) *

SUBJECT#	GROUP	BASELINE	FINAL
1	S	23.0	22.3
2	S	32.5	10.9
3	S	62.5	19.9
4	S	8.2	69.8
5	S	46.5	15.1
6	S	47.2	16.1
7	S	30.7	21.5
8	S	29.2	42.3
9	S	69.9	47.7
10	S	73.0	58.8
11	S	35.4	25.5
12	A	50.3	21.1
13	A	32.7	38.0
14	A	56.1	72.8
15	A	60.2	29.1
16	A	55.0	32.8
17	A	7.5	22.0
18	A	9.7	29.5
19	A	6.9	49.3
20	A	16.8	70.9
21	A	60.5	54.8
22	A	39.4	70.5
23	A	71.5	91.1
24	A	33.6	91.1
25	A	26.3	26.6
26	A	56.2	107.4
27	A	0.8	16.1
28	A	6.6	22.0

* Determined using Inductively Coupled Plasma Spectroscopy

Table of Subjects' Daily Urinary Boron (ICP) (ug)*

SUBJECT#	GROUP	BASELINE	FINAL
1	S	.203	.320
2	S	.472	.150
3	S	1.020	.196
4	S	.088	.902
5	S	.908	1.471
6	S	.442	.147
7	S	.570	1.349
8	S	.509	1.484
9	S	.807	3.026
10	S	.819	.597
11	S	.523	.340
12	A	.721	.683
13	A	.685	4.150
14	A	.718	1.021
15	A	.810	.280
16	A	.862	.449
17	A	.398	1.106
18	A	.205	.762
19	A	.131	3.374
20	A	.341	3.866
21	A	1.337	2.625
22	A	.495	2.532
23	A	.780	.515
24	A	.505	4.520
25	A	.655	.380
26	A	2.129	4.481
27	A	.574	.287
28	A	.970	2.506

* Determined using Inductively Coupled Plasma Spectroscopy

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