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To the Graduate Council:

I am submitting herewith a dissertation written by Glenda Sue Thompson entitled "The Effects of Dietary Supplements on Bone Density and Nutritional Status of Elderly Women." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Nutrition.

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130

THE EFFECTS OF DIETARY SUPPLEMENTS ON BONE DENSITY AND NUTRITITIONAL STATUS OF ELDERLY WOMEN

A Dissertation

Presented to

the Graduate Council of

The University of Tennessee

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

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by

Glenda Sue Thompson

August 1973

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ABSTRACT

Seventy-six ambulatory women with a mean age of 68 years participated in a 12-month study of the effects of fluoride, with and without multivitamin and calcium phosphate supplements, on nutritional status including bone density. Mean daily nutrient intakes calculated from 4- to 7-day weighed food intakes were 1519 kcal including 15% from protein and 36% from fat; others were 797 mg calcium, 1000 mg phosphorus, and 8.6 mg iron. Mean intakes by 59 women who were not receiving vitamin supplements were 5800 IU of vitamin A and 35 mg of ascorbic acid. Mean blood hemoglobin was 13.9 g/100 ml; serum calcium and phosphorus level means were 9.60 and 3.35 mg/100 ml, respectively. A mean of 1.92 mg of phosphorus was released by alkaline phosphatase, while the mean cholesterol level was 222 mg/100 ml. Mean serum levels of total protein and albumin were 6.77 and 3.67 g/100 ml, respectively, and the A/G ratio was 1.22. Plasma ascorbic acid averaged 0.34 mg/100 ml and serum vitamin A, 50.4 μg/100 ml for the women who were not receiving vitamin supplements.

The mean bone density index of the left phalanx 5-2 was 0.819 g equivalents of alloy per cc of bone, while mean height and weight were 156.3 cm and 65.4 kg, respectively. Canonical correlation showed an age-associated decrease in bone density, height, weight, and intake of protein, calcium, phosphorus, and ascorbic acid.

The group of 76 women was divided into 4 groups, approximately equal in number, matched for bone density, age, and weight. Fluoride was administered at a level of 0.25 mg/kg body weight/day as a single

therapeutic agent to one group and in combination with 464 mg calcium and 360 mg phosphorus as calcium phosphate and 25,000 IU vitamin A and 200 mg ascorbic acid in a multivitamin capsule to another group. The diets of another group of women were supplemented with 464 mg calcium and 360 mg phosphorus, while the diets of 19 of the women were not supplemented. Subgroups of 4 subjects from each experimental group participated in metabolic balance studies. Metabolic studies and bone density measurements were repeated after 4 months of supplementation and all tests were repeated after 12 months.

After supplementation, minor differences in nutrient intakes and blood levels occurred in the groups receiving fluoride. Consumption of calcium decreased in the group which received fluoride, although the final mean intake was 761 mg per day. In the group receiving fluoride in combination with calcium phosphate and vitamin supplements, increases in plasma ascorbic acid and serum vitamin A and decreases in serum total protein, calcium, and phosphorus were obtained. Nevertheless, all values remained within normal ranges.

Supplementation with fluoride alone resulted in a significant increase (10%) in bone density, and fluoride in combination with calcium phosphate and vitamins produced a 4% increase. Insignificant increases in bone density were observed when the diet was unsupplemented (2%) or supplemented with calcium phosphate (2.5%).

Nitrogen balances remained positive throughout the study. An age-associated defect in calcium absorption was indicated in all experimental groups. Changes in neither calcium nor phosphorus balances correlated with those in bone density. Positive balances of these

nutrients were shown when fluoride accompanied the supplements of calcium, phosphorus, and vitamins but bone density decreased in 2 of the 4 subjects in that subgroup. All members of the balance group receiving fluoride alone exhibited negative balances of calcium and phosphorus while bone density increased.

No adverse effects of fluoride supplementation were observed in any of the women during the study. The biological significance of a decrease in serum calcium has not been elucidated; a shift of serum calcium to bone, or a reduction in calcium mobilized from bone (possibly lowering serum calcium), may be advantageous. Increases in bone density in response to fluoride without alterations in dietary intake or blood constituents, other than serum calcium, tends to encourage further exploration of the prophylactic as well as therapeutic benefits of low-level fluoride supplementation.

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CHAPTER I

INTRODUCTION

Aging is a physiological process beginning at conception and continuing until death. The elderly person is not only the product of his genetic inheritance, but also of the lifestyle he has established for himself through the activities he has chosen, food preferences he has cultivated, illnesses he has overcome, and a multiplicity of other phenomena. Longevity of women is greater than that of men; thus more women live to experience the physiological, psychological, and social problems which accompany old age. Some of these maladies may result, in part, from decreased nutrient intakes, altered nutrient requirements, or impaired nutrient absorption.

Decreased bone density and a tendency for spontaneous fracture occur with increasing frequency in women after about age 50 years; however, it has been reported that loss of bone is of less magnitude and occurs less frequently in individuals in regions where the fluoride content of the drinking water is high.

In clinical cases, administration of fluoride at 1 mg/kg body weight/day has been consistently reported as both safe and beneficial in the treatment of bone diseases such as Paget's disease, multiple myeloma, and osteoporosis.

In this study, sodium fluoride was administered as a single therapeutic agent to 20 elderly women and in combination with calcium phosphate and vitamins to a similar number of participants in an effort to

increase bone density. For comparative purposes, calcium phosphate was provided for 17 participants and the diets of 19 women remained unsupplemented.

Bone density, nutrient intakes, blood constituents, and utilization of calcium, phosphorus, and nitrogen were evaluated before and after 12 months of supplementation. In addition, metabolic balances and bone density measurements were repeated about 4 months after supplementation was initiated to ascertain any early changes attributable to the supplements.

That dietary supplements such as sodium fluoride, calcium phosphate, and multiple vitamins would aid in maintenance of normal bone density indices without causing undesirable side effects was the hypothesis of this study. The objectives of the study were:

1. To ascertain the nutritional status of a female geriatric population.
2. To administer sodium fluoride at relatively low levels to a number of elderly women to test its effectiveness in increasing bone density.
3. To administer calcium phosphate and multiple vitamins with and without sodium fluoride to delineate the separate effects of these supplements on bone density.
4. To assess whether or not the supplements have any other effects on nutritional status, including nutrient intake and blood levels of selected constituents.
5. To study relationships between metabolic balances and bone density.

CHAPTER II

REVIEW OF LITERATURE

In formulating nutrition policy for the seventies, Watkin (1) recognized both short- and long-range objectives. The short-range objective was to improve the status of those who are old; the long-range objective was to alter nutritional patterns and health of the young in such a manner that physiological aging and its accompanying disabilities are deferred to the oldest possible age.

Aging has intrigued man for centuries. Poets have eulogized age, historians have recorded it, families have worshipped it, and philosophers have been humbled by it. Explorers have searched for a natural fountain of youth, medicine has been dedicated in its effort to permit mankind to attain old age, society has tried to adjust to the needs of the aged, and today, science is attempting to delineate the aging process. Biochemists, biophysicists, physiologists, biologists, nutritionists, and physicians (2) are spending thousands of hours and dollars as they endeavor to isolate the causes of the changes, and to discover means for delaying these changes.

Nutrient Intake

The diversity of environmental conditions, economic situations, and personal food prejudices makes generalizations about dietary intakes of aged individuals difficult. For example, nutrient intakes by elderly persons living in a retirement home which employed the services of a consulting dietitian had better nutrient intakes than those living in

their own homes (3); elderly hospitalized patients who were able to feed themselves generally had lower nutrient intakes than chronically ill patients who consumed the foods selected for them by staff members (4); and socially isolated older persons who lived alone, were unemployed, and ate most of their meals alone consumed lesser quantities of nutrients than did those who were living with a spouse, working past the retirement age, and eating with their families (5). Guggenheim and Margulec (6) found that food consumed by 49 women living alone was similar to that consumed by 27 women living with their families; however, mean nutrient intakes, by the two groups, of energy, protein, calcium, iron, vitamin A, and ascorbic acid met the Recommended Dietary Allowances (7) only at the 50th percentile.

Decreased nutrient intake with advancing age is generally recognized. In the San Mateo study, Steinkamp et al. (8) reported significantly reduced intakes of calories, protein, calcium, iron, vitamin A, ascorbic acid, and the B vitamins after age 75. Intakes of animal protein were not decreased with increasing age. Swanson (9) reported, from a survey of 1072 women aged 30 to 90 years, a downward trend in intakes of energy, protein, and calcium; Fry et al. (10), after studying both weighed (by 6 women) and recorded (by 26 women) food consumption, also reported a downward trend with increasing age in intake of all nutrients except calcium and riboflavin. From dietary records obtained for 252 men aged 20 to 99 in the Baltimore Longitudinal Study, daily intakes of energy, protein, fat, calcium, iron, vitamin A, and the B vitamins decreased with increasing age; intakes of ascorbic acid increased as age increased (11). From 7-day records of food consumption kept by 2808 women aged 30 to 80

who participated in the nutritional status studies of the 1950's (12), decreased intakes of energy, protein, calcium, iron, and the B vitamins with advancing age were shown. Lyons and Trulson (13) have reported that one-fourth of the 100 men and women aged 65 and over, who participated in a nutrient intake study, were consuming less than 75% of the recommended allowance for energy, riboflavin, iron, and ascorbic acid. The recent Ten-State Nutrition Survey (14) revealed that in the population surveyed, persons 60 years of age and older consumed insufficient food to meet recommended allowances. Elderly persons living in the low income-ratio states (Kentucky, Louisiana, South Carolina, Texas, West Virginia) had lower dietary intakes than those in high income-ratio states; however, dietary intake was not closely related to income. Males were found to consume more nutrients than females and Blacks to consume less than Whites. Body weight and nutrient intake were consistently related.

Biochemical Studies

A survey of research on serum vitamin A, carotene, and ascorbic acid levels in the aged population failed to yield significant variation with age (15). Brin et al. (16), from a nutritional status study of 234 elderly men and women, reported a mean plasma ascorbic acid of 0.84 mg/100 ml for women; no mean for serum vitamin A was calculated, but 58% of the women had serum levels of vitamin A greater than 58 µg/100 ml. Brewer et al. (17) reported greater variability in hemoglobin and serum vitamin A concentrations in persons past 50 years of age than in younger persons although the means appeared to be within the range of normal. A

mean serum protein concentration of 7.13 g/100 ml was within the range of normal.

In the Ten-State Nutrition Survey (18), hemoglobin values increased in women past childbearing age but remained lower than those for men. Serum albumin and total protein were within the lower limits of normal. Serum vitamin A levels increased with age. Some deficient and low values for serum ascorbic acid were observed in the elderly.

Mineral absorption studied using isotopes of calcium decreased with advancing age (19), although Smith et al. (20) could detect no indication of impaired absorption except in one group of diagnosed osteoporotics aged 61 to 70 years. Avioli et al. (19) found no difference between calcium absorbed by the osteoporotic and the normal.

No changes in collagen metabolism as indicated by urinary hydroxyproline excretion were observed in osteoporotic and non-osteoporotic aged persons when compared with normal young adults (21). Data from balance studies reported by Ohlson et al. (22) indicate that the need for protein and calcium is not decreased with age.

Recommended Dietary Allowances for the Aged

The National Research Council's reference woman for which adult dietary allowances are recommended is 22 years old and weighs 58 kg. The environment in which she lives has a mean temperature of 20° and she is of normal activity (7).

The dietary allowances for a woman 55 years of age or older differs little from that recommended by the National Research Council for a woman 22 years of age. The allowance for iron is lowered for the post-

menopausal years. The caloric allowance is reduced in keeping with the approximate decline of basal metabolism rate and reduction in physical activity. The dietary allowance per day is thus: 1700 kilocalories, 55 g protein, 5000 IU vitamin A, 25 IU vitamin E, 55 mg ascorbic acid, 0.4 mg folacin, 13 mg equivalents niacin, 1.5 mg riboflavin, 1.0 mg thiamin, 2.0 mg vitamin B_6, 5 μg vitamin B_{12}, 800 mg calcium, 800 mg phosphorus, 80 μg iodine, 10 mg iron, and 300 mg magnesium (7).

Bone Density

The skeletal system exhibits changes with advancing age. Frost (23) has indicated that up to 40% of the skeleton is lost between youth and old age. That the changes are undesirable is supported by the work of Iskrant and Smith (24) from which the authors estimated that of the one million fractures experienced annually by women over 45 years of age 700,000 were incurred by those with bone densities so low that they were classified as osteoporotic. Gitman and Kamholtz (25) have determined that 65.8% of the women over 65 have radiographically manifest osteoporosis. Guggenheim et al. (26) detected some degree of osteoporosis in 94% of the women over age 60 examined in Jerusalem. The literature is in good agreement that, not only does bone density decrease with age, but also the fifth decade is the usual time of onset (27, 28, 29, 30, 31).

From rather extensive studies involving radiographic bone measurements from more than 15,000 subjects (26, 27, 32, 33), it is evident that women lose bone more rapidly than do men. For example, Garn et al. (27) found the loss of bone to occur twice as fast in women as in men. Reduced bone density in old age is not peculiar to any one population,

race, or geographical area, although variations in frequencies are observed. Nordin (34) found bone loss to start earlier among Japanese and Indian women than in British or American women. Chalmers and Ho (35) reported a higher incidence of hip fracture in women from Sweden than in those from Great Britain, China, or Africa (Bantu). Blacks have bones which are more dense than Whites (32) and Moldawer et al. (36) found the incidence of compression fracture to be greater in Whites than Blacks. Engh et al. (37) reported that fracture due to minimum trauma is greatest in Whites residing in institutions.

The entire female skeleton apparently loses bone at about the same rate. Analysis of bone from cadavers (32) showed that the density (weight/volume) of long bones from a skeleton did not differ from one another. Dequeker et al. (38) found that changes in the second metacarpal paralleled those occurring throughout the skeleton. Nordin (39) has recently reported that the loss of trabecular bone is greater than that of cortical bone. Smith and Walker (40) previously observed that as cortical thickness decreased, the diameter at the midshaft increased. Reduction of bone density with advancing age is accepted as a widespread, or perhaps universal, phenomenon. It has been suggested (41) that once bone loss begins, the average amount of bone diminishes linearly with age and values for the amount of bone at each age have a normal distribution. It was also suggested by Newton-John and Morgan (41) that since no greater variation in the amount of bone appeared in the aged than in youth, classification of an osteoporotic group within the aged population was not justified. Goldsmith et al. (42) found that osteoporotic women lost bone mass before the fifth decade but in the succeeding three decades,

the non-osteoporotics lost mineral twice as rapidly as did osteoporotics. By the eighth decade the two populations could not be distinguished from each other.

A longitudinal study was conducted by Adams et al. (43) in which second observations were made 11 years after the first. At the time of the first measurements the age range was 55 to 64 years. Bone loss of the second metacarpal was shown to be characteristic of the individual. Although there was an overall loss of bone in both sexes, some individuals lost little or no bone. Women tended to lose bone at a faster rate than men. While backache, loss of height, and fractures of the vertebrae and long bones have been identified as clinical symptoms of osteoporosis, the authors could not equate reduced bone density with the clinical symptoms. Tillinghast (44) found that demineralization of spinal bone frequently occurred without associated pathology.

Formation and Remodeling of Bone

Bone remodeling occurs throughout life in foci of the cortex and trabeculae. It appears to be greatest in infancy and lowest in the fourth decade (45). With aging, osteoporosis which is characterized by a reduced amount of bone tissue per unit volume of bone frequently occurs. Although reduced in quantity, the bone appears normal in its chemical composition (46, 47). Where bone is formed, osteoblasts that participate in the synthesis of matrix are found. Osteocytes develop from osteoblasts and have nuclei with prominent nucleoli and cytoplasm with well-formed Golgi zones indicating that they are active cells. Formation of collagen takes place close to the osteoblasts. Nutrients cannot diffuse easily through dense

bone although it is necessary that nutritive flow to the osteoblasts be maintained. The Haversian system of canals or tunnels which contains mesenchymal cells (osteons), nerves, and blood vessels provides a passage-way through which nutrients can diffuse. The average number of osteoblasts, the average rate at which each makes new bone matrix and the average functional lifetime of the osteoblasts change little with age (48). Osteoclasts are giant multinucleated cells which participate in the resorption of bone. The Haversian systems are formed and reconstructed by the erosive action of osteoclasts. Thus in response to a stimulus, undefined at present, there is proliferation of osteons into osteoclasts for the initiation of bone resorption. Resorption subsides after about two months and osteoblasts appear and begin to construct new bone. About three months are required to replace the bone removed by the osteoclasts (23).

Bone remodeling is a complex phenomenon. At present, there is no clear-cut explanation for decreased bone density with age. Trabecular bone which is covered with endosteal (bone resorbing) surfaces is lost faster than is cortical bone when bone formation occurs. Decreased bone density of the vertebrae may be detected earlier than in the extremities because the endosteal surface-to-bone-volume ratio is higher for trabecula than for cortex (23). If, as concluded by Rodan and Anbar (49), remodeling is regulated by the turnover of matrix, reduced bone density and osteoporosis may be attributed to disease of the matrix as reported by Shapiro (50). Other work (51) indicates an increased synthesis of proteolytic enzymes in bone with aging. Keating et al. (52) have found that serum calcium levels in men are reduced with aging while in women

high levels are maintained at the expense of bone calcium. Jowsey (53) has suggested that decreased bone density may result from the efforts of the body to maintain serum calcium. Hypocalcemia has been associated with formation and mineralization of bone matrix (54).

Long-term exposure to water-borne fluoride has been associated with increases in bone mineral (55, 56). Epker (57) found that in dogs, high levels of fluoride promoted active bone formation and thus counteracted the loss of cortical bone with aging. It was further shown in rats (58), that fluoride decreased the ease with which the skeleton could be induced to undergo resorption. Human biopsies from three patients receiving fluoride therapy showed new bone formation (59). However, in the absence of sufficient calcium and vitamin D, the new bone was not calcified. Zipkin (60) detected a homeostatic deposition of fluoride in bone; smaller bones of rats pair-fed at 60% of the consumption of control animals retained more of the fluoride than did the controls fed ad libitum. Phosphate supplementation increased the porosity of rabbit tibias and also resulted in soft-tissue calcification (61). Several studies are reported which show a relationship between nutritional status and bone density. Meema and Meema (62) found osteoporosis to be most prevalent among old, non-diabetic women with low body weight. Nordin (39) observed that the incidence of osteoporosis is lowest where the availability of calcium is highest.

Interrelation of Diet and Bone Density

With radiographic and histological evidence that bone density decreases with age and that clinically diagnosed osteoporosis increases

with age, numerous studies have been conducted in an attempt to explain and correct the phenomenon. Researchers have attempted to interrelate several of the parameters including dietary intake, metabolic balances, serum levels of nutrients, urinary constituents, bone density, and bone morphology. Several species including humans have been utilized in experimental studies.

In humans, low bone density was found to be associated with a low product of serum calcium times serum phosphorus, low vitamin D intake, and elevated serum alkaline phosphatase. Nutrient intakes of those with low bone densities did not always differ from those with high densities (63).

In a Bantu male population where intake of inorganic iron was extremely high due to consumption of alcoholic beverages prepared in iron kettles, a significant inverse correlation between bone mineral density and the concentration of iron stored in the liver was observed. Increased bone resorption space was observed in the osteoporotic Bantu subjects. Subsequent studies with guinea pigs showed that an iron overload reduced the hepatic concentration of ascorbic acid and decreased the percentage of bone forming surface as well as increasing areas of bone resorption. Injection of ascorbic acid prevented these changes in bone (64).

Protein-calorie deficiency induced in pigs resulted in a reduced rate of proliferation and maturation of cartilage and decreased osteoblastic activity; in addition, generalized rarefaction of the bone occurred (65). In rats maintained on a low-protein diet, decreased radiographic density of bone was observed (66).

In three patients with osteoporosis, calcium balances were slightly negative during periods of low calcium intake (67). Long-term calcium supplementation failed to improve the calcium balance to the extent it was achieved in non-osteoporotics. Fecal excretion of calcium was higher in osteoporotic than in non-osteoporotic patients. Radioisotope data revealed that little of the calcium excretion was endogenous. The authors concluded that osteoporotics have poor absorption of calcium. In other studies, Spencer et al. (68) reported that addition of calcium to low-calcium diets of persons with osteoporosis resulted in positive calcium balances without altering phosphorus or nitrogen balances. When sodium fluoride was given to nine patients for 22 to 42 days, ^{47}calcium absorption studies revealed that intestinal absorption of calcium and the calcium balances did not increase (69).

Addition of fluoride to the diet of monkeys increased the accretion rate of calcium and the size of calcium pool in animals maintained on an adequate protein diet. When added to low-calcium diets, fluoride promoted increased retention of dietary calcium and phosphorus (70). In eight patients with osteoporosis, fluoride improved their balances of both phosphorus and nitrogen but failed to alter calcium retention. Kinetic studies showed that fluoride reduced the exchangeable calcium without reducing bone formation (71).

Therapeutic Dietary Regimens for Reducing Bone Loss

Osteoporosis has been associated with diets consistently low in calcium (72, 73, 74). The earliest therapy for osteoporosis consisted of the administration of calcium salts. Loss of calcium from bone after

menopause has been related to elevated levels of calcium in serum and urine (75). Failure by osteoporotics to reduce urinary calcium in response to a diet low in calcium has been reported (76, 77). In fact, only at high levels of dietary calcium (40 mg/kg body weight) was more calcium retained by osteoporotics than by controls (78).

Recently, case studies of osteoporotic patients treated with sodium fluoride have been described (79, 80, 81, 82). In a study reported by Higgins et al. (79) two of three patients on sodium fluoride supplements of 60 mg/day for 1 to 4 months experienced relief from pain although there were no changes in calcium, phosphorus, or nitrogen balances. In four osteoporotic patients treated with sodium fluoride at levels ranging from 0.15 to 1.7 mg/kg body weight/day, bone biopsies taken after 7 to 30 months showed that new bone was formed but remained uncalcified unless calcium and vitamin D were administered concurrently (59, 82). Both an increase in bone density and retention of calcium were achieved within two years by a patient on 88 mg sodium fluoride/day (80). Some calcium retention and increases in bone density were experienced in patients treated with 10 to 66 mg fluoride/day for about a year (81), especially where the supplements included vitamin D.

In 11 patients administered sodium fluoride at 30 to 90 mg/day in addition to calcium and vitamin D for one year, Jowsey et al. (83) reported an increase in new bone formation in patients receiving at least 45 mg sodium fluoride/day while 600 mg calcium/day decreased bone resorption. Only Rose (84) failed to observe clinical remission with fluoride supplementation.

CHAPTER III

EXPERIMENTAL PROCEDURE

I. GENERAL PLAN

Seventy-six ambulatory women, aged 41 to 84, residing at Eastern State Psychiatric Hospital in Knoxville, Tennessee, were participants in this study. Medical records of potential subjects were screened for presence of any chronic illness such as cardiac problems, diabetes, or malignancy. Participation was permitted with the approval of the individual, the clinical director of the hospital, and the person's nearest living relative or her legal guardian (Appendix A). A physical examination by one of the hospital's physicians preceded the study and the participants were under constant surveillance by the hospital staff.

Sixty women were housed in three dormitories while 16 lived in home-like cottages for all or part of the study. Sixteen women, representative of the entire group but housed in one wing of a dormitory, were selected for 7-day metabolic balance studies. Evaluations of dietary intake, selected blood constituents, anthropometric measurements, metabolic balances, and bone density were made in the initial phase of the study. Nutrient intakes of some of the women were supplemented with vitamins, calcium phosphate, and sodium fluoride. The initial measurements were repeated on 60 subjects after 12 months of supplementation. In addition, metabolic balances and bone density measurements were obtained after the first four months of supplementation. Twelve of the

16 selected for metabolic balance studies were available to participate in all three of the collection periods.

Most women ate their meals in one of the hospital cafeterias. Each participant consumed those foods she chose from her plate which was served on a cafeteria line. In the home-like cottages, each woman was permitted to serve herself. Menus were planned by a registered dietitian employed by the hospital and no changes were made in the hospital menus. Food preparation was under the direction of a food service supervisor.

After the initial tests, each woman was assigned to one of four experimental groups. Subjects in each group were matched for bone density, age, and weight. Group 1 (19 women) received no vitamin or mineral supplements. Group 2 (17 women) was continued on a vitamin capsule (Table 1) which had been prescribed by the hospital medical staff and calcium phosphate (Table 2) in wafer form daily was added. Twenty women in Group 3 were given fluoride[1] as its sodium salt in capsule form at the level of 0.25 mg F/kg body weight/day. To achieve a range as narrow as possible from the level of 0.25 mg F/kg body weight/day, some women received the supplement for only six days per week (Table 3). Twenty women in Group 4 received a vitamin capsule and calcium phosphate as administered to Group 2 and sodium fluoride as given to Group 3. Four women assigned to each group participated in the metabolic studies.

---

[1]Davies Rose Hoyt, Pharmaceutical Division of the Kendall Company. Needham, Mass. Obtainable in increments of 5 mg fluoride.

TABLE 1

COMPOSITION OF VITAMIN SUPPLEMENT[1]

	Daily
Vitamin A palmitate (USP units)	25,000
Vitamin D (USP units)	400
Thiamine mononitrate (mg)	10
Niacinamide (mg)	100
Riboflavin (mg)	10
Pyridoxine hydrochloride (mg)	5
d-α-tocopheryl acid succinate (IU)	15
Cyancobalamin (μg)	5
Calcium panothenate (mg)	20
Ascorbic acid (mg)	200

[1]Interstate Drug Exchange, Plainview, L.I., N.Y. One capsule daily.

TABLE 2

COMPOSITION OF CALCIUM PHOSPHATE SUPPLEMENT[1]

	Daily
Calcium (mg)	464
Phosphorus (mg)	360
Vitamin D_2 (USP units)	400

[1]Abbott Laboratories, North Chicago, Ill. Dibasic calcium phosphate, hydrous, 1 g in wafer form twice daily.

TABLE 3

INDIVIDUAL FLUORIDE SUPPLEMENTS

Identification Number	Body Weight (kg)	Fluoride (mg)	Frequency
01	71.9	20	Mon.-Sat.
03	60.4	15	Daily
04	48.4	15	Mon.-Sat.
05	85.2	20	Daily
06	46.1	15	Mon.-Sat.
09	56.4	15	Daily
10	45.4	15	Mon.-Sat.
18	36.8	10	Daily
23	57.6	15	Mon.-Sat.
27	69.5	20	Mon.-Sat.
29	95.6	25	Daily
31	38.2	10	Daily
35	75.4	20	Daily
37	42.5	10	Daily
40	69.4	20	Mon.-Sat.
41	69.6	20	Mon.-Sat.
47	56.3	15	Daily
54	76.0	20	Daily
55	50.0	15	Mon.-Sat.
56	61.7	15	Daily
59	74.3	20	Mon.-Sat.
60	72.7	20	Daily
61	71.8	20	Mon.-Sat.
65	79.7	20	Daily
69	99.0	25	Daily
71	54.0	15	Mon.-Sat.
75	50.9	15	Mon.-Sat.
77	51.5	15	Mon.-Sat.
79	48.6	15	Mon.-Sat.
81	61.5	15	Daily
83	67.0	20	Mon.-Sat.
84	76.3	20	Daily
87	132.3	30	Daily
88	65.1	20	Mon.-Sat.
90	77.7	20	Daily
92	111.3	30	Daily
93	56.1	15	Daily
94	95.0	25	Daily
96	90.5	25	Mon.-Sat.
99	57.7	15	Daily

II. COLLECTION METHODS

Dietary Information

Food served to each woman was weighed to the nearest gram on a top-loading balance as each item was placed on the plate. The weight of each unconsumed portion of food was obtained and the quantity consumed was calculated by difference (Appendix B). Average daily consumption was based on intakes for four to seven consecutive days. The average daily nutritive intake of energy, protein, fat, calcium, phosphorus, iron, vitamin A, and ascorbic acid was obtained for each individual (85). Careful records of snacks consumed during the dietary studies were kept and included in the day's nutrient intake. During the 7-day balance studies, a sample of each snack item and each food served was immediately frozen in airtight plastic cartons for analysis of nitrogen, calcium, and phosphorus.

Urine and Feces

To assist with urinary and fecal collections, a technician was available 24 hours/day at each restroom on the ward where the metabolic study was carried out. Those few women who left the ward were reminded to return to the ward to use the bathroom. Excreta were collected in plastic bowls lined with plastic collection bags and inserted into the toilets. Urine was removed from the collection bag to a 4 liter, acid rinsed container. At the end of each 24-hour period, the urine was mixed thoroughly and its volume was measured in a stoppered graduated cylinder. A 5% aliquot was removed, added to the composite, and frozen immediately for later determination of nitrogen, calcium, phosphorus

and hydroxyproline. In addition, approximately 5 ml of each daily collection was frozen separately for daily creatinine determinations. Feces was retained in the collection bag and inserted into a plastic carton for immediate freezing on the cold plate of a freezer. After the feces was frozen to a solid state, it was transferred from the collection bag into a large plastic carton and the composite was stored at -10°.

Blood

Fasting blood samples, 25 ml, were drawn by a registered nurse. Approximately 10 ml was immediately transferred from the syringe to an oxalated test tube. Oxalated tubes were prepared by placing 0.1 ml of a 10% potassium oxalate solution (w/v) into each test tube, dispersing it evenly in a thin layer around the tube, and drying at 90 - 100° (86). Blood was mixed with the potassium oxalate by corking the tubes and gently but rapidly inverting them. The remaining 15 ml was removed from the syringe to a test tube and permitted to coagulate. Hemoglobin was determined on the uncoagulated blood sample. Separation of both plasma and serum was accomplished by centrifugation in an International Clinical Centrifuge at 1500 x g for 10 minutes. Plasma and serum were removed with dropping pipettes. Plasma ascorbic acid levels were determined immediately. One ml samples of serum for determination of alkaline phosphatase activity were set in an ice bath. The remaining serum tubes were stoppered and placed in larger tubes for freezing. They were packed in ice for transport to the laboratory within an hour. Syringes were washed, packaged in sterile envelopes, and autoclaved at 120° for 20 minutes. Disposable sterile needles were used. Blood samples from 6 to 10 patients were drawn per day.

Anthropometric Measurements

The height and weight of each participant were obtained. If shoes other than flat slippers were worn, height was corrected for heels. Each subject was encouraged to stand as tall as possible, looking straight ahead, while the measurement was taken. Sweaters or jackets were removed before weight was recorded.

Bone Density Measurements

A bone densitometer, developed by the Department of Nutrition in the College of Home Economics at The University of Tennessee, Knoxville, was used to estimate bone density. The instrument utilized x-ray energy to scan the left phalanx 5-2, as the hand was automatically moved through a collimated x-ray beam. Linear absorption curves were traced directly onto graph paper for anteroposterior and lateral finger positions. A trace of the alloy standard was superimposed on the lateral tracing and a bone density index was calculated from measurements of these tracings (87).

III. ANALYSIS OF FOOD, FECES, AND URINE

Ashing

Fecal and urine composites and samples of food consumed during the metabolic balance periods were stored at -10° in sealed polyethylene cartons until they could be analyzed. Sample preparation included blending an equal weight of hot distilled water with feces and blending one entire serving of each food to a homogenous mass.

Duplicate aliquots of all foods, feces, and urine were placed in Coors #6 crucibles. Either 1 or 2 g of food was ashed depending on

the predicted quantity of calcium and phosphorus in the food (85). Two g of the fecal slurry (1 g feces) or 3 ml aliquots of urine were dried for approximately 12 hours under a heat lamp. Ashing was carried out in a muffle furnace at 550° for 20 hours. The furnace was allowed to cool between ashings.

After removal from the furnace, each sample was moistened with 1 ml of demineralized water and then was dissolved in 4 ml of concentrated hydrochloric acid (2 ml was used when the final volume was to be 50 ml). Each sample was treated for silica (88) by evaporation to dryness over water, followed by continued heating over water for 1 hour. After treatment for silica, the ash was again moistened with 1 ml of demineralized water and dissolved in 6 ml of concentrated hydrochloric acid (3 ml was used when the final volume was to be 50 ml).

Ashes were dissolved over low heat; food ashes were transferred to either a 50 or 100 ml volumetric flask depending on the predicted quantity of calcium and phosphorus in the food (85). Fecal and urine ashes were diluted to 100 ml. Polyethylene bottles were used for storage of the ash solutions.

Acid Rinsing

Polyethylene bottles used for storage of the ash solutions and all glassware utilized in ashing and in calcium and phosphorus analyses were rinsed for 30 minutes in a dilute hydrochloric acid solution (1 part concentrated hydrochloric acid and 9 parts water).

Instruments

In all analyses, unless otherwise specified, the International Centrifuge Model SBB #50092 H was utilized for centrifugation and the Beckman Model B Spectrophotometer was used for determination of sample absorbances. The Perkin-Elmer Atomic Absorption Spectrophotometer Model 303 was used for determination of sample transmittance. Absorbance was obtained by referring each transmittance to a table of absorbances which was prepared to accompany the instrument.

Calcium

Aliquots of ash solutions from food, feces, and urine were diluted with lanthanum oxide to depress the interference of phosphorus, and calcium was determined by atomic absorption spectrophotometry (89).

Reagents

1. Calcium stock standard, 0.5 mg/ml: 0.3121 g Ca_2CO_3 was diluted to 250 ml with water after 1 ml concentrated HCl had been added to enhance solubility of the Ca_2CO_3.

2. Calcium working standard, 0.05 mg/ml: 10 ml of the stock standard was diluted to 100 ml with water.

3. Lanthanum oxide, 5%: 5.9 g La_2O_3 was mixed with about 50 ml of water in a 100 ml volumetric flask. Twenty-five ml of concentrated HCl was added and the contents of the flask were cooled before being diluted to volume with water. The solution was filtered and used within 1 hour after preparation.

Method. Duplicate aliquots of ash solutions from food, feces, or urine estimated to contain between 0.02 and 0.10 mg calcium (85) were

placed in acid rinsed 15 x 125 mm test tubes and water was added to a volume of 9 ml. Standards were prepared from the working standard to contain 0.00 to 0.10 mg calcium and diluted to 9 ml with water. The lanthanum solution was prepared and 1 ml was added to each sample and standard. Mixing was by mechanical mixer. Calcium was aspirated into an acetylene flame and determined at 420.2 nm with an atomic absorption spectrophotometer equipped with a calcium-zinc lamp. Absorbances (A) were referred to a standard curve from which the quantity of calcium in each sample was determined.

Calculations.

$$\text{mg Ca/day} = \text{mg Ca/sample} \times \text{dilution factor} \times \text{mean daily urine volume or mean daily weight of food or feces}$$

Phosphorus

Phosphorus in the ash solutions of food, feces, and urine was reacted with ammonium molybdate and sulfuric acid. The phosphomolybdic acid thus formed was reduced by N-phenyl-p-phenylenediamine monohydrochloride with the development of a blue color which was measured with a spectrophotometer (90).

Reagents.

1. Phosphorus stock standard, 1 mg/ml: 438.1 mg of KH_2PO_4 was dissolved and diluted to 100 ml with water.

2. Phosphorus working standard, 10 µg/ml: 1 ml of the stock standard was diluted to 100 ml with water.

3. Sulfuric acid, 5 N: 138 ml concentrated H_2SO_4 was added very slowly to about 750 ml water in a liter flask. After cooling, the solution was diluted to volume with water.

4. Ammonium molybdate, 0.025 M: 30.9 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ was diluted with water to 1 liter with the inclusion of 1 ml concentrated H_2SO_4 to facilitate solubility. It was stored in a polyethylene container.

5. N-phenyl-p-phenylenediamine monohydrochloride (PPD)[2], 0.5% in 1% sodium bisulfite: 50 mg PPD was moistened with 95% ethanol followed by about 50 ml water and 1 g $NaHSO_3$ before the solution was diluted to 100 ml with water. It was filtered into a brown bottle for storage.

Method. Duplicate quantities of ash solutions estimated to contain 2 to 10 µg phosphorus (85) were placed in 12 x 100 spectrophotometer tubes. The volumes were diluted to 1 ml with water. Standards were also prepared to contain 0 to 10 µg of phosphorus in 1 ml. One ml of 5 N sulfuric acid was added to each sample and standard followed by the addition of 1 ml of 0.025 M ammonium molybdate. The tube contents were mixed with a mechanical mixer both before and after the addition of 2 ml of PPD. Color was permitted to develop for 10 minutes prior to determination of the absorbances (A) on a spectrophotometer at 770 nm.

Calculations.

$$\text{mg P/day} = \text{µg P/sample} \times \text{dilution factor} \times \text{mean daily urine volume or mean daily weight of food or feces}$$

$$\text{Where: µg P/sample} = \text{A of sample} \times \frac{\text{conc of std}}{\text{A of std}}$$

[2]Eastman Organic Chemicals, Rochester, N.Y. Catalogue #2043.

Nitrogen

A macro-Kjeldahl method was used for determination of nitrogen in food, feces, and urine. After digestion, the ammonia was distilled, collected in boric acid, and titrated with 0.1 N hydrochloric acid (91). All analyses were done in triplicate.

Reagents.

1. Sodium sulfate: Na_2SO_4.

2. Concentrated sulfuric acid: H_2SO_4.

3. Cupric sulfate: $CuSO_4$.

4. Hengar crystals.

5. Sodium hydroxide, 50%: 500 g of NaOH was dissolved in 750 ml water in an ice bath.

6. Boric acid, 4%: 40 g of H_3BO_3 was dissolved in 1 liter of water with heating.

7. Hydrochloric acid, 0.1 N: 8.3 ml of concentrated HCl was diluted to 1 liter and standardized.

8. Methyl red - methylene blue indicator: 2 parts of 0.2% alcoholic methyl red was mixed with 1 part of 0.2% alcoholic methylene blue.

Method. Analyses were performed on samples of blended food estimated to contain 10 to 15 mg nitrogen, on 3 ml samples of urine, and on 1 g samples of fecal slurry. Food homogenates and fecal slurry samples were weighed in small paper cups and the cup with its contents was placed in a Kjeldahl flask. The blank determination for the food and fecal samples included a paper cup. Liquid foods and urine were pipetted directly into the Kjeldahl flasks. To each flask was added 5 g sodium sulfate,

20 ml concentrated sulfuric acid, 0.3 g cupric sulfate, a Hengar crystal, and 2 glass beads. Flask contents were digested until the solutions were clear and then they were cooled for approximately 30 minutes. The digests were diluted with 200 ml of water, mixed well, and again cooled. Fifty ml of 50% sodium hydroxide was poured down the side of the flask so that it layered under the digest but did not mix prior to connection of the flask with the condenser and its placement on the distilling rack. The flask contents were swirled to mix, the burners ignited, and the nitrogen collected in Erlenmeyer flasks containing 50 ml of 4% boric acid and methyl red-methylene blue color indicator. The contents of each flask were titrated back to their original shade of purple with standardized hydrochloric acid. The average volume of acid used to titrate the blank was subtracted from the average volume used to titrate the sample.

Calculations.

$$\text{g N/day} = \text{ml HCl} \times \text{N HCl} \times 0.014 \times \text{dilution factor} \times \text{mean daily urine volume or mean daily weight of feces or food}$$

Creatinine in Urine

Creatinine in urine reacted with picrate in an alkaline solution to form an intense orange color which was measured spectrophotometrically (92).

Reagents.

1. Sodium hydroxide, 10%: 10 g of NaOH was diluted to 100 ml with water.

2. Picric acid, 1%: 1 g of $C_6H_3N_3O_7$ was diluted to 100 ml with water.

3. Alkaline picrate: 100 ml of 10% NaOH was mixed with 100 ml of 1% picric acid and diluted to 1 liter with water.

4. Creatinine stock standard, 1 mg/ml: 1.6016 g of crystalline creatinine zinc chloride was diluted to 1 liter with 0.1 N HCl.

5. Creatinine working standard, 0.05 mg/ml: 5 ml of the stock standard was diluted to 100 ml with water.

Method. One-tenth ml of urine was diluted to 20 ml in a 100 ml volumetric flask. Twenty ml of alkaline picrate was added and mixed gently. After standing for exactly 15 minutes, the contents were diluted to volume. Standards were prepared to contain 0.00 mg to 0.25 mg in 20 ml. Absorbances (A) of the standards and samples were determined in 12 x 100 spectrophotometer tubes with a spectrophotometer at 520 nm.

Calculations.

$$\text{mg creatinine/24 hours} = \text{A of sample} \times \frac{\text{conc of std}}{\text{A of std}} \times \text{dilution factor} \times \text{daily urine vol}$$

Hydroxyproline in Urine

Hydroxyproline (HOP) from acid hydrolyzed urine was oxidized to a pyrrole by Chloramine T. The pyrrole was condensed with p-dimethylamino-benzaldehyde (Ehrlich's reagent) to produce a red chromophore which was measured spectrophotometrically (93).

Reagents.

1. Sodium carbonate, 5%: 25 g Na_2CO_3 was diluted to 500 ml with water.

2. Citrate buffer, 0.1 M, pH 6: 58.8 g of sodium citrate ($Na_3C_6H_5O_7 \cdot H_2O$) was diluted to 2 liter with water and 19.2 g citric acid ($H_3C_6H_5O_7 \cdot H_2O$) was diluted to 1 liter with water. Two liters of

sodium citrate solution were mixed with 150 ml of the citric acid solution and the pH of the buffer was adjusted to 6.0.

3. Oxidizing reagent: 1 part Chloramine T and 4 parts buffer solution were mixed just prior to use.

A. Chloramine T: 3.5 g Chloramine T was dissolved, diluted to 50 ml with water, and stored in the refrigerator.

B. Buffer solution, pH 6: 57 g of sodium acetate ($CH_3COONa \cdot 3H_2O$), 37.5 g of sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$), 5.5 g of citric acid ($H_3C_6H_5O_7 \cdot H_2O$), and 385 ml of isopropyl alcohol were diluted to 1 liter with water.

4. Ehrlich's reagent: 20 g p-dimethylaminobenzaldehyde was dissolved in 22 ml of concentrated HCl in a steam bath after which 128 ml of isopropyl alcohol was added. This reagent was prepared just prior to use.

5. Hydroxyproline stock standard, 100 µg/ml: 25 mg of L-hydroxyproline[3] was diluted to 250 ml with 0.001 N HCl.

6. Hydroxyproline working standard, 10 µg/ml: 10 ml of the stock standard was diluted to 100 ml with water. The working standard was prepared daily.

Method. Duplicate urine samples to which an equal volume of concentrated hydrochloric acid had been added were hydrolyzed for 18 hours at 125°. After cooling, samples were filtered through a sintered glass funnel. One ml aliquots of each filtrate were neutralized with 6 ml

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[3]Nutritional Biochemicals Corporation, Cleveland, Ohio.

of 5% sodium carbonate and 3 ml of 0.1 M citrate buffer. Two ml of isopropyl alcohol and 0.5 ml oxidizing reagent were added to 1.5 ml of the neutralized filtrate. After 4 minutes at room temperature, the samples were chilled in an ice bath and 5 ml of Ehrlich's reagent was added. The samples were heated in boiling water for 2 minutes and then cooled immediately to room temperature in an ice bath. After standing at room temperature for 90 minutes, the absorbance (A) of each sample was determined on a spectrophotometer at 565 nm. Standards containing 0 to 10 µg of hydroxyproline were prepared under the same conditions.

Calculations.

$$\text{mg HOP/24 hours} = \text{A of sample} \times \frac{\text{conc of std}}{\text{A of std}} \times \text{dilution factor} \times \text{mean daily urine vol}$$

IV. ANALYSIS OF BLOOD CONSTITUENTS

Hemoglobin in Oxalated Blood

Hemoglobin was determined by the cyanmethemoglobin method utilizing the Hycel standard[4] and cyanmethemoglobin reagent[4]. Absorbance was determined spectrophotometrically.

Reagents.

1. Cyanmethemoglobin reagent: The reagent was obtain, pre-weighed, in a sealed vial. Using several rinsings of water, the contents of the vial were transferred to a 1 liter volumetric flask and after vigorous mixing, the solution was diluted to volume.

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[4]Hycel, Inc., Houston, Texas.

2. Cyanmethemoglobin standard, 80 mg/100 ml: Each vial as purchased contained sufficient standard for a curve ranging from 0 to 20 g/100 ml.

3. Hemoglobin controls: Lysed human erythrocytes[4] at concentrations comparable to low, normal, and high hemoglobin levels were used as controls.

Method. Five ml of the cyanmethemoglobin reagent was pipetted into 12 x 100 mm spectrophotometer tubes prior to drawing of the blood. Pipettes calibrated to contain 0.02 ml were used to deliver duplicate samples of the blood to tubes containing cyanmethemoglobin reagent. Thorough mixing was accomplished by inversion. Absorbances (A) were determined on a spectrophotometer at 540 nm against a reagent blank set at zero absorbance. A standard curve was prepared each time a new reagent was used. Instrumentation and technique were checked for each day's determination by use of the prepared solutions of lysed human erythrocytes. These controls were treated exactly as were the blood samples. Hemoglobin tests were made within 1 hour after blood was drawn.

Calculations.

$$\text{g hemoglobin/100 ml} = \text{A of sample} \times \frac{\text{conc of std}}{\text{A of std}} \times \text{dilution factor}$$

Ascorbic Acid in Plasma

When deproteinated plasma was added to a dye, the dye was reduced by the ascorbic acid in the plasma. This reduction produced a color change which was measured on a spectrophotometer. The method was a modification of that by Mindlin and Butler (94).

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[4]Hycel, Inc., Houston, Texas.

Reagents.

1. Meta-phosphoric acid, 2.5%: 2.5 g of HPO_3 was dissolved and diluted to 100 ml with water. It was stored in a brown bottle at 10° and a fresh solution was prepared every 2 weeks. This solution was used to determine the proper dye concentration.

2. Meta-phosphoric acid, 6%: 60 g of HPO_3 was dissolved and diluted to 1 liter with water. It was stored in a brown bottle at 10° and a fresh solution was prepared every 2 weeks.

3. Sodium acetate, pH 7: 22.65 g of $NaC_2H_5O_2 \cdot 3H_2O$ was dissolved in 500 ml water. It was adjusted to pH 7 with 1% acetic acid.

4. Dye solution: A few crystals of 2,6-dichloroindophenol were dissolved in a small amount of distilled water in a test tube. The solution was heated in a steam bath to 85 - 90° then filtered while hot.

5. Dye-acetate solution: About 5 ml of the dye solution was diluted to 500 ml with water. The proper concentration was adjusted as follows: 2 ml of the dye solution was pipetted into a 15 x 150 mm calibrated tube. Two ml of the sodium acetate and 4 ml of 2.5% HPO_3 were added. The absorbance of the solution was determined at 520 nm after setting the instrument to zero absorbance using 8 ml of water as a blank. The absorbance of the trial solution should be between 0.25 and 0.35. The dye solution was altered by the addition of more water if the solution had absorbance greater than 0.35, or more dye if it was less than 0.25. After the dye solution was altered, the procedure was repeated until the absorbance fell within the proper range. The dye-acetate solution was prepared by mixing equal volumes of the diluted dye

and the sodium acetate solution. Stored in a brown bottle at 10°, the solution was acceptable for 10 days.

6. Ascorbic acid standard, 0.0125 mg/ml: 50 mg of ascorbic acid[3] was placed in a 500 ml volumetric flask and dissolved. After vigorous mixing, 25 ml was pipetted into a 200 ml volumetric flask and diluted to volume. The standard was used immediately.

7. Blanking solution: 5 ml of 6% HPO_3 was saturated with ascorbic acid.

Method. Prior to drawing of the blood samples, 7 calibrated tubes, 15 x 150 mm, for the standards and one for each sample were placed in a test tube rack wrapped in black cloth. Into each tube was pipetted 4 ml of the dye-acetate solution. In a second test tube rack, to 7 tubes for the standards and one for each sample was added 4 ml of 6% meta-phosphoric acid. To tubes for the samples, 2 ml of water was also added. For each sample, a test tube fitted with a funnel and Whatman #42 filter paper was placed in another black-cloth-wrapped test tube rack to receive the plasma-acid mixture.

Two ml of plasma was deproteinated when added to the tubes containing 4 ml meta-phosphoric acid. The contents were mixed and filtered into dry test tubes already set up in the wrapped rack.

A spectrophotometer was set at 520 nm. To prepare for determination of sample absorbances, 4 ml from the zero standard and 4 ml of each plasma-meta-phosphoric acid filtrate was pipetted into a calibrated tube containing the dye-acetate solution. After mixing, each absorbance was determined

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[3]Nutritional Biochemicals Corporation, Cleveland, Ohio.

then a drop of the blanking solution was added and the absorbance was redetermined. The absorbance of the blanked sample was subtracted from that of the sample.

The ascorbic acid standard was prepared and a standard curve was determined immediately after sample absorbance had been obtained. A standard curve was prepared with a range of 0.00125 to 0.00625 mg/ml. After thorough mixing, 4 ml of each standard was removed, added to the dye-acetate solution, and treated in the same manner as were the samples. Absorbances (A) of the standards were plotted against the ascorbic acid concentrations.

Calculations.

ascorbic acid mg/100 ml =

$$\frac{\text{A of zero conc std} - \text{A of sample}}{\text{A of zero conc std} - \text{A of std}} \times \text{conc of std} \times \text{dilution factor}$$

Alkaline Phosphatase in Serum

The activity of alkaline phosphatase in serum was estimated by the method of Dryer et al. (90) in which the enzyme was allowed to release phosphorus from a glycerophosphate substrate at 37° for 1 hour. Phosphorus was converted to phosphomolybdic acid which could be reduced by N-phenyl-p-phenylenediamine monohydrochloride with the development of a blue chromophore proportional to the quantity of phosphorus present. Controls were used to obtain a pre-incubation level of phosphorus.

Reagents.

1. Trichloroacetic acid (TCA), 30%: 30 g TCA was dissolved in 100 ml water.

2. Trichloroacetic acid (TCA), 10%: 10 g TCA was dissolved in 100 ml water.

3. Glycerophosphate substrate, pH 9.3: 0.5 g sodium beta-glycerophosphate and 0.424 g of sodium barbital were dissolved in 50 ml of warm water. The solution was cooled, transferred to a 100 ml volumetric flask, and diluted with water.

4. Ammonium molybdate, 0.008 M: 9.8 g of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ was dissolved in 100 ml water. One-tenth ml of concentrated H_2SO_4 was added to aid solubility. Storage was in a polyethylene bottle.

5. N-phenyl-p-phenylenediamine monohydrochloride (PPD)[2], 0.5% in 1% sodium bisulfite: 50 mg PPD was moistened with 95% ethanol followed by about 50 ml water and 1 g $NaHSO_3$ before the solution was diluted to 100 ml. It was filtered into a brown bottle for storage.

6. Phosphorus stock standard, 1 mg/ml: 438.1 mg of KH_2PO_4 was dissolved in 100 ml of 10% TCA.

7. Phosphorus working standard, 10 µg/ml: 1 ml of the stock standard was diluted to 100 ml with 10% TCA.

Method. Duplicate centrifuge tubes containing 1.8 ml of the substrate for each sample were placed in a water bath at 37° for 10 minutes. Two-tenth ml of serum was added and the tubes were incubated for 1 hour after which the enzyme was inactivated by the addition of 0.5 ml of 30% TCA. The mixture was allowed to stand for 10 minutes to assure complete precipitation of protein and then centrifuged for 10 minutes at 900 x g. The controls were prepared with the same quantities of reagents but the

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[2]Eastman Organic Chemicals, Rochester, N.Y. Catalogue #2043.

0.5 ml of 30% TCA was added before the 0.2 ml of serum to assure immediate inactivation of the enzyme.

After centrifugation, 1 ml of the clear supernatant fluid was placed in a 12 x 100 mm spectrophotometer tube followed by the addition of 0.2 ml of molybdate solution. The contents were mixed, 2 ml of PPD was added, and color was developed for 10 minutes before absorbance (A) was determined with a spectrophotometer at 770 nm against a reagent blank set at zero absorbance. Since each sample was prepared in duplicate, the average of the serum blank was subtracted from the average of the incubated sample. A standard curve ranging from 0 to 10 μg phosphorus per ml was prepared and utilized in calculating the mg of inorganic phosphorus released by the enzyme.

Calculations.

$$\text{mg P/100 ml serum} = \left(\begin{matrix}\text{A of}\\ \text{sample}\end{matrix} - \begin{matrix}\text{A of}\\ \text{blank}\end{matrix}\right) \times \frac{\text{conc of std}}{\text{A of std}} \times \begin{matrix}\text{dilution}\\ \text{factor}\end{matrix}$$

Total Protein, Albumin, and A/G Ratio in Serum

Serum proteins were reacted with a biuret reagent to produce a purple color, which had an absorbance proportional to the amount of protein present. Albumin was determined similarly after removal of the globulin by salt fractionation (95).

Reagents.

1. Sodium sulfite, 28%: After anhydrous Na_2SO_3 was dried at 100° overnight, 280 g was placed in a liter volumetric flask and mixed with about 800 ml water prior to dilution to 1 liter. Two days were generally required for the Na_2SO_3 to go completely into solution.

2. Ether-span: About 2 ml of Span-20[5] was diluted to 200 ml with ether.

3. Biuret reagent: 1.5 g of $CuSO_4 \cdot 5H_2O$ was dissolved in 500 ml water followed by the solution of 6 g potassium sodium tartrate ($KNaC_2H_6O_6 \cdot 4H_2O$), and 40 g NaOH. After the addition of 1 g of KI the reagent was diluted to 1 liter, mixed, and stored in a polyethylene bottle.

4. Protein standard: Versatol[6] was purchased for use as a standard. It was hydrated in the vial with 5 ml water according to manufacturer's instructions and treated exactly like a serum sample during the analyses.

Method. For the standard and each sample, 12 ml of the 28% sodium sulfite was pipetted into glass-stoppered 15 ml centrifuge tubes. Five-tenths ml of Versatol or of serum was added to each. The contents were mixed by very gentle inversion 5 times. Duplicate 2 ml aliquots of the suspension were pipetted into 12 x 100 mm spectrophotometer tubes and identified as total protein. To the balance of the suspension in the centrifuge tube, 2 ml of ether-span was added. The tubes were stoppered tightly and inverted 15 times in 20 seconds to separate out the globulin. After centrifugation at 500 x g for 15 minutes, duplicate 2 ml aliquots of the albumin solution were withdrawn, placed in spectrophotometer tubes, and identified as albumin. After the addition of 4 ml biuret, color

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[5]Atlas Chemical Industries, Inc., Wilmington, Delaware.

[6]Scientific Products Division, Chamblee, Georgia.

was developed in a 37° water bath for 15 minutes. The absorbance (A) was determined on a spectrophotometer at 550 nm against a biuret blank set at zero absorbance.

Calculations.

$$\text{g total protein/100 ml serum} = \text{A sample total protein} \times \frac{\text{conc of std}}{\text{A of std}}$$

$$\text{g albumin/100 ml serum} = \text{A sample albumin} \times \frac{\text{conc of std}}{\text{A of std}}$$

$$\text{A/G ratio} = \frac{\text{albumin}}{\text{total protein} - \text{albumin}}$$

Cholesterol in Serum

Cholesterol in serum was saponified with alcoholic potassium hydroxide. The saponified cholesterol was extracted into petroleum ether, an aliquot was evaporated, and the residue was treated with the Lieberman-Burchard reagent. The absorbance of the green color was proportional to the quantity of cholesterol present (96).

Reagents.

1. Ethanol, 95%.

2. Petroleum ether, boiling range, 30 - 60°.

3. Potassium hydroxide, 33%: 10 g of KOH was dissolved in 20 ml water.

4. Alcoholic potassium hydroxide: This reagent was prepared just prior to use by placing 6 ml of 33% KOH in a 100 ml volumetric flask and diluting to volume with 95% ethanol.

5. Cholesterol standard, 0.4 mg/ml in ethanol: 20 mg of cholesterol[3] was dissolved in 50 ml of 95% ethanol.

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[3]Nutritional Biochemicals Corporation, Cleveland, Ohio.

6. Lieberman-Burchard reagent: 20 volumes of acetic anhydride was chilled to 10°. One volume of concentrated sulfuric acid was added, mixed, and kept cold for 10 minutes. Ten volumes of glacial acetic acid was added and the mixture was warmed to room temperature (25°). The reagent was used within 1 hour.

Method. Duplicate 15 ml glass-stoppered centrifuge tubes were used for each sample. In each tube 0.25 ml serum was mixed with 2.5 ml of alcoholic potassium hydroxide. Triplicate standards were prepared containing 2.5 ml of the standard and 0.15 ml of 33% potassium hydroxide. Each tube was stoppered and mixed by several inversions. Samples were placed in a 37° water bath for 55 minutes. After cooling to 25°, 5 ml of petroleum ether and 2.5 ml water were added and mixed with tube contents. The tubes were mixed vigorously by inversion for 1 minute. Separation of components was accomplished by centrifugation at 500 x g for 5 minutes. Using a pipette equipped with a rubber bulb, 5 ml of the petroleum ether layer was removed from each sample and placed in a small dry bottle. From the tubes containing the standard, duplicate 1, 2, and 3 ml aliquots (containing 0.2, 0.4, and 0.6 mg cholesterol respectively) were removed. The petroleum ether aliquot was evaporated to dryness in a 60° water bath. A small dry bottle, containing no cholesterol, was used for a zero standard.

The instability of the chromophore formed necessitated timed readings after the reagent was added. Thus, prior to color development, the blank, one set of standards, each pair of samples, and the second set of standards were placed in order and 6 ml of the Lieberman-Burchard reagent

was added to the bottles at 30 second intervals. The bottles were capped and their contents swirled for mixing. Color was developed at 25°. Thirty minutes after the reagent was added to the blank bottle, its contents were transferred to a 12 x 100 mm spectrophotometer tube and the spectrophotometer was set to zero absorbance at 620 nm. Similarly, the absorbance of each standard and sample was determined in order at 30 second intervals.

Calculations.

$$\text{mg cholesterol/100 ml serum} = \text{A of sample} \times \frac{\text{conc of std}}{\text{A of std}} \times \text{dilution factor}$$

Calcium in Serum

From a deproteinated serum prepared for calcium determination, serum calcium was determined by atomic absorption spectrophotometry after lanthanum had been added to depress the phosphate (89).

Reagents.

1. Trichloroacetic acid (TCA), 10%: 10 g of TCA was diluted to 100 ml with water.

2. Lanthanum oxide, 5%: This reagent was used, as prepared, for the calcium standards and was diluted for the samples. Final concentration of lanthanum in both standards and samples was 0.005 mg/ml. To prepare the 5% solution, 5.9 g of La_2O_3 was placed in a 100 ml volumetric flask. About 50 ml of water was added, followed by 25 ml concentrated HCl. After solubility was achieved, the mixture was cooled, diluted to volume, filtered, and used within 1 hour.

3. Lanthanum oxide, dilution for samples: 20 ml of the 5% lanthanum solution was diluted to 100 ml with water. This solution was also used within 1 hour.

4. Calcium stock standard, 0.5 mg/ml: In a 250 ml volumetric flask, 0.3121 g of Ca_2CO_3 was moistened with about 50 ml water. One ml of concentrated HCl was added to assure solubility before the standard was diluted to volume with water.

5. Calcium working standard, 0.05 mg/ml: 10 ml of the stock standard was diluted to 100 ml with water.

Method. To deproteinate the serum, 3.6 ml of 10% TCA was mixed with 0.4 ml serum in 15 ml conical centrifuge tubes. Duplicates for each sample were prepared. After standing for 10 minutes, the samples were centrifuged at 700 x g for 10 minutes (90). Two ml of the deproteinated serum solution was removed from each centrifuge tube and placed in a 15 x 125 mm acid-rinsed test tube. One ml of diluted lanthanum was added to each sample. Standards were prepared to contain 0.00 to 0.10 mg calcium and 1 ml of 5% lanthanum was added to each standard. All tubes were mixed by a mechanical mixer. The standards and samples were aspirated into an acetylene flame of an atomic absorption spectrophotometer equipped with a calcium-zinc lamp and their transmittancies determined at 431.2 nm. The standard curve was plotted and the calcium absorbances of the samples were referred to the curve to obtain their concentrations.

Calculations.

mg Ca/100 ml serum = mg Ca from graph x dilution factor

Phosphorus in Serum

Deproteinated serum was treated with ammonium molybdate to convert inorganic phosphorus to phosphomolybdate which was reduced by N-phenyl-p-phenylenediamine monohydrochloride. The absorbances were determined spectrophotometrically (90).

Reagents.

1. Trichloroacetic acid (TCA), 10%: 10 g TCA was dissolved and diluted to 100 ml with water.

2. Ammonium molybdate, 0.008 M: 9.8 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ was dissolved in 100 ml water. One tenth ml of concentrated H_2SO_4 was added to aid solubility. The solution was stored in a polyethylene container.

3. N-phenyl-p-phenylenediamine monohydrochloride (PPD)[2], 0.5% in 1% sodium bisulfite: 50 mg PPD was moistened with 95% ethanol followed by about 50 ml water and 1 g $NaHSO_3$ before the solution was diluted to 100 ml. It was filtered into a brown bottle for storage.

4. Phosphorus stock standard, 1 mg/ml: 438.1 mg of KH_2PO_4 was diluted to 100 ml with 10% TCA.

5. Phosphorus working standard, 10 µg/ml: 1 ml of the stock standard was diluted to 100 ml with 10% TCA.

Method. Serum was deproteinated by the addition of 0.2 ml serum to 1.8 ml of 10% trichloroacetic acid in duplicate 15 ml conical centrifuge tubes. After standing for 10 minutes, samples were centrifuged at 700 x g for 10 minutes. One ml of the deproteinated serum was removed

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[2]Eastman Organic Chemicals, Rochester, N.Y. Catalogue #2043.

from each centrifuge tube and delivered to a 12 x 100 mm spectrophotometer tube. Each aliquot was then treated with 0.2 ml ammonium molybdate followed by 2 ml PPD. After standing for 10 minutes to develop the blue chromophore , the absorbance (A) was determined on a spectrophotometer at 770 nm. A standard curve was prepared to contain 0 to 10 µg/ml phosphorus.

Calculations.

$$\text{mg P/100 ml} = \text{A of sample} \times \frac{\text{conc of std}}{\text{A of std}} \times \text{dilution factor}$$

Carotene and Vitamin A in Serum

Serum proteins were precipitated with alcohol and then carotene and vitamin A were extracted into petroleum ether. After the absorbance of the carotene was determined, the solvent was evaporated. The intensity of the blue chromophore produced by the addition of trifluoroacetic acid to the samples was also measured on a spectrophotometer to determine vitamin A concentration. The method was a modification of that used by Neeld et al. (97).

Reagents.

1. Ethanol, 95%.
2. Petroleum ether, boiling range 37 - 44°.
3. Chloroform.
4. Acetic anhydride.
5. Trifluoroacetic acid-Chloroform chromagen: The chromagen was prepared just prior to use by mixing 1 volume of trifluoroacetic acid with 2 volumes of chloroform.

6. Carotene standard, 5 µg/ml: 50 mg beta-carotene[7] was dissolved in 5 ml chloroform and diluted to 100 ml with petroleum ether. The concentration was 0.5 mg/ml. One ml was further diluted with petroleum ether to 100 ml for use in the preparation of the standard curve. Carotene is stable in petroleum ether for just a few hours.

7. Vitamin A standard, 3.75 µg/ml: 50 mg of Vitamin A acetate in cottonseed oil[8] was dissolved in 5 ml chloroform and diluted to 50 ml with petroleum ether. For the final concentration, 12.5 ml was diluted to 100 ml with petroleum ether. The vitamin A standard was stable under refrigeration for 2 days.

Method. Samples were prepared in glass-stoppered centrifuge tubes by mixing 2 ml serum, 2 ml ethanol, and 4 ml petroleum ether followed by vigorous agitation for 2 minutes. Samples were centrifuged for 3 minutes at 500 x g. Three ml aliquots were removed and placed in 12 x 100 mm spectrophotometer tubes. Absorbances of samples and carotene standards ranging in concentration from 0.00 to 2.50 µg/ml were determined on a spectrophotometer at 450 nm using petroleum ether as a blank. Then samples, carotene standards, and vitamin A standards ranging from 0.25 to 1.25 µg/ml were evaporated to dryness in a 60° water bath. Residues were dissolved in 0.3 ml chloroform followed by the addition of 0.3 ml acetic anhydride. Three ml of trifluoroacetic acid-chloroform chromagen was added and 5 seconds later the absorbances (A) were determined at 620 nm. A carotene correction factor obtained from the carotene standards treated as vitamin A was calculated to correct for the contribution of carotene to the vitamin A chromophore.

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[7]Hoffmann-LaRoche Company, Nutley, N.J.

[8]United States Pharmacopeial Convention, Inc. 34.4 mg all-trans retinyl acetate equivalent to 30.0 mg retinol per gram in cottonseed oil.

Calculations.

μg carotene/100 ml serum = A of sample$_{450}$ x C

Under conditions of this laboratory:

$$C = \frac{\text{conc of std}}{\text{A of std}} \times \text{dilution factor} = 1234$$

μg vitamin A/100 ml serum = (sample A_{620} - carotene A_{620}) x C

Under conditions of this laboratory:

$$C = \frac{\text{conc of vitamin A std}}{\text{A of vitamin A std}} \times \text{dilution factor} = 507, \text{ and}$$

carotene A_{620} = carotene A_{450} x carotene correction factor

Where:

$$\text{carotene correction factor} = \frac{\mu g \text{ carotene in std}}{\text{carotene } A_{620} \text{ in std}} = 0.198$$

V. STATISTICAL ANALYSES

Correlations between Measurements

Simple correlation coefficients were calculated between bone density and age, weight, and height at the initial test period.

Description of Canonical Correlation and Factor Analysis

Both canonical correlation and factor analysis are methods of multivariate analyses. Canonical correlation coefficients measure the simultaneous relationship between 2 groups of variables where variables within each group are correlated. Canonical variables, defined as the sums of products of each measurement within each group and its normalized characteristic vector, are produced by a canonical correlation analysis.

The first canonical correlation represents the maximum correlation between linear combinations of measurements within each group. The Chi-Square test for significance is utilized with canonical correlations.

By factor analysis, a large number of correlated variables are reduced to a few uncorrelated variables for the purpose of identifying those measurements which are most influential in summarizing variability and covariability of the measurements employed (98).

Multivariate Analyses

Canonical correlation analyses were performed on physical and dietary, physical and blood, and dietary and blood variables estimated in the initial phase of the study. All measurements for the 17 women who were receiving vitamin supplements were excluded from the canonical correlations. In the first analysis the physical measurements of bone density, age, weight, and height were related to dietary energy, protein, fat, calcium, phosphorus, iron, vitamin A, and ascorbic acid. The second analysis related the physical measurements to serum levels of ascorbic acid, hemoglobin, total protein, albumin, the A/G ratio, cholesterol, vitamin A, calcium, phosphorus, and alkaline phosphatase. A third analysis indicated the relationships between the dietary measurements and serum levels of selected blood constituents. The Statistical Analysis System (a computer software package) developed by Barr and Goodnight (99) was used for calculating the canonical correlations.

Tests for Significance of Differences.

With the rationale that each individual was her best control, a difference was calculated for each individual's measurements, for example, final test value minus initial test value. The paired t-test was used to evaluate whether the mean difference of a given group was significantly

different from zero. To test whether the mean differences between the final and initial tests of the 4 groups were significantly different from each other, a least squares analysis of variance and Duncan's New Multiple-Range test were employed (100).

CHAPTER IV

RESULTS

I. NUTRITIONAL STATUS

Physical Measurements

The mean bone density index of the 76 aged women was 0.819 ± 0.022 g equivalents of alloy per cc of bone (Table 4). The mean weight of the women was 65.4 ± 2.1 kg and the mean height was 156.3 ± 1.0 cm. The mean age was 68.0 ± 0.9 years.

Nutrient Intake

Nutrient intakes from food of the women were consistent with those recommended by the National Research Council (7). Mean intakes for the 8 nutrients evaluated are shown in Table 5. Energy intake per day was 1519 ± 42 kcal. Approximately 15 and 36% of the kcal were obtained from protein and fat respectively. The mean intake of protein was 58 ± 2 g and that of fat was 62 ± 2 g. The mean calcium intake was 797 ± 27 mg; that of phosphorus was 1001 ± 29 mg. The mean intake of iron was 8.6 ± 0.2 mg/day. Vitamin A and ascorbic acid consumed from the diet by the 59 women who were not receiving vitamin supplements showed daily means of 5831 ± 665 IU and 35 ± 2 mg, respectively. The 17 women for whom a vitamin supplement had been prescribed were receiving 25,000 IU and 200 mg of vitamin A and ascorbic acid respectively, from that source.

Blood Levels of Selected Constituents

Mean levels of blood constituents are shown in Table 6. Vitamin levels are exclusive of values for 17 persons whose diets were supplemented

TABLE 4

PHYSICAL MEASUREMENTS OF ELDERLY WOMEN AT THE INITIAL TEST PERIOD (n=76)

Measurement	Mean ± SE
Age (years)	68.0 ± 0.9
Height (cm)	156.3 ± 1.0
Weight (kg)	65.4 ± 2.1
Bone Density Index[1]	0.819 ± 0.022

[1]Gram equivalents of alloy per cc of bone.

TABLE 5

DAILY NUTRIENT INTAKE OF ELDERLY WOMEN AT THE INITIAL TEST PERIOD (n=76)

Nutrient	RDA[1]	Mean[2] ± SE
Energy (kcal)	1700	1519 ± 42
Protein (g)	55	58 ± 2
Fat (g)	--	62 ± 2
Calcium (mg)	800	797 ± 27
Phosphorus (mg)	800	1001 ± 29
Iron (mg)	10	8.6 ± 0.2
Vitamin A (IU)	5000	5831[3] ± 665
Ascorbic Acid (mg)	55	35.2[3] ± 1.8

[1]Recommended Dietary Allowance (7).

[2]Mean of 4 or 7 days.

[3]Mean is exclusive of 17 women who were receiving vitamin supplements.

TABLE 6

BLOOD LEVELS OF SELECTED CONSTITUENTS OF ELDERLY WOMEN AT THE INITIAL TEST PERIOD (n=76)

Constituent	Acceptable Values[1]	Mean ± SE
Blood Hemoglobin (g/100 ml)	12.0 - 16.0	13.93 ± 0.11
Plasma Ascorbic Acid (mg/100 ml)	0.3 - 1.4	0.34[2] ± 0.03
Serum Total Protein (g/100 ml)	6.0 - 8.0	6.77 ± 0.05
Serum Albumin (g/100 ml)	4.0 - 5.5	3.67 ± 0.03
Serum A/G Ratio (g/100 ml)	1.8 - 2.5	1.22 ± 0.03
Serum Cholesterol (mg/100 ml)	120 - 260	222.0 ± 4.4
Serum Calcium (mg/100 ml)	9.0 - 11.0	9.60 ± 0.08
Serum Phosphorus (mg/100 ml)	3.0 - 4.0	3.35 ± 0.09
Serum Vitamin A (µg/100 ml)	25 - 90	50.36[2] ± 2.06
Serum Alkaline Phosphatase (mg/100 ml)[3]		1.92 ± 0.08

[1]Normal laboratory values of clinical importance (101).

[2]Mean is exclusive of 17 women who were receiving vitamin supplement.

[3]Inorganic phosphorus liberated from glycerophosphate substrate in 1 hour at 37°.

with vitamins prior to their participation in the study. The mean level of serum ascorbic acid for women who were not receiving vitamin supplements was 0.34 ± 0.03 mg/100 ml. A mean serum level of 50.36 ± 2.06 µg vitamin A/100 ml was observed for the same women. When values for the 17 supplemented women were included the means of plasma ascorbic acid and serum vitamin A were elevated by 50 and 2% respectively. With a mean of 13.93 ± 0.11 g/100 ml, hemoglobin was within the range of normal for adults as was cholesterol with a mean of 222.0 ± 4.4 mg/100 ml (101). Serum total protein had a mean of 6.77 ± 0.42 g/100 ml; the mean albumin content was 3.67 ± 0.03 g/100 ml. The albumin to globulin (A/G) ratio was 1.22 ± 0.03. Serum levels of calcium and phosphorus had means of 9.60 ± 0.08 and 3.35 ± 0.09 mg/100 ml respectively. The activity of the enzyme, alkaline phosphatase, was estimated to be 1.92 ± 0.08 mg/100 ml.

Interrelations of Dietary Intake, Blood Levels of Selected Constituents, and Physical Measurements

A significant negative correlation between age and bone density ($r = 0.4$, $P < 0.01$) indicated that the bone density decreased with advancing age. Significant positive correlations were observed between bone density and height ($r = 0.5$, $P < 0.001$) and weight ($r = 0.5$, $P < 0.001$) as presented in Table 7.

Canonical correlation between nutrient intakes and physical measurements of the subjects who were not receiving vitamin supplements (Table 8) revealed that increased age of the women was accompanied by decreased body weight and a reduction in consumption of nutrients such as protein, calcium, phosphorus, and ascorbic acid ($r = 0.7$, $P < 0.0006$).

TABLE 7

CORRELATION OF BONE DENSITY[1] WITH PHYSICAL MEASUREMENTS AT THE INITIAL TEST PERIOD (n=76)

Relationship Tested	r value	P value
Age	-0.3655	<0.01
Height	0.4621	<0.001
Weight	0.5141	<0.001

[1]Gram equivalents of alloy per cc of bone.

TABLE 8

CANONICAL VARIABLE CORRELATIONS OF THE RELATIONSHIP OF PHYSICAL AND DIETARY MEASUREMENTS AT THE INITIAL TEST PERIOD (n=59)

	Canonical Variables[1]	
	1	2
Canonical correlations	0.7	0.6
Chi-square		
Degrees of freedom	32	21
Probability chi-square	0.0006	0.06
Correlation coefficients of the canonical variable with each variable of the group		
Physical		
Bone density	-0.47	0.62
Age	0.91	-0.18
Height	-0.43	0.30
Weight	-0.92	-0.13
Dietary		
Energy	-0.36	-0.04
Protein	-0.55	-0.04
Fat	-0.35	-0.12
Calcium	-0.41	-0.04
Phosphorus	-0.50	-0.06
Iron	-0.19	0.11
Vitamin A	-0.25	0.54
Ascorbic Acid	-0.57	0.44

[1]Canonical variables are the sums of the products of the criterion variables and the normalized characteristic vector.

An additional significant correlation ($r = 0.6$, $P < 0.07$) independent of the first canonical variable indicated a relationship between decreases in bone density and height and reduction in intakes of vitamin A and ascorbic acid. The canonical correlation presented in Table 9 revealed a relationship ($r = 0.7$, $P < 0.007$) between weight and serum levels of ascorbic acid, hemoglobin, alkaline phosphatase, and phosphorus; however a subsequent factor analysis showed that at best only 20% of the correlative effects could be attributed to levels of serum constituents. Another significant canonical correlation ($r = 0.8$, $P < 0.05$) was shown between nutrient intakes and serum levels of selected nutrients (Table 10) attributable primarily to a relationship of dietary vitamin A ($r = 0.9$ in the dietary canonical variable) to serum ascorbic acid ($r = 0.9$ in the blood variable).

Metabolic Balances and Urinary Constituents

Utilization of nitrogen, calcium, and phosphorus by the 16 women who participated in the metabolic studies is shown in Table 11. A mean apparent nitrogen balance of 2.30 g per day was estimated in the initial phase of this study. The nitrogen which was consumed in the diet was analyzed to be 9.00 g daily. Of that intake, mean values of 6.04 and 0.67 were recovered from the urinary and fecal excretions respectively. Nitrogen intake was positively correlated with nitrogen balance at $r = 0.7$, $P < 0.001$. A negative apparent calcium balance of -52 mg per day was registered and fecal calcium accounted for more than three-fourths of the calcium consumed; however, a significant positive correlation ($r = 0.6$, $P < 0.01$) between intake and balance of calcium was observed. A positive

TABLE 9

CANONICAL VARIABLE CORRELATIONS OF THE RELATIONSHIP OF PHYSICAL AND BLOOD MEASUREMENTS AT THE INITIAL TEST PERIOD (n=59)

	Canonical Variable[1] 1
Canonical correlation	0.7
Chi-square	
Degrees of freedom	40
Probability chi-square	0.007
Correlation coefficients of the canonical variable with each variable of the group	
Physical	
Bone density	0.28
Age	-0.08
Height	-0.12
Weight	0.67
Blood	
Ascorbic Acid	-0.39
Hemoglobin	0.56
Total Protein	0.21
Albumin	0.06
A/G Ratio	-0.15
Cholesterol	-0.14
Calcium	-0.08
Phosphorus	-0.56
Vitamin A	0.09
Alkaline Phosphatase	0.44

[1]Canonical variables are the sums of the products of the criterion variables and the normalized characteristic vector.

TABLE 10

CANONICAL VARIABLE CORRELATIONS OF THE RELATIONSHIP OF DIETARY AND BLOOD MEASUREMENTS AT THE INITIAL TEST PERIOD (n=59)

	Canonical Variable[1] 1
Canonical correlation	0.8
Chi-square	
Degrees of freedom	80
Probability chi-square	0.05
Correlation coefficients of the canonical variable with each variable of the group	
Dietary	
Energy	0.01
Protein	0.08
Fat	0.11
Calcium	-0.17
Phosphorus	-0.04
Iron	0.22
Vitamin A	0.87
Ascorbic Acid	0.29
Blood	
Ascorbic Acid	0.92
Hemoglobin	0.03
Total Protein	-0.07
Albumin	0.18
A/G Ratio	0.17
Cholesterol	-0.07
Calcium	-0.10
Phosphorus	-0.19
Vitamin A	-0.03
Alkaline Phosphatase	-0.09

[1]Canonical variables are the sums of the products of the criterion variables and the normalized characteristic vector.

TABLE 11

METABOLIC MEASUREMENTS AT THE INITIAL TEST PERIOD FOR PARTICIPANTS IN THE BALANCE STUDIES (n=16)

Constituent	Mean[1] + SE
Nitrogen (g/day)	
Intake	9.00 ± 0.66
Urinary	6.03 ± 0.47
Fecal	0.67 ± 0.08
Balance	2.30 ± 0.27
Calcium (mg/day)	
Intake	770 ± 69
Urinary	139 ± 27
Fecal	683 ± 76
Balance	-52 ± 85
Phosphorus (mg/day)	
Intake	1038 ± 69
Urinary	417 ± 28
Fecal	363 ± 31
Balance	258 ± 52
Hydroxyproline, Urinary (μg/24 hours)	24.06 ± 1.72
Creatinine, Urinary (mg/24 hours)	607 ± 46

[1]Mean of 7 days.

apparent phosphorus balance of 258 mg per day was obtained. A highly significant correlation between intake and balance of phosphorus was observed ($P < 0.001$). Urinary creatinine was found to have a mean daily value of 607 ± 47 mg. The mean daily urinary excretion of hydroxyproline was 24.06 ± 1.72 μg. The interrelations of the metabolic balances with bone density and hydroxyproline excretion of these subjects have been described and discussed in detail in a thesis by MacDuff (102).

II. EFFECTS OF DIETARY SUPPLEMENTATION

Although groups were matched for bone density, age, and weight at the onset of supplementation, re-evaluation of the initial bone densities after deletion of values for those who did not complete the study showed that withdrawals from the study had impaired the initial matching. To compensate for the unmatched groups, final minus intermediate bone density measurements were regressed on initial measurements and this test of homogeniety indicated that changes in bone density in response to supplements was not affected by the levels of the initial measurements. Response to dietary supplements was therefore evaluated by a least squares analysis of variance of the difference between the initial and final measurements. Changes in each group between the initial and final measurements were tested by the paired t-test. Significant alterations in nutrient intake, blood constituents, and physical measurements between groups were subsequently determined using the Duncan's New Multiple-Range Test (100).

Bone Density and Physical Measurements

Supplementation with sodium fluoride (Group 3) resulted in a significant ($P < 0.01$) increase between the initial and final bone density indices for that group (Table 12). There were no significant changes in bone density measurements of the other 3 groups. The multiple-range test showed that the increased bone density of Group 3 was significantly greater than that of Groups 1 and 2 (Table 13); the same testing indicated that fluoride alone was more effective in increasing bone mass than was fluoride given in combination with vitamins and calcium phosphate. There had been no significant alteration of bone density at the intermediate test period. No significant correlations were found between the changes in bone density and changes in nutrient intake or levels of blood constituents evaluated during the experimental period. Neither height nor weight changed significantly during the course of this study.

Nutrient Intake

A decrease in calcium intake from food between the initial and final test periods registered for Group 3 was significant ($P < 0.05$) as indicated in Table 14. Calcium and phosphorus intakes increased, as was expected, in Groups 2 and 4 by the addition of calcium phosphate supplements to the diets ($P < 0.001$). The multiple-range test as shown in Table 15 indicated that, although the increased intakes of Groups 2 and 4 were significantly greater than those of the other groups, the decreased intake of calcium by Group 3 was not significantly different from that of Group 1.

TABLE 12

PHYSICAL MEASUREMENTS AT THE INITIAL AND FINAL TEST PERIODS BY GROUP

	Group and Supplement			
	1	2	3	4
	None (n=17)	Ca,P (n=13)	F (n=18)	F,Ca,P, Vitamins (n=12)
Bone Density Index[1]				
Initial	0.807[2] ±0.044	0.824 ±0.041	0.820 ±0.056	0.808 ±0.053
Intermediate	0.813 ±0.044	0.841 ±0.044	0.832 ±0.046	0.822 ±0.048
Final	0.823 ±0.040	0.842 ±0.046	0.906 ±0.053	0.842 ±0.056
Difference[3]	0.016	0.019	0.086[4]	0.033
Height (cm)				
Initial	153.6 ±1.4	154.4 ±1.2	158.5 ±1.8	154.5 ±2.6
Final	153.9 ±1.4	153.5 ±1.4	157.6 ±1.9	153.5 ±2.4
Difference	0.3	-0.9	-1.0	-1.0
Weight (kg)				
Initial	65.7 ±4.5	59.1 ±3.5	67.5 ±6.1	64.1 ±3.4
Final	68.3 ±4.8	58.6 ±4.1	66.4 ±6.0	61.8 ±3.0
Difference	2.6	-0.4	-1.1	-2.4

[1]Gram equivalents of alloy per cc of bone.

[2]Mean ± SE.

[3]Mean of individual differences; final minus initial measurement.

[4]Significantly different from zero by paired t-test at $P<0.01$.

TABLE 13

DIFFERENCES IN PHYSICAL MEASUREMENTS AND THEIR SIGNIFICANCE AMONG GROUPS[1,2,3]

	Group and Supplement			
	1	2	3	4
	None (n=17)	Ca,P (n=13)	F (n=18)	F,Ca,P, Vitamins (n=12)
Bone Density Index[4]	0.016[b] ±0.020	0.019[b] ±0.024	0.086[a] ±0.020	0.033[ab] ±0.024
Height (cm)	0.28 ±0.16	-0.93 ±0.71	-0.96 ±0.45	-0.96 ±0.53
Weight (kg)	2.65 ±1.88	-0.45 ±1.52	-1.11 ±1.33	-2.37 ±1.84

[1]Difference equals final minus initial measurement by subject.

[2]Values expressed as mean ± SE.

[3]Means in a row without a common superscript are significantly different from each other at $P<0.05$.

[4]Gram equivalents of alloy per cc of bone.

TABLE 14

NUTRIENT INTAKES AT THE INITIAL AND FINAL TEST PERIODS BY GROUP

	Group and Supplement			
	1	2	3	4
	None (n=17)	Ca,P (n=13)	F (n=18)	F,Ca,P, Vitamins (n=12)
Energy (kcal)				
Initial	1462[1] ±119	1527 ±74	1677 ±84	1563 ±58
Final	1428 ±71	1608 ±95	1562 ±73	1486 ±80
Difference[2]	-34	81	-115	-76
Protein (g)				
Initial	57 ±5	57 ±2	63 ±4	62 ±2
Final	54 ±4	56 ±3	62 ±4	59 ±4
Difference	-3	-2	-1	-3
Fat (g)				
Initial	58 ±4	65 ±3	67 ±3	67 ±2
Final	58 ±3	67 ±4	66 ±4	66 ±4
Difference	0	2	-1	-1
Calcium (mg)				
Initial	713 ±50	819 ±52	874 ±59	851 ±43
Final	712 ±34	1241 ±43	761 ±62	1207 ±51
Difference	0	422[3]	-113[4]	356[3]

TABLE 14 (continued)

	Group and Supplement			
	1	2	3	4
	None (n=17)	Ca,P (n=13)	F (n=18)	F,Ca,P Vitamins (n=12)
Phosphorus (mg)				
Initial	947 ±68	1018 ±47	1090 ±64	1054 ±38
Final	951 ±48	1367 ±53	1048 ±65	1349 ±60
Difference	4	348[3]	-42	295[3]
Iron (mg)				
Initial	8.5 ±0.8	8.8 ±0.5	9.3 ±0.6	9.1 ±0.4
Final	8.7 ±0.7	8.8 ±0.6	9.5 ±0.5	9.1 ±0.6
Difference	0.2	0	0.2	0
Vitamin A (IU)				
Initial	5045 ±544	29795 ±469	6215 ±1436	5251 ±482
Final	7844 ±1593	30620 ±595	5712 ±1172	31473 ±1043
Difference	2799	565	-504	26222[5]
Ascorbic Acid (mg)				
Initial	32.3 ±2.8	231.9 ±3.0	38.4 ±4.2	37.5 ±2.5
Final	38.5 ±5.2	241.5 ±3.9	36.4 ±3.2	236.0 ±4.7
Difference	6.2	9.8[3]	-1.9	198.5[5]

[1]Mean ± SE.

[2]Mean of individual differences: final minus initial measurement.

[3]Significantly different from zero by paired t-test at $P<0.01$.

[4]Significantly different from zero by paired t-test at $P<0.05$.

[5]Significantly different from zero by paired t-test at $P<0.001$.

TABLE 15

DIFFERENCES IN NUTRIENT INTAKES AND THEIR SIGNIFICANCE AMONG GROUPS[1,2,3]

	Group and Supplement			
	1	2	3	4
	None (n=17)	Ca,P (n=13)	F (n=18)	F,Ca,P Vitamins (n=12)
Nutrient				
Energy (kcal)	-34.11 ±93.48	81.38 ±106.98	-114.79 ±98.82	-76.08 ±111.27
Protein (g)	-2.63 ±3.48	-1.56 ±3.98	-0.72 ±3.38	-3.42 ±4.14
Fat (g)	0.36 ±3.96	1.51 ±4.53	-1.40 ±3.84	-1.22 ±4.71
Calcium (mg)	-0.06[b] ±49.40	422.46[a] ±56.53	-112.62[b] ±47.99	355.66[a] ±58.80
Phosphorus (mg)	4.24[b] ±58.98	348.46[a] ±67.50	-42.17[b] ±57.30	295.24[a] ±70.21
Iron (mg)	0.19 ±0.63	0.04 ±0.72	0.17 ±0.61	-0.02 ±0.75
Vitamin A (IU)	2799[b] ±1369	565[b] ±1630	-504[b] ±1330	26221[a] ±1630
Ascorbic Acid (mg)	6.18[b] ±4.03	9.88[b] ±4.80	-1.92[b] ±3.91	198.48[a] ±4.80

[1]Difference equals final minus initial measurement by subject.

[2]Values expressed as mean ± SE.

[3]Means in a row without a common superscript are significantly different from each other at $P<0.05$.

Increases in the mean intakes of vitamin A and ascorbic acid in subjects in Group 4 were significant ($P < 0.001$) as a result of the vitamin supplement which each member of this group received. Since Group 2 was receiving the vitamin supplement prior to the study and that supplement was maintained throughout the study, there were relatively small changes in intakes of vitamin A and ascorbic acid by that group. Dietary intakes of energy, protein, fat, and iron were not significantly altered during the course of this study in any of the groups.

Blood Constituents

Plasma levels of ascorbic acid were increased in Groups 1, 3, and 4 between the initial and final test periods (Table 16); the paired t-test showed that the increases were significant. The increase in Group 1 was significant at $P < 0.01$ while that of Group 3 was significant at $P < 0.05$, and that of Group 4 was significant at $P < 0.001$. Those in Group 2 who were receiving vitamin supplements at the initial test period had a plasma level of ascorbic acid at 1.15 ± 0.17 mg/100 ml and it did not change appreciably during the study. The multiple range test revealed that the increase in plasma level of Group 4 was significantly different from those in the other groups. Group 2 showed a slight decrease in plasma ascorbic acid and the change was significantly different from those of Groups 1 and 3 (Table 17).

Group 2 showed a significant increase in serum total protein ($P < 0.01$) and albumin ($P < 0.05$)without significant alteration of the A/G Ratio (Table 16). Serum albumin increased significantly in Group 1 ($P < 0.05$) and Group 3 ($P < 0.01$). The elevation of serum albumin altered the A/G Ratio significantly ($P < 0.01$) in Group 3 and the ratio

TABLE 16

BLOOD VALUES AT THE INITIAL AND FINAL TEST PERIODS BY GROUP

	Group and Supplement			
	1	2	3	4
	None (n=17)	Ca,P (n=13)	F (n=18)	F,Ca,P Vitamins (n=12)
Plasma Ascorbic Acid (mg/100 ml)				
Initial	0.29[1] ±0.04	1.15 ±0.17	0.38 ±0.08	0.29 ±0.03
Final	0.61 ±0.08	1.10 ±0.14	0.73 ±0.11	1.34 ±0.04
Difference[2]	0.32[3]	-0.08	0.35[4]	1.05[5]
Blood Hemoglobin (g/100 ml)				
Initial	14.10 ±0.19	13.20 ±0.31	14.03 ±0.24	14.15 ±0.35
Final	14.26 ±0.22	13.60 ±0.51	14.59 ±0.27	14.36 ±0.39
Difference	0.16	0.40	0.56	0.21
Serum Total Protein (g/100 ml)				
Initial	6.79 ±0.11	6.74 ±0.11	6.78 ±0.09	6.98 ±0.10
Final	6.93 ±0.10	7.06 ±0.12	6.85 ±0.10	6.80 ±0.08
Difference	0.14	0.32[3]	0.07	-0.16
Serum Albumin (g/100 ml)				
Initial	3.69 ±0.06	3.58 ±0.07	3.65 ±0.07	3.74 ±0.06
Final	3.85 ±0.04	3.78 ±0.08	3.90 ±0.06	3.88 ±0.08
Difference	0.16[4]	0.19[4]	0.26[3]	0.14

TABLE 16 (continued)

	Group and Supplement			
	1	2	3	4
	None (n=17)	Ca,P (n=13)	F (n=18)	F,Ca,P Vitamins (n=12)
Serum A/G Ratio (g/100 ml)				
Initial	1.21 ±0.05	1.21 ±0.08	1.19 ±0.06	1.18 ±0.06
Final	1.28 ±0.05	1.18 ±0.07	1.36 ±0.06	1.33 ±0.08
Difference	0.06	-0.02	0.16[3]	0.15[4]
Serum Cholesterol (mg/100 ml)				
Initial	214.1 ±9.6	234.9 ±11.7	221.6 ±10.5	224.6 ±8.2
Final	217.9 ±13.3	246.8 ±15.4	219.0 ±10.7	228.2 ±12.1
Difference	3.9	11.9	-2.6	3.7
Serum Calcium (mg/100 ml)				
Initial	9.49 ±0.13	9.83 ±0.21	9.68 ±0.10	10.19 ±0.16
Final	9.51 ±0.09	9.75 ±0.14	9.60 ±0.11	9.38 ±0.13
Difference	0.01	-0.08	-0.09	-0.81[5]
Serum Phosphorus (mg/100 ml)				
Initial	3.25 ±0.11	3.75 ±0.44	3.34 ±0.09	3.49 ±0.10
Final	3.13 ±0.09	3.08 ±0.15	3.03 ±0.09	2.86 ±0.11
Difference	-0.12	-0.67	-0.31[4]	-0.63[3]

TABLE 16 (continued)

	Group and Supplement			
	1 None (n=17)	2 Ca,P (n=13)	3 F (n=18)	4 F,Ca,P, Vitamins (n=12)
Serum Vitamin A (µg/100 ml)				
Initial	51.90 ±4.69	52.98 ±4.68	46.47 ±3.55	51.82 ±3.56
Final	51.00 ±2.28	60.53 ±7.92	49.59 ±2.74	63.58 ±3.32
Difference	-0.93	7.78	3.12	11.75[3]
Serum Alkaline Phosphatase (mg/100 ml)[6]				
Initial	1.79 ±0.14	1.74 ±0.16	2.04 ±0.19	2.18 ±0.31
Final	2.48 ±0.23	2.88 ±0.39	3.55 ±0.24	3.33 ±0.38
Difference	0.70[5]	1.14[3]	1.51[5]	1.14[4]

[1]Mean ± SE.

[2]Mean of individual differences: final minus initial measurement.

[3]Significantly different from zero by paired t-test at $P<0.01$.

[4]Significantly different from zero by paired t-test at $P<0.05$.

[5]Significantly different from zero by paired t-test at $P<0.001$.

[6]Inorganic phosphorus liberated from glycerophosphate substrate in 1 hour at 37°.

TABLE 17

DIFFERENCES IN BLOOD LEVELS OF SELECTED CONSTITUENTS AND THEIR SIGNIFICANCE AMONG GROUPS[1,2,3]

	Group and Supplement			
	1	2	3	4
	None (n=17)	Ca,P (n=13)	F (n=18)	F,Ca,P, Vitamins (n=12)
Constituent				
Plasma Ascorbic Acid (mg/100 ml)	0.32[b] ±0.12	-0.08[c] ±0.14	0.35[b] ±0.12	1.05[a] ±0.14
Blood Hemoglobin (g/100 ml)	0.16 ±0.29	0.40 ±0.34	0.56 ±0.29	0.21 ±0.35
Serum Total Protein (g/100 ml)	0.14[ab] ±0.09	0.32[a] ±0.10	0.07[ab] ±0.09	-0.17[b] ±0.11
Serum Albumin (g/100 ml)	0.16 ±0.07	0.19 ±0.08	0.26 ±0.06	0.14 ±0.08
Serum A/G Ratio (g/100 ml)	0.06 ±0.05	-0.02 ±0.06	0.16 ±0.05	0.15 ±0.06
Serum Cholesterol (mg/100 ml)	3.88 ±8.38	11.92 ±9.58	-2.61 ±8.14	3.66 ±9.97
Serum Calcium (mg/100 ml)	0.01[b] ±0.15	-0.08[b] ±0.17	-0.09[b] ±0.15	-0.80[a] ±0.18
Serum Phosphorus (mg/100 ml)	-0.12 ±0.19	-0.67 ±0.22	-0.31 ±0.19	-0.63 ±0.23
Serum Vitamin A (μg/100 ml)	-0.94 ±4.63	7.78 ±5.51	3.12 ±4.50	11.77 ±5.51
Serum Alkaline Phosphatase (mg/100 ml)[4]	0.70 ±0.26	1.14 ±0.29	1.51 ±0.25	1.14 ±0.31

[1]Difference equals final minus initial measurement by subject.

[2]Values expressed as mean ± SE.

[3]Means in a row without a common superscript are significantly different from each other at $P<0.05$.

[4]Inorganic phosphorus released from glycerophosphate substrate in 1 hour at 37°.

was also increased significantly in Group 4. The multiple-range test revealed a significant difference between the increase in serum level of total protein experienced by Group 2 and the decrease registered by Group 4. Neither of the changes by Groups 2 or 4 was significantly different from Group 1 which received no supplement or from Group 3 which received fluoride (Table 17).

Serum calcium ($P < 0.001$) and phosphorus ($P < 0.01$) decreased significantly between the initial and final tests in Group 4 as indicated in Table 16. Group 3 also showed a decrease in serum phosphorus during the same period ($P < 0.05$). The decrease in serum calcium in Group 4 was significantly greater ($P < 0.05$) than changes by any of the other groups (Table 17). The changes in serum phosphorus of the 4 groups were not significantly different from each other.

Group 4 experienced a significant ($P < 0.01$) increase in serum vitamin A during the supplemental period, but the multiple-range test revealed that this change was not significantly different from those of the other groups.

Alkaline phosphatase activity increased in all groups: in Group 1 ($P < 0.001$), in Group 2 ($P < 0.01$), in Group 3 ($P < 0.001$), and in Group 4 ($P < 0.05$). The multiple-range test (Table 17) showed that the increases by the groups were not significantly different from each other, however. Neither blood hemoglobin nor serum cholesterol changed significantly between the initial and final tests for any of the groups.

Metabolic Balances and Urinary Constituents

Of the 16 women who were evaluated metabolically at the initial test period, only 12 were able to participate in the balance phase of

the study at all 3 evaluation periods. However, 3 of the 4 women with incomplete balance data were present at 2 of the test periods. These 3 women had all been assigned to the unsupplemented diet (Group 1).

The mean utilization of nitrogen is shown by group in Table 18 and by individual subject in Table 22 in Appendix C. All participants in the metabolic studies were in positive apparent nitrogen balance at the initial and final test periods. However, nitrogen intake tended to decrease during the study with both intake and balances being lowest at the intermediate test period.

Utilization of calcium is shown by group in Table 19 and by individual participant in Table 23 in Appendix C. Calcium intakes decreased slightly at the final test period in Groups 1 and 3 which did not receive calcium phosphate supplements. The calcium intakes of those in Groups 2 and 4 were higher at the intermediate and final test periods than were intakes of those in the groups where calcium supplements were not provided. Large quantities of calcium were recovered from the feces of those in Group 2. Mean urinary calcium decreased in all groups when final measurements were compared to initial values. A significant negative correlation ($P < 0.0001$) was found between fecal calcium and calcium balance on an overall basis and within each group.

Apparent phosphorus balances were decreased during the course of this study in Groups 1, 2, and 3. Group 4 which received fluoride in addition to calcium phosphate showed an increase in its mean apparent phosphorus balance as indicated in Table 20. All groups were in positive balance at the initial test period, but Group 2 was in negative balance at the final period although each participant in that group received 360

TABLE 18

NITROGEN UTILIZATION AT THE THREE TEST PERIODS BY GROUP

	Group and Supplement			
	1	2	3	4
	None (n=4)[1]	Ca,P (n=3)	F (n=4)	F,Ca,P Vitamins (n=4)
Intake (g/day)				
Initial	7.23[2] ±0.96	8.83 ±0.72	10.11 ±2.55	9.52 ±0.95
Intermediate	6.16 ±1.75	5.99 ±0.77	9.19 ±2.25	7.55 ±1.20
Final	5.38 ±0.55	7.85 ±0.74	9.75 ±1.59	7.70 ±0.76
Urinary (g/day)				
Initial	4.98 ±0.98	5.65 ±0.52	7.18 ±1.36	5.94 ±0.71
Intermediate	3.91 ±1.27	4.49 ±0.50	6.59 ±1.49	6.62 ±2.40
Final	3.78 ±0.47	5.49 ±0.42	6.86 ±0.76	4.68 ±0.81
Fecal (g/day)				
Initial	0.44 ±0.09	0.69 ±0.39	0.76 ±0.10	0.84 ±0.09
Intermediate	0.41 ±0.17	0.70 ±0.16	1.28 ±0.53	1.02 ±0.12
Final	0.35 ±0.07	0.89 ±0.20	1.04 ±0.26	0.70 ±0.11
Balance (g/day)	1.81 ±0.38	2.50 ±0.22	2.18 ±0.98	2.73 ±0.41
Intermediate	1.85 ±0.62	0.80 ±0.49	1.32 ±0.43	-0.10 ±1.41
Final	1.25 ±0.02	1.48 ±0.17	1.85 ±0.78	2.33 ±0.45

[1]For Group 1 n equals 3 and 2 for the intermediate and final test periods, respectively.

[2]Mean ± SE.

TABLE 19

CALCIUM UTILIZATION AT THE THREE TEST PERIODS BY GROUP

	Group and Supplement			
	1	2	3	4
	None (n=4)[1]	Ca,P (n=3)	F (n=4)	F,Ca,P Vitamins (n=4)
Intake (mg/day)				
Initial	617[2] ±37	757 ±102	885 ±265	786 ±66
Intermediate	485 ±3	951 ±48	770 ±236	1037 ±82
Final	596 ±46	1124 ±64	850 ±195	1106 ±76
Urinary (mg/day)				
Initial	117 ±32	144 ±61	99 ±17	116 ±37
Intermediate	42 ±16	61 ±7	65 ±12	80 ±24
Final	48 ±22	69 ±33	70 ±24	60 ±23
Fecal (mg/day)				
Initial	677 ±61	424 ±220	715 ±81	896 ±216
Intermediate	390 ±120	811 ±282	1095 ±419	859 ±77
Final	589 ±29	1582 ±293	960 ±180	708 ±79
Balance (mg/day)				
Initial	-177 ±75	188 ±251	71 ±210	-226 ±171
Intermediate	53 ±108	78 ±321	-390 ±195	98 ±102
Final	-40 ±96	-528 ±293	-179 ±74	338 ±131

[1]For Group 1 n equals 3 and 2 for the intermediate and final test periods, respectively.

[2]Mean ± SE.

TABLE 20

PHOSPHORUS UTILIZATION AT THE THREE TEST PERIODS BY GROUP

	Group and Supplement			
	1	2	3	4
	None (n=4)[1]	Ca,P (n=3)	F (n=4)	F,Ca,P Vitamins (n=4)
Intake (mg/day)				
Initial	880[2]	964	1165	1102
	±41	±68	±266	±63
Intermediate	711	1070	1035	1203
	±123	±90	±264	±125
Final	719	1280	1145	1238
	±63	±102	±190	±93
Urinary (mg/day)				
Initial	323	443	485	396
	±23	±100	±50	±43
Intermediate	242	418	590	445
	±55	±48	±123	±42
Final	272	501	511	448
	±10	±111	±26	±117
Fecal (mg/day)				
Initial	374	262	390	425
	±63	±117	±41	±43
Intermediate	227	528	638	509
	±56	±187	±264	±34
Final	329	1031	622	443
	±14	±189	±130	±69
Balance (mg/day)				
Initial	182	260	290	281
	±50	±92	±201	±71
Intermediate	242	124	-193	250
	±127	±440	±142	±86
Final	119	-251	12	347
	±87	±176	±54	±132

[1]For Group 1 n equals 3 and 2 for the intermediate and final test periods, respectively.

[2]Mean ± SE.

mg of phosphorus in supplemental form. Individual utilization of phosphorus is shown in Table 24 in Appendix C.

Urinary constituents evaluated as part of the metabolic studies are shown in Table 21 by group and in Table 25 in Appendix C by individual participant. Mean excretion of hydroxyproline decreased by 36% in Group 1 during the study but remained stable in the other 3 groups.

Maximum variation in the mean excretion of creatinine was an increase of 15% in Group 2. Creatinine excretion increased by 6% in Group 3 and was unchanged in Groups 1 and 4.

TABLE 21

URINARY CONSTITUENTS AT THE THREE TEST PERIODS BY GROUP

	Group and Supplement			
	1	2	3	4
	None (n=4)[1]	Ca,P (n=3)	F (n=4)	F,Ca,P Vitamins (n=4)
Hydroxyproline (µg/24 hours)				
Initial	23.4[2] ±4.7	25.0 ±3.8	23.1 ±3.0	22.1 ±2.8
Intermediate	21.8 ±8.4	24.1 ±1.8	24.8 ±4.3	26.3 ±9.3
Final	14.8 ±0.2	26.5 ±3.3	23.4 ±4.3	21.4 ±5.4
Creatinine (mg/24 hours)				
Initial	537 ±109	583 ±23	646 ±92	545 ±64
Intermediate	594 ±147	703 ±50	727 ±98	634 ±115
Final	519 ±148	670 ±37	688 ±24	550 ±97

[1]For Group 1 n equals 3 and 2 for the intermediate and final test periods, respectively.

[2]Mean ± SE.

CHAPTER V

DISCUSSION

I. NUTRITIONAL STATUS PRIOR TO DIETARY SUPPLEMENTATION

Bone Density

Previous studies conducted by the Department of Nutrition in the College of Home Economics at the University of Tennessee, Knoxville, utilizing the bone densitometer described by Williams and Mason (103) and involving females aged 3 to 90 years, showed that bone density indices ranged from 0.72 to 1.21. Odland et al. (30) reported that from a survey of 296 females aged 3 to 90 years, bone density indices increased from a mean index of 0.72 for preschool girls aged 3 to 5 to a mean index of 1.10 for adolescents aged 16 to 20. Bone density indices continued to increase throughout the early adult years and the greatest index, 1.21, was exhibited by women aged 41 to 50 years. Thereafter the bone density index decreased to 0.74 for women 80 to 90 years of age.

A comparison of bone density indices obtained in the present study with those from other laboratories is complicated by the variation in standards and units of measurement, the particular bone of the skeleton selected for evaluation, methods of compensation for flesh, and lack of established norms for quantities of bone at various age levels (104). Although absolute values cannot be compared with those from different laboraties, bone density trends may be observed.

Decreases in bone density with advancing age as observed in this study have also been reported by Morgan et al. (29). Additional evidence that adult women lose bone as they age has been provided by Arnold et al. (105) from their analysis of bone for ash.

In addition to reduction in bone density with age, age-associated decrements in height and weight were observed in this study in agreement with the findings of others (43,106,107).

Nutrient Intakes and Blood Constituents

A unique feature of this study was that nutrients consumed were calculated from weighed food intakes. Most large-scale nutrition studies have derived nutrient intakes from interviews and questionnaires such as those used in the Ten-State Nutrition Survey (14), from diet histories as employed by Odland et al. (3), or from food records as utilized by Fry et al. (10) and Bransby and Osborne (108).

Variations in the activities which participants in the various dietary studies enjoy, their association with other people, the geographical location where they make their homes, and the state of their general health make comparisons between studies difficult and, on occasion, confusing.

Age related decrements in consumption of protein, calcium, phosphorus, and ascorbic acid were observed in this study; however, mean nutrient intakes of protein, calcium, and phosphorus compared favorably with the recommended allowances (7). Food consumed provided nutrient levels similar to those of women evaluated in other studies (4,10,14,108).

Food choices influenced consumption of iron, calcium, vitamin A, and ascorbic acid as indicated by the lack of correlation between

consumption of these nutrients and that of other nutrients obtained from food. The consumption of vitamin A, however, was related to that of ascorbic acid. Food preferences did not appear to alter the consumption of kilocalories, protein, fat, and phosphorus; regardless of the foods an individual chose to eat, these nutrients were included in her daily food intake as indicated by their canonical correlations.

Mean quantities of nutrients consumed by women in this study were neither as high nor as low as other levels which have been reported (3,5,14,112). Intakes of kilocalories, iron, and ascorbic acid were below those quantities recommended by the National Research Council (7). However, a glance at the mean weight, which was 7 kg higher than the mean weight established by National Research Council for adult women, as well as higher than means reported by Master et al. (107), Gillum et al. (109), Durnin (110), and the Ten-State Nutrition Survey (18) suggests that the reduced intake of kilocalories was beneficial to these ambulatory but rather sedentary women.

The recommended allowance (7) for iron from the food consumed is 10 mg per day and intakes of 10 mg have been reported by several who have studied dietary patterns of aged persons (8,10,13,108,109,110). The mean consumption of iron by women in this study at 8.6 mg daily appears to be low; however, mean hemoglobin levels of 13.9 g/100 ml were within the range of normal (101). In addition, hemoglobin levels lower than 13.9 g have been reported by others. Gillum et al. (109) found a mean of 13.4 g/100 ml for 296 women aged 50 to 80; Brewer et al. (17) reported a mean of 13.5 for 52 hospitalized women aged 44 to 98. A slightly lower mean of 13.3 g/100 ml was obtained for

the 2185 women older than 59 years whose nutritional status was assessed in the Ten-State Nutrition Survey (18).

Dietary intake of ascorbic acid was lower than the recommended allowances although mean plasma levels were within the lower limits of normal. The mean value of 35 mg per day consumed by women on this study who were not receiving vitamin supplements came within 1 mg of meeting two-thirds of the recommended allowance of 55 mg (7). The adequacy of a nutrient at this level has been discussed by Whitfield (111). Ascorbic acid intake by women over 60 years of age has been evaluated in several studies. Hospitalized patients were found to consume about 30 mg ascorbic acid per day (4,112). In the Ten-State Nutrition Survey (14), a mean intake of 67 mg per day was observed while Morgan et al. (113) reported a mean of 86 mg per day which was more than twice as high as the mean intake of ascorbic acid in this study. Odland et al. (3) found a mean intake of 122 mg per day. While still others (6,8,10,13) have reported intakes higher than that observed in this study, it is of interest to observe that Bransby and Osborne (108) reported a mean daily intake of only 21 mg for 178 healthy women aged 60 to 80. A normal range of plasma ascorbic acid is 0.3 to 1.5 mg/100 ml; the mean level for women participating in this study exclusive of those on vitamin supplements was 0.34 mg/100 ml. When women who were receiving vitamin supplements were included, the mean plasma level increased to 0.52 mg/100 ml. Either mean is somewhat low when compared to the desirable range; they are also low when compared to means presented from studies of other aged populations. Only Barrows and Beauchene (114) reported

a mean of 0.43 mg/100 ml which was intermediate between our supplemented and unsupplemented means. A mean level of 0.69 mg/100 ml was reported for 47 hospitalized women aged 44 to 98 (17); Brin et al. (16) found a mean level of 84 mg/100 ml for 103 women of approximately 73 years. Morgan et al. (113) have determined a mean of 1.07 mg/100 ml for 293 women aged 50 to 75; however, the range was 0.07 to 2.53 mg/100 ml. In the Ten-State Nutritional Survey (18), serum ascorbic acid fluctuated with the income index; a mean of 0.72 mg/100 ml for 326 women with a low income index was obtained in contrast to a mean of 1.08 mg for 858 women with a high income index. All women were older than 59 years.

Dietary protein, calcium, phosphorus, and vitamin A consumed by the women met the recommended allowance for women past 55 years of age (7). Serum levels of the corresponding nutrients were also within the ranges of normal (101). It should be recognized, however, that in this study dietary intake was a poor predictor of serum levels of the constituents observed. Serum levels were, in turn, poor predictors of physical measurements. The level of a nutrient in serum represents the balance between its entrance into and removal from the circulatory system (115). Correlation between dietary nutrients and serum constituents would be anticipated only when dietary intakes were low for lengthy periods of time.

The enzyme, alkaline phosphatase, is associated with liberation of phosphorus from organic complexes. The units by which the activity of the enzyme is estimated are varied and include units devised by King and Armstrong, King-Armstrong units, or by Bodansky, Bodansky units

(116). Other units of measurement include IU, units/liter, and mg of phosphorus released per 100 ml serum. When units from other studies were extrapolated to mg of phosphorus released per 100 mg serum, the activity of the enzyme as measured in this study appeared to be low. While the activity may be less than that evaluated by other techniques, reduced activity appears to be of little concern. A low level of enzyme activity recorded for the women suggests that prior to supplementation limited remodeling of bone was occurring. The enzyme becomes hyperactive in metabolic diseases of the bone such as rickets and Osteitis deformans (Paget's disease).

II. EFFECT OF SUPPLEMENTATION WITH VITAMINS, CALCIUM PHOSPHATE, AND SODIUM FLUORIDE

The levels of supplements, added to the diets of the subjects were low since dietary intake, unsupplemented, compared favorably with the recommended allowances and levels of blood constituents were within the ranges of normal. Metabolic balances of nitrogen and phosphorus were positive and the calcium balance was just slightly negative (-52 mg). Fluoride was administered at a level of 0.25 mg/kg body weight/day, which was relatively low in contrast to the levels that have been utilized in other studies (80,81,117,118). None of the women were required to discontinue use of the supplement as a result of adverse effects and no symptoms of fluoride toxicity were observed in any of the women during the present study.

Bone Density

With fluoride therapy, increases in bone as evaluated from

microradiograph appearances and osteoid tissue measurements of 4 patients were reported (59,82). Similar increases have been noted in 1 subject by Cass et al. (118) and in 4 subjects by Bernstein and Cohen (81). In a case study by Rich and Ivanovich (80) an increase of approximately 13% in bone density was attained using 40 to 50 mg fluoride per day. The level employed in this study ranged from 10 to 30 mg per day. Bernstein and Cohen (81) found that patients treated with levels greater than 44 mg fluoride per day exhibited lumbar spine x-ray films which showed sharper, thicker, and better demarcated cortices with more uniform and thicker trabeculae than were observed in pretreatment films. Jowsey et al. (59) found that treatment of 3 patients with sodium fluoride at levels up to 0.77 mg F/kg body weight/day resulted in formation of new bone. Administration of tetracycline at specific times during the fluoride therapy identified newly formed bone. Rib biopsies from the 3 patients showed that bone formed during the administration of fluoride covered an average of 53% of the bone surface. Although bone formation was increased to a level much greater than normal, the new tissue was not always adequately mineralized (59). Later work by Jowsey et al. (83) resulted in a recommendation of 50 mg of sodium fluoride (22.7 mg F) per day accompanied by at least 900 mg of calcium per day and 50,000 IU of vitamin D twice weekly for restoration of bone mass in osteoporotic subjects.

The bone densities of 15 of the 18 subjects increased in this study in response to fluoride without the ingestion of calcium phosphate and vitamin supplements. When calcium phosphate and vitamin

supplements accompanied fluoride, 10 of 12 women increased their bone densities. Although deposition of mineral was necessary for increased density to be measured, the quality of the bone cannot be described since histological studies were not included.

Nutrient Intake

Insignificant changes in mean nutrient consumption from food were observed during the study; when minerals and vitamins were added as a part of the supplement regimen, the intake of those nutrients was significantly increased. Since Group 4 received supplements of calcium, phosphorus, vitamin A, and ascorbic acid, intakes of those 4 nutrients were significantly increased. Group 2 experienced significant increases in calcium and phosphorus consumption. Since these women were already receiving vitamin supplements at the beginning of the study and were maintained on them, no significant change in their intake was observed. Calcium consumption by the women in Group 3, who received fluoride without additional vitamin and mineral supplements, decreased at the final test period; however, the mean change in intake was not significantly different from that of Group 1. The mean amount of dietary calcium consumed by Group 3 in the final test period, 761 mg, was only 5% lower than the recommended intake, and it seems unlikely that the fluoride supplement was responsible for the decrease in calcium consumption.

Blood Constituents

Serum levels of both calcium and phosphorus decreased in the 2 groups which received a fluoride supplement; however, the decrement

was significant ($P<0.05$) only for Group 4. Similar decreases in serum calcium accompanying fluoride treatment have been reported by Jowsey et al. (59,83), Rich et al. (117), and Cass et al. (118). Although the decrease in the serum calcium level of Group 4 was significant, the final level (9.4 mg/100 ml) remained within the range of normal. As anticipated, due to supplementation of the diet, those in Group 4 increased their plasma ascorbic acid and serum vitamin A levels significantly at the final test period. A decrease in plasma ascorbic acid by those in Group 2 cannot be logically explained. An insignificant decrease in serum protein between the initial and final test periods was experienced by Group 4. Levels of serum protein increased in Groups 1 and 2 and the increase in Group 2 was of such a magnitude that the change differed significantly from that of Group 4. The change by Group 4 was not significantly different from that of the other group receiving fluoride (Group 3). The decrease in serum protein levels of Group 4 could not be attributed to fluoride supplementation.

Metabolic Balances

Several changes were observed in the metabolic balance studies although biological variability, innate errors of such studies, and the limitation in the number of subjects participating tended to obliterate any statistically significant effects.

The mean nitrogen intake at the initial test period had decreased by approximately 14% by the final test period. Decreases occurred not only in analyzed nitrogen intake of those participating in the balance

studies, but also in the calculated protein intake of the larger group of 60. Mean decreases in apparent nitrogen balance were experienced by all 4 groups, although 2 of the subjects in Group 4 increased their balances. None of the participants was in negative nitrogen balance at the final test period although apparent nitrogen balance decreased by 25% during the study.

Administration of calcium supplements to Group 2 resulted in greater amounts of fecal calcium. Subjects in that group lost almost 30% more calcium in the feces than they were consuming (diet plus supplement) at the final test period. Such losses are not completely illogical since 300 to 800 mg of calcium may enter the intestinal tract endogenously each day (119). The mean positive balance of Group 2 decreased from 188 to -528 mg/day. Two of the 3 persons experienced drastic increases in fecal calcium. This increase was not observed at the intermediate test period. Three members of the group receiving only fluoride supplements (Group 3) were in negative calcium balance at the initial test period (mean equal to 7 mg/day) and at the final test period all 4 members were in negative calcium balance. At the intermediate test period the mean apparent calcium balance had decreased to -390 mg/day. Balances were less negative (mean of -179 mg) at the final period. Cass et al. (118) observed that with fluoride therapy the negativity of calcium balance decreased from -300 mg/day to -48 mg/day in 8 months.

Three members of Group 4 were in negative calcium balance at the initial test period with a mean apparent balance of -226 mg/day. Balances increased throughout the study and a final mean apparent balance of 338 mg/day was observed.

Varying responses to calcium supplements, especially in combination with sodium fluoride, have been reported. Purves (120) found that at 60 mg F/day, 4 of 5 patients changed from negative to positive calcium balance in a 3 month period of time; Rose (84) found no change in response to fluoride in a 6 to 12 month period, and Bernstein and Cohen (81) reported that calcium balances were decreased initially, then became higher with longer fluoride therapy. Increasing calcium intake without adding fluoride did not alter calcium balances in 3 osteoporotic women to the extent that was observed in young non-osteoporotics (67). Varied response to calcium supplements has been described in several additional studies (43,81,117).

A correlation ($P<0.0001$) between fecal calcium and calcium balance, regardless of calcium intake or bone density, is not inconsistent with an age-associated defect in calcium absorption. Hurxthal and Vose (121) reported decreased calcium absorption and the necessity for consumption of large quantities of calcium to maintain positive balance in elderly subjects. Spencer et al. (67) indicated that calcium absorption is poor in patients with osteoporosis.

Phosphorus balances fluctuated in a pattern quite similar to that observed for calcium. Decreases in urinary excretion as observed in this study with administration of fluoride also have been reported by Jowsey et al. (83) and Spencer et al. (122).

Fluoride Overview

In Group 3, increased bone density was accompanied by decreases

in the positive balances of nitrogen and phosphorus. Calcium balance was slightly negative at the last test period; however, it was less negative than it had been at the intermediate period. Rich et al. (117) reported a reduced tendency to store calcium during fluoride therapy as compared to storage during calcium therapy; his patients, however, were able to remain in positive calcium balance with fluoride supplementation, in contrast to the results obtained in the present study. Spencer et al. (122) found that absorption of calcium did not increase when dietary calcium was increased in the presence of fluoride. In earlier work, these same authors (69) reported that calcium asorption decreased with fluoride therapy in 9 men regardless of calcium intake. In this study serum levels of both calcium and phosphorus were reduced in those persons receiving fluoride supplements. A similar decrease in blood levels of these nutrients with fluoride therapy has been observed by others (59,83,117,118).

The lack of a statistically significant increase in bone density by the subjects receiving vitamins, calcium phosphate, and sodium fluoride supplements was somewhat unexpected (Group 4). However, 10 of 12 subjects increased their bone densities which may be biologically significant for the individual subject. Increases in serum ascorbic acid and vitamin A levels were stimulated by the vitamin supplements. Serum levels of calcium and phosphorus significantly decreased ($P<0.01$) as was the tendency for Group 3 which received the fluoride supplement. The mean calcium balance which was negative at the onset of supplementation was positive 330 mg by the end of the study. The positive phosphorus balance was tripled during the

12 months of supplementation. Perhaps, if supplementation had been extended beyond 12 months, statistically significant increases in bone density would have been observed.

Increases in bone density could not be related to changes in nutrient intake, blood constituent levels, or metabolic balances measured in this study. Adams et al. (43) could not relate bone loss to similar parameters. While Cass et al. (118) failed to attain positive calcium balances with fluoride therapy, biopsy of the iliac crest showed greatly increased amounts of osteoid material.

It has long been known that calcium absorption and its balance varies between individuals and periodically in the same person (123). It is possible that bone mineral behaves in a similar pattern, thus rendering both balance information and bone density measurements difficult to interpret.

The failure to achieve an increase in bone density in Group 4 of as large a magnitude as that in Group 3 was not expected. It is possible that supplementation with calcium and fluoride (Group 4) simultaneously, could lead to the formation of insoluble calcium fluoride, thus making both elements unavailable to the extent that the salt was formed. It seems quite certain, however, that it was not the unavailability of the calcium that was the influencing factor. Even when the calcium was administered without supplemental fluoride (Group 2) the increase in bone density obtained was not significant. Thus, if the complex formed, it must have been the relative unavailability of the fluoride that was important. That an increase (4%) in bone density was achieved in Group 4 might have been due to

the partial availability of the fluoride ion. Free fluoride is known to be rapidly absorbed from the intestinal tract (124).

From all indications, fluoride supplementation had no important effects on the overall nutritional status of the subjects except for improvements in their bone densities. The fact that bone density was most increased in the group receiving fluoride alone as the dietary supplement certainly argues in favor of the use of the element in the treatment of osteoporosis and/or in the maintenance of bone density of elderly women. In addition, it is tempting to speculate that the element must serve a unique role in metabolism of bone.

CHAPTER VI

SUMMARY

The nutritional status of 76 women with a mean age of 68 years was assessed before and after a 12-month experimental period in which diets were unsupplemented or supplemented with fluoride (0.25 mg/kg body weight/day), with calcium phosphate (464 mg calcium and 360 mg phosphorus daily), or with a combination of fluoride, calcium phosphate and vitamins. Approximately 18 women over 50 years of age were assigned to each group.

Prior to supplementation, weighed dietary intakes were evaluated for 8 nutrients. Mean energy intake per day was 1519 kilocalories with 15% derived from protein and 36% from fat. Mean daily intakes included 797 mg of calcium, 1000 mg phosphorus, and 8.6 mg of iron. Mean dietary intakes of the 59 women who were not receiving vitamin supplements were 5800 IU and 35 mg for vitamin A and ascorbic acid respectively. The mean intakes of vitamin A and ascorbic acid increased by 75 and 135% respectively when values for 17 women receiving dietary supplements of these 2 nutrients were included.

Blood levels of 10 constituents were also determined. Mean blood hemoglobin was 13.9 g/100 ml and serum calcium and phosphorus levels had means of 9.60 and 3.35 mg/100 ml, respectively. A mean of 1.92 mg inorganic phosphorus was released from a glycerophosphate substrate by alkaline phosphatase, while the mean cholesterol level was 222 mg per 100 ml. Mean serum levels of total protein and albumin were 6.77 and 3.67 g/100 ml, respectively, and the mean A/G ratio was

1.22. At the initial test the plasma ascorbic acid averaged 0.34 mg/100 ml and serum vitamin A, 50.4 μg/100 ml; the means were increased by 50 and 2% respectively when values for the 17 subjects receiving vitamin supplements were included. In addition, mean excretion of hydroxyproline was 24.06 μg/24 hours and creatinine excretion was 607 mg/24 hours.

The mean bone density index of the left phalanx 5-2 was 0.819 g equivalents of alloy per cc of bone, while mean height and weight were 156.3 cm and 65.4 kg, respectively.

Canonical correlations showed an age-associated decrease in bone density, height, weight, and intake of protein, calcium, phosphorus, and ascorbic acid. Significant correlations existed between dietary intakes of energy, protein, fat, and phosphorus and also between vitamin A and ascorbic acid consumption. The initial measurements indicated that the women represented a typical geriatric population with reduced nutrient intakes, bone densities, heights, and weights.

Although minor differences in nutrient intakes and blood levels occurred in the groups receiving fluoride, the changes could not be attributed to fluoride supplementation. Consumption of calcium decreased in the group which received fluoride, although the final mean intake was 761 mg per day. In the group receiving fluoride in combination with calcium phosphate and vitamin supplements, increases in plasma ascorbic acid and serum vitamin A and decreases in serum total protein, calcium and phosphorus were obtained. Nevertheless, all values remained within normal ranges.

Supplementation with fluoride resulted in a significant 10%

increase in bone density and fluoride in combination with calcium phosphate and vitamins produced a 4% increase in bone density. Insignificant increases in bone density were observed when the diet was unsupplemented (2%) or supplemented with calcium phosphate (2.5%).

Metabolic balance studies revealed mean increases in balances of calcium and phosphorus by those receiving fluoride in combination with calcium phosphate and vitamins. An age-associated defect in calcium absorption was indicated in all experimental groups. Nitrogen balances of all women remained positive throughout the study. Changes in neither calcium nor phosphorus balances correlated with those in bone density. Positive balances of these nutrients were shown when fluoride accompanied the supplements of calcium, phosphorus, and vitamins but bone density decreased in 2 of the 4 subjects in that balance group. All members of the group receiving fluoride exhibited negative balances of calcium and phosphorus while bone density increased.

No adverse effects or fluoride toxicity symptoms were observed in any of the women during the study. The biological significance of a decrease in serum calcium has not been elucidated; a shift of serum calcium to bone, or a reduction in calcium mobilized from bone (possibly lowering serum calcium), may be advantageous. Increases in bone density in response to fluoride without alterations in dietary intake or serum levels of the constituents evaluated in this study, other than serum calcium, tends to encourage further exploration of the prophylactic as well as therapeutic benefits of low-level fluoride supplementation.

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APPENDICES

APPENDIX A

LETTERS AND CONSENT FORMS

Dear (Patient)

The Nutrition Department at The University of Tennessee is inviting you to participate in a nutrition study. The purpose of the study is to evaluate the medical-nutritional status of a group of selected women patients at Eastern State Psychiatric Hospital and then to evaluate the effectiveness of dietary supplements in changing their nutritional status.

Participation in the study consists of allowing us to assign you to a group receiving for twelve months either the regular hospital diet or the hospital diet supplemented with vitamins and/or minerals and to:

1. Record food intake for a week
2. Take a blood sample for analysis
3. Collect urine and fecal output for a week
4. Determine bone density by a harmless x-ray procedure similar to dental x-rays

The initial tests (1,2,3,4) will be made in January and will be repeated at six month intervals.

The dietary supplementation will be in the form of multiple vitamin and mineral capsules. The mineral supplements in which we are particularly interested are calcium phosphate and sodium fluoride. Calcium phosphate has been frequently added to the diets of the elderly to prevent bone loss. Recent studies have indicated that sodium fluoride added to the diet may decrease the amount of mineral lost from bone during aging. Sodium fluoride has long been known to reduce tooth decay when added to drinking water. With age, bones generally become weaker and they may suddenly break. We feel that supplementation of the diet with sodium fluoride may be of value in reducing bone mineral losses and susceptibility to fractures. The level of fluoride used will be well below that which has caused any toxic effects. In rare instances supplementation of the diet with high levels of sodium fluoride has produced side effects such as nausea and joint pain. However, the majority of people who have received sodium fluoride indicate that the mineral is well tolerated and that it tends to reduce joint pains.

We think this study will provide very useful information concerning the nutritional status of older people and we hope that you will desire to help us.

Sincerely,

Dear

The Department of Nutrition at The University of Tennessee is making a study of the nutritional status of elderly women in which _____ has indicated a desire to participate. Enclosed is a copy of a letter explaining the study, which we have given to her. The study has also been explained to her personally. A copy of a signed consent form indicating her desire to participate is also enclosed. We need your permission for her to participate. The study will interfere very little with the schedule which she is now following at Eastern State Psychiatric Hospital. To give us your permission, please sign the Permission Form in the space indicated by the red X, write in the date, and return the form to us in the enclosed self-addressed and stamped envelope at your earliest convenience. If you have questions regarding the study, please contact us by writing to:

Dr. Roy E. Beauchene
Department of Nutrition
The University of Tennessee
Knoxville, Tennessee 37916

If you prefer, you may call the Nutrition Department at The University of Tennessee, Knoxville (974-3491). Dr. Beauchene, Mrs. Mason, Miss Whitfield, or Miss Thompson will be glad to answer your questions.

Thank you very much for your interest and cooperation.

Sincerely,

Sue Thompson

CONSENT FORM

I, _____, would like to participate in the University of Tennessee, Department of Nutrition, study. I understand that I will receive either the regular hospital diet or the hospital diet supplemented by vitamins or minerals and that (1) my food intake will be recorded for a week, (2) a blood sample will be taken, (3) urine and feces will be collected for a week, and (4) bone density will be determined by a painless and harmless x-ray procedure. I also understand that these tests will be repeated at six month intervals during the study.

Signed _____

Date _____

PERMISSION FORM

I am willing for _____ to participate in the University of Tennessee, Department of Nutrition, study. I understand that she will be given either the regular hospital diet or the hospital diet plus a vitamin or mineral supplement and that (1) food intake will be recorded for a week, (2) a blood sample will be taken, (3) urine and feces will be collected for a week, and (4) bone density will be determined by a painless and harmless x-ray procedure. I also understand that these tests will be repeated at six month intervals during the study.

Signed _____

Date _____

APPENDIX B

SYSTEM FOR DETERMINING THE WEIGHT OF FOOD SERVED AND CONSUMED

Sheet 1
Food Served

Lunch 6/1/71

		Plate Plus				Bowl Plus			
Name of Subject	Plate	Fried Fish	Buttered Potatoes	Green Beans	Cole Slaw	Bowl	Apple Betty	Bread	Milk
	N	g	g	g	g	N	g	g	g
Green	1[1]	493	612	660	723	1[2]	289	25	190[3]

Sheet 2
Food Unconsumed

Lunch 6/1/71

		Plate Plus				Bowl Plus			
Name of Subject	Plate	Fried Fish	Buttered Potatoes	Green Beans	Cole Slaw	Bowl	Apple Betty	Bread	Milk
	N	g	g	g	g	N	g	g	g
Green	1[1]	444	400	400	400	1[2]	206	0	6

Sheet 3
Food Eaten

Lunch 6/1/71

Name of Subject	Plate	Fried Fish	Buttered Potatoes	Green Beans	Cole Slaw	Bowl	Apple Betty	Bread	Milk
	N	g	g	g	g	N	g	g	g
Green	1[1]	49	119	48	63	1[2]	83	25	185

---

[1] Plate #1 weighed 400 g.

[2] Bowl #1 weighed 200 g.

[3] Paper cup (8 oz.) weighed 5 g.

APPENDIX C

NUTRIENT UTILIZATION TABLES AND STATISTICAL TABLES

TABLE 22

NITROGEN UTILIZATION BY INDIVIDUAL SUBJECTS

Subject number	Test period	Intake	Urinary loss	Fecal loss	Balance
		g/day			
Group 1 (no supplement)					
1	Initial	8.99	7.52	0.58	0.89
	Intermediate	9.65	6.38	0.28	2.99
2	Initial	5.02	2.96	0.17	1.89
	Intermediate	4.07	2.19	0.18	1.70
3	Initial	6.23	4.04	0.50	1.69
	Final	4.83	3.32	0.28	1.23
4	Initial	8.68	5.38	0.53	2.77
	Intermediate	4.77	3.15	0.76	0.85
	Final	5.94	4.25	0.42	1.27
Group 2 (Ca,P supplement)					
5	Initial	7.38	4.65	0.36	2.37
	Intermediate	5.43	3.84	0.58	1.00
	Final	6.88	4.83	0.59	1.46
6	Initial	9.57	6.40	0.24	2.93
	Intermediate	7.51	5.48	0.50	1.53
	Final	9.31	6.26	1.27	1.79
7	Initial	9.54	5.89	1.46	2.19
	Intermediate	5.04	4.16	1.02	-0.14
	Final	7.35	5.38	0.80	1.18

TABLE 22 (continued)

Subject number	Test period	Intake	Urinary loss	Fecal loss	Balance
		g/day			
Group 3 (F supplement)					
8	Initial	16.50	10.49	0.92	5.09
	Intermediate	16.32	10.99	2.80	2.54
	Final	14.10	8.11	1.82	4.16
9	Initial	8.10	5.67	0.85	1.58
	Intermediate	6.79	5.07	1.12	0.61
	Final	8.74	6.62	0.73	1.39
10	Initial	9.71	8.21	0.46	1.04
	Intermediate	7.56	5.81	0.42	1.33
	Final	9.62	7.90	0.72	1.00
11	Initial	6.15	4.35	0.80	1.00
	Intermediate	6.09	4.50	0.76	0.81
	Final	6.55	4.79	0.91	0.84
Group 4 (F,Ca,P Vitamins supplement					
12	Initial	11.99	7.47	1.04	3.48
	Intermediate	10.80	13.79	1.03	-4.02
	Final	8.55	5.38	0.38	2.79
13	Initial	8.01	5.47	0.95	1.59
	Intermediate	6.40	5.14	0.97	0.28
	Final	9.38	6.47	0.72	2.20
14	Initial	8.02	4.19	0.69	3.14
	Intermediate	5.26	3.82	0.75	0.69
	Final	6.15	4.14	0.88	1.12
15	Initial	10.06	6.65	0.70	2.71
	Intermediate	7.74	3.74	1.33	2.69
	Final	6.71	2.71	0.80	3.20

TABLE 23

CALCIUM UTILIZATION BY INDIVIDUAL SUBJECTS

Subject number	Test period	Supplement	Intake[1]	Urinary loss	Fecal loss	Balance
			mg/day			
Group 1 (no supplement)						
1	Initial	---	560	149	722	-311
	Intermediate	---	490	74	196	220
2	Initial	---	573	44	560	-31
	Intermediate	---	481	20	609	-148
3	Initial	---	722	187	600	-65
	Final	---	551	69	618	-135
4	Initial	---	613	87	827	-301
	Intermediate	---	483	32	366	86
	Final	---	642	26	560	56
Group 2 (Ca,P supplement)						
5	Initial	---	603	41	331	231
	Intermediate	464	888	46	866	-25
	Final	464	1020	26	1752	-757
6	Initial	---	951	253	97	601
	Intermediate	464	1045	68	297	680
	Final	464	1240	136	1984	-881
7	Initial	---	716	137	845	-266
	Intermediate	464	920	68	1271	-418
	Final	464	1111	46	1012	54

TABLE 23 (continued)

Subject number	Test period	Supplement	Intake[1]	Urinary loss	Fecal loss	Balance
		mg/day				
Group 3 (F supplement)						
8	Initial	---	1680	103	893	684
	Intermediate	---	1476	64	2323	-911
	Final	---	1431	114	1459	-142
9	Initial	---	600	112	751	-263
	Intermediate	---	521	100	752	-331
	Final		721	110	669	-58
10	Initial	---	598	131	502	-35
	Intermediate	---	525	54	436	34
	Final	---	623	34	985	-396
11	Initial	---	662	50	715	-103
	Intermediate	---	556	42	868	-354
	Final	---	627	21	725	-119
Group 4 (F,Ca,P Vitamins supplement)						
12	Initial	---	901	107	778	16
	Intermediate	464	1246	81	771	393
	Final	464	1196	54	489	653
13	Initial	---	892	99	1526	-733
	Intermediate	464	1004	135	938	-69
	Final	464	1270	123	700	448
14	Initial	---	636	218	541	-123
	Intermediate	464	850	87	693	71
	Final	464	945	50	822	72
15	Initial	---	716	40	740	-64
	Intermediate	464	1047	17	1033	-3
	Final	464	1013	14	823	177

[1] Supplement included.

TABLE 24

PHOSPHORUS UTILIZATION BY INDIVIDUAL SUBJECTS

Subject number	Test period	Supplement	Intake[1]	Urinary loss	Fecal loss	Balance
		----------	mg/day	----------		
Group 1 (no supplement)						
1	Initial	---	932	340	516	76
	Intermediate	---	957	333	129	496
2	Initial	---	763	262	277	224
	Intermediate	---	571	141	325	105
3	Initial	---	884	322	260	302
	Final	---	656	282	343	32
4	Initial	---	940	369	443	128
	Intermediate	---	605	252	226	127
	Final	---	782	261	315	206
Group 2 (Ca,P supplement)						
5	Initial	---	828	256	234	339
	Intermediate	360	973	324	566	83
	Final	360	1113	304	1239	-430
6	Initial	---	1039	599	75	365
	Intermediate	360	1250	480	186	584
	Final	360	1466	690	1200	-424
7	Initial	---	1026	475	477	75
	Intermediate	360	986	450	831	-294
	Final	360	1261	508	654	100

TABLE 24 (continued)

Subject number	Test period	Supplement	Intake[1]	Urinary loss	Fecal loss	Balance
		mg/day				
Group 3 (F supplement)						
8	Initial	---	1952	575	490	887
	Intermediate	---	1823	957	1417	-551
	Final	---	1694	578	955	162
9	Initial	---	910	467	425	18
	Intermediate	---	766	507	486	-227
	Final	---	1009	529	470	10
10	Initial	---	1002	548	315	139
	Intermediate	---	836	452	244	140
	Final	---	1060	464	692	-96
11	Initial	---	796	350	330	116
	Intermediate	---	716	446	404	-134
	Final	---	817	474	370	-27
Group 4 (F,Ca,P Vitamins supplement)						
12	Initial	---	1228	410	377	441
	Intermediate	360	1531	523	552	456
	Final	360	1330	351	309	670
13	Initial	---	1102	458	551	93
	Intermediate	360	1081	513	413	156
	Final	360	1453	796	421	235
14	Initial	---	930	271	360	299
	Intermediate	360	953	373	510	70
	Final	360	1048	358	635	54
15	Initial	---	1148	445	413	290
	Intermediate	360	1247	371	560	316
	Final	360	1122	287	406	429

[1]Supplement included.

TABLE 25

URINARY CONSTITUENTS FOR INDIVIDUAL SUBJECTS

Subject number	Test period	Hydroxyproline	Creatinine
		µg/day	mg/day
Group 1 (no supplement)			
1	Initial	37.27	820
	Intermediate	38.60	870
2	Initial	16.64	320
	Intermediate	13.94	368
3	Initial	18.16	426
	Final	15.02	371
4	Initial	21.64	583
	Intermediate	12.89	545
	Final	14.53	667
Group 2 (Ca,P supplement)			
5	Initial	17.41	564
	Intermediate	20.49	780
	Final	31.45	660
6	Initial	28.91	630
	Intermediate	26.59	719
	Final	27.77	739
7	Initial	28.56	556
	Intermediate	25.18	609
	Final	20.22	612

TABLE 25 (continued)

Subject number	Test period	Hydroxyproline	Creatinine
		µg/day	mg/day
Group 3 (F supplement)			
8	Initial	27.28	627
	Intermediate	36.15	1010
	Final	32.84	707
9	Initial	25.52	504
	Intermediate	25.34	574
	Final	26.72	687
10	Initial	25.48	910
	Intermediate	22.39	619
	Final	21.45	736
11	Initial	14.01	544
	Intermediate	15.40	706
	Final	12.44	621
Group 4 (F,Ca,P Vitamins supplement)			
12	Initial	25.59	714
	Intermediate	31.27	939
	Final	30.49	633
13	Initial	26.45	447
	Intermediate	50.41	684
	Final	31.12	784
14	Initial	14.30	445
	Intermediate	12.77	451
	Final	12.61	422
15	Initial	22.17	574
	Intermediate	10.72	462
	Final	11.56	363

TABLE 26

LEAST SQUARES ANALYSIS OF VARIANCE FOR DIFFERENCE[1] IN PHYSICAL MEASUREMENTS BY GROUP

Source of Variation	df	SS	MS	F
Bone Density[1] Index[2]				
Group	3	0.0549	0.0183	2.2282[4]
Error	56	0.4602	0.0082	
Total	59	0.5151		
Bone Density[3] Index[2]				
Group	3	0.0008	0.0003	0.0445[5]
Error	56	0.3323	0.0059	
Total	59	0.3331		
Height (cm)				
Group	3	18.54	6.18	1.9707[6]
Error	56	175.66	3.14	
Total	59	194.20		
Weight (kg)				
Group	3	210.79	70.26	1.7056[6]
Error	56	2306.99	41.20	
Total	59	2517.78		

[1]Difference equals final minus initial measurement by subject.

[2]Gram equivalents of alloy per cc of bone.

[3]Difference equals intermediate minus initial measurement by subject.

[4]Significant, $P<0.09$.

[5]Not significant, $P>0.10$.

[6]Not significant, $P>0.05$.

TABLE 27

LEAST SQUARES ANALYSIS OF VARIANCE FOR DIFFERENCE[1] IN DIETARY MEASUREMENTS BY GROUP

Source of Variation	df	SS	MS	F
Energy (kcal)				
Group	3	308238	102746	0.6913[2]
Error	56	8322829	148622	
Total	59	8631067		
Protein (g)				
Group	3	63	21	0.1020[2]
Error	56	11521	206	
Total	59	11584		
Fat (g)				
Group	3	82	27	0.1024[2]
Error	56	14918	266	
Total	59	15000		
Calcium (mg)				
Group	3	3070010	1023337	24.6561[3]
Error	56	2324249	41504	
Total	59	5394259		
Phosphorus (mg)				
Group	3	1747694	582565	9.8456[3]
Error	56	3313539	59170	
Total	59	5061233		
Iron (mg)				
Group	3	0.43	0.14	0.0215[2]
Error	56	375.24	6.70	
Total	59	375.67		
Vitamin A (IU)				
Group	3	6196663836	2065554612	64.7946[3]
Error	55	1753316484	31878482	
Total	58	7949980320		
Ascorbic Acid (mg)				
Group	3	362648	120883	437.9301[3]
Error	55	15182	276	
Total	58	377830		

[1]Difference equals final minus initial measurement by subject.

[2]Not significant, $P>0.05$.

[3]Significant, $P<0.0001$.

TABLE 28

LEAST SQUARES ANALYSIS OF VARIANCE FOR DIFFERENCE[1] IN BLOOD CONSTITUENTS BY GROUP

Source of Variation	df	SS	MS	F
Blood Hemoglobin (g/100 ml)				
Group	3	1.6566	0.5522	0.3734[2]
Error	56	82.8108	1.4788	
Total	59	84.4673		
Plasma Ascorbic Acid (mg/100 ml)				
Group	3	8.0188	2.6729	10.8343[5]
Error	55	13.5690	0.2467	
Total	58	21.5878		
Serum Total Protein (g/100 ml)				
Group	3	1.54	0.51	3.7078[3]
Error	56	7.77	0.14	
Total	59	9.31		
Serum Albumin (g/100 ml)				
Group	3	0.1218	0.0406	0.5234[2]
Error	56	4.3440	0.0776	
Total	59	4.4658		
Serum A/G Ratio (g/100 ml)				
Group	3	0.3235	0.1078	2.1460[2]
Error	56	2.8138	0.0502	
Total	59	3.1373		
Serum Cholesterol (mg/100 ml)				
Group	3	1596	532	0.4460[2]
Error	56	66812	1193	
Total	59	68408		
Serum Calcium (mg/100 ml)				
Group	3	5.5978	1.8659	4.7580[4]
Error	56	21.9615	0.3922	
Total	59	27.5593		
Serum Phosphorus (mg/100 ml)				
Group	3	3.0930	1.0310	1.6441[2]
Error	56	35.1168	0.6271	
Total	59	38.2098		

TABLE 28 (continued)

Source of Variation	df	SS	MS	F
Serum Vitamin A (µg/100 ml)				
Group	3	1296	432	1.1853[2]
Error	55	20048	364	
Total	58	21344		
Serum Alkaline Phosphatase (mg/100 ml)[6]				
Group	3	5.7634	1.9211	1.7017[2]
Error	56	63.2209	1.1289	
Total	59	68.9843		

[1]Difference equals final minus initial measurement by subject.

[2]Not significant, P>0.05.

[3]Significant, P<0.02.

[4]Significant, P<0.005.

[5]Significant, P<0.0001.

[6]Inorganic phosphorus released from glycerophosphate substrate in 1 hour at 37°.

VITA

Glenda Sue Thompson was born in Sherman, Texas, on September 8, 1936. A 1955 graduate of Howe High School, Howe, Texas, she received a Bachelor of Science degree in Home Economics from Baylor University, Waco, Texas, in 1959. She accepted a teaching position at Iowa Park High School, Iowa Park, Texas, where she taught Home Economics until enrolling in Texas Technological College, Lubbock, Texas. In August, 1964, she received a Master of Science degree with a major in Food and Nutrition. She was employed by State College of Arkansas, Conway, Arkansas, prior to enrolling for full-time graduate studies at The University of Tennessee in September, 1968. She received the Doctor of Philosophy degree with a major in Nutrition in August, 1973, and accepted a position as Professor and Chairman of the Home Economics Department at State College of Arkansas. She is a member of American Dietetic Association, Gerontological Society, American Home Economics Association, American Council of Administrators of Home Economics, and Phi Upsilon Omicron.