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Effect of Age, Caloric Restriction, and Dietary Protein on Bone Composition of Male Rats

Melany H. Looper
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To the Graduate Council:

I am submitting herewith a thesis written by Melany H. Looper entitled "Effect of Age, Caloric Restriction, and Dietary Protein on Bone Composition of Male Rats." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Nutrition.

Roy E. Beauchene, Major Professor

We have read this thesis and recommend its acceptance:

Betty Ruth Carruth, Nina Marable

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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EFFECT OF AGE, CALORIC RESTRICTION, AND DIETARY PROTEIN ON BONE COMPOSITION OF MALE RATS

A Thesis

Presented for the

Master of Science

Degree

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Melany H. Looper

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ABSTRACT

Thirty-day old male Wistar rats were fed 1 of 8 purified diets. Three groups were fed diets containing either 12, 20, or 28% casein (protein) ad libitum. Three other groups were fed diets containing 18, 30, or 42% casein with a feed intake restricted to two-thirds of that consumed by the ad libitum groups. In addition, two groups were initially fed diets containing 20% casein, then the casein level was either gradually (0.8%/month) decreased to 12% or increased to 28% by 1 year of age. At either 1 or 2 years of age, the rats were sacrificed, and their femurs were analyzed for length, wet weight, dry weight, fat-free dry weight, fat and ash. The femurs of the ad libitum groups were longer ($p < 0.0001$), heavier (wet bone wt, $p < 0.0001$), and contained greater amounts ($p < 0.0001$) of absolute ash than those of restricted rats. Increased age of the rats in both the ad libitum and restricted groups was also associated with increases in the previously listed responses, but they were of lesser magnitude. Restricted-feeding ($p < 0.0001$) and aging ($p < 0.006$) were associated with increased percentages of water. There was a significant diet by age interaction ($p < 0.002$) for the percentage fat in the femurs. Ash expressed as a percentage of fat-free dry bone was neither significantly affected by caloric or protein restriction nor by age when the ad libitum group was compared to the restricted group. Length and wet weight of the femurs were remarkably similar regardless of whether the protein was constant or changing. Ash expressed in an absolute amount was greater

($p < 0.01$) in the changing rather than in the constant protein ad libitum group but only approached significance when expressed as a percent of fat-free dry femur weight. Within the changing group, the 20 → 12% protein group had femurs which were shorter ($p < 0.001$) and weighed less (wet bone wt, $p < 0.001$) than the 20 → 28% group, but percent water was not significantly different.

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CHAPTER I

INTRODUCTION

A major concern of today's society is the nutritional practices necessary for optimal health and longevity (1). Since many elderly individuals are in "good" health, there are implications that degenerative changes may be prevented or at least postponed if nutrition and other environmental factors are optimized throughout the lifespan. The amount of dietary protein required for growth and development versus that required for maintenance of normal bodily functions throughout the lifespan is still a controversial issue (2, 3).

In the United States where the diet contains a nonspecific high level of total protein, there is also a high incidence of osteoporosis (4-6). Although osteoporosis is due to a number of interacting factors over time, it has been suggested that a high protein intake may be related to demineralization of the bone and loss of the supporting bone matrix (7, 8). Contrary to this theory, other researchers have found no relationship between bone composition and high protein feeding (9, 10). Therefore, there is disagreement concerning the effects dietary protein may have on osteoporosis.

General malnutrition has been shown to be accompanied by osteopenia in both humans and experimental animals (11, 12). Furthermore, it has been shown that the effect of semistarvation on bone composition may be osteoporotic rather than osteomalacic, and the degeneration may

be a result of reduced bone turnover (13). This research is an attempt to provide further insight into the effect of age, caloric restriction, and level of dietary protein on bone composition and the implications for optimal skeletal development and maintenance over the lifespan.

CHAPTER II

REVIEW OF LITERATURE

General Nutrition, Aging, and Longevity

There is evidence that diet and nutritional status have effects on the rate of the aging process and life expectancy but not on maximum lifespan (14). Munro (2) suggested three main subcategories for the relationships between human nutrition and aging: age-related physiological changes, chronic diseases related to the aging process with nutritional implications, and the nutrient requirements of the elderly population. Although it has been noted in a review (15) that longevity could be influenced by the quantity and quality of the diet, Masoro et al. (16) and Young (17) emphasized that the underlying mechanisms remained controversial issues, e.g. the roles of energy, protein, carbohydrate, and/or fat. Watkins (3) also reported that there have been no satisfactory dietary allowances proposed for protein and many of the other nutrients related to the elderly as opposed to the young adult population.

Age Changes in Bone

Bone loss has been of general interest because of the indication that long-term nutrition may be associated with age-related changes and the speculation that diet can modify the rate of bone loss with age even if it cannot prevent totally the occurrence over time (18). Newton-John and Morgan (19) noted three major disorders of bone loss

as defined by their clinical appearances: osteomalacia--most of the bone tissue was uncalcified, osteitis fibrosa--the bone was eroded by osteoclasts and replaced with fibrous tissue, and osteoporosis--the bone was fully calcified and otherwise normal except for a decrease in the overall mass. Evidence of bone loss has been shown to begin during the middle ages in both sexes (20, 22), 40-45 years of age in women and 50-65 years of age in men, and continues throughout the aging process although the bone loss may not be diagnosed as osteoporotic or one of the other types of pathological bone diseases. Saville (23) found that maximum bone losses were seen in the sixth decade of life in women and the eighth decade in men. Munro (1) reported human males had an average loss of 12% in bone density by 90 years of age and females of 25%. Krishnarao and Draper (24) investigated the use of mice as experimental models in studies on bone loss as a function of age; mice femurs showed bone losses with age comparable to those of humans while smaller changes were seen in the mice tibias. In another study, Indritz and Hegarty (25) expressed caution if findings about bone composition were expressed on a wet bone weight basis and the rat femur was used as the experimental model. They found that the rat femur differed significantly in moisture content from other bones assayed regardless of age.

Caloric and Protein Restriction and Aging

Researchers have shown that when experimental animals were restricted-fed, there was a significant lengthening in lifespan (26-29).

In a study by Everitt et al. (30), an increase in mean lifespan was observed when the experimental rats were restricted slightly or when feed restriction was introduced gradually later in life. In a more recent investigation by Everitt et al. (31), a mild restriction increased lifespan while severe restriction decreased the lifespan compared to the ad libitum group. Furthermore, Stuchlikova et al. (32) and Beauchene et al. (33) observed that feed restriction increased lifespan when employed only in youth or only after maturity. Although dietary restrictions do not have to be a life-long process to increase longevity, it has been established that longevity depends on when the restriction begins and the degree of the restriction (34, 35).

In two studies by Leto et al. (36, 37), it was observed that when protein restriction was the type of dietary manipulation implemented, a significant lengthening of the lifespan occurred. In an attempt to explain the relationship between low protein diets and increased lifespan, oxygen consumption and body temperature were determined. They concluded that changes in body temperature and oxygen consumption did not account for all of the increase in lifespan brought about by feeding a low protein diet. However, they did propose that the increase in lifespan may have been due to a decreased use of genetic material in the restricted-fed animals. They hypothesized that continuous use of the genetic material in ad libitum-fed animals may have led to an increased risk of imperfections in transcription of information and to an acceleration of the aging process.

Caloric Restriction and Bone

Researchers began to explore the effects of dietary restriction on bone integrity (38) since bone loss was identified as a degenerative process that occurred with increasing age and could be related to nutrition. In a study of the effects of semistarvation on bone composition (39), the ability to maintain some bone growth was noted even though there was a failure to gain body weight. Although some bone growth did occur, general malnutrition was accompanied by osteopenia in a human study by Garn et al. (12) and in an experimental animal study by Platt and Stewart (11). In another study by Shires et al. (13), the effects of semistarvation on mineral homeostasis and skeletal morphology were osteoporotic rather than osteomalacic, and the defect was reportedly reduced bone turnover. Therefore, caloric restriction had a detrimental effect although the pathogenesis was not clearly defined.

Calcium Metabolism and Hypercalciuria

The effect of dietary protein on calcium metabolism and calciuria has been a very controversial issue in both nutritional and medical research laboratories. Some investigators observed hypercalciuria and negative calcium balance in normal subjects fed a high-protein diet (40, 41). Other researchers suggested that protein-induced hypercalciuria was due to an increased glomerular filtration rate, a decreased fractional renal tubular reabsorption of calcium, and/or depletion of calcium from the bone (6, 42-45). On the contrary, Spencer et al. (46) showed that the loss of calcium associated with a high-protein diet

depended on the type of protein because the intake of large amounts of meat protein did not increase urinary calcium while some other types did; these findings were confirmed in a more recent study from the same laboratory (47). Furthermore, Mahalko et al. (48) and Spencer et al. (49) concluded that the naturally high-protein intake given as meat did not cause an increase in urinary calcium or calcium loss because the meat was high in phosphorous which may have decreased the excretion of urinary calcium.

Calcium Metabolism and Bone

Because it is a major dietary component, protein was manipulated to investigate its effect on calcium metabolism and bone turnover (7, 50) but the findings were both controversial and inconclusive. Spencer et al. (49) suggested that meat protein was not a contributing factor to osteoporosis and that bone loss with aging was inevitable. In studies by Calvo et al. (9), Whiting and Draper (51), and Allen and Hall (52), high protein diets did not promote either bone resorption or bone loss in rats. In contrast, in an investigation by Licata et al. (4) and in another by Anand and Linkswiler (53), it was reported that high dietary protein was associated with increased calciuria and negative calcium balance. Since dietary calcium remained constant and calcium absorption did not change significantly, it was suggested that loss of calcium from bone contributed to the hypercalciuria. Other researchers have also hypothesized that bone loss occurred when there was an imbalance between bone resorption and bone formation as

a result of more calcium being released from the skeleton than was being absorbed (8, 54-56). Furthermore, reduced calcium retention and hypercalciuria were linked to an osteopenia observable in skeletal radiographs and bone densitometric measurements (57-59).

Acid-Base Balance and Bone Metabolism

In a study by Whiting and Draper (60), a normal protein diet acidified by adding sulfur (in the form of either methionine plus cystine or inorganic sulfate) was fed to adult rats to determine its effect on bone metabolism. They found evidence of bone loss when additional methionine and cystine were incorporated into the diet, and in another study, the effect was proportional to the sulfur amino acid content of the diet (61). Wachman and Berstein (62) indicated that the age-related increase of osteoporosis was partially a result of life-long buffering by the basic salts of the bone to maintain pH homeostasis. Newell and Beauchene (63) and Dekanic (64) also studied the effect of chronic acidosis on bone composition and noted that the acid loaded animals developed bones similar in dimensions to those of the controls but were deficient in the mineral component.

Omnivores vs Vegetarians: Effect on Human Bones

In some human studies comparing vegetarians and omnivores, it was found that the incidence of osteoporosis was higher in the omnivores than in the vegetarians (65-68). Marsh et al. (69) observed that lacto-ovo-vegetarian women 50-89 years of age lost 18% of bone mineral mass while omnivorous women of the same ages lost 35%, but there were

no significant differences during the third, fourth, or fifth decades of life. Other researchers indicated that bone loss occurred as a function of age regardless of nutritional implications (70, 71), and still others (72, 73) suggested nutrition as an important determinant of bone mass in young adults while having no effect on age-related bone loss. According to Marx (74), settling this controversial issue of the optimum quantity and quality of protein and the relationship between a high-meat protein diet and the increased incidence of osteoporosis in the United States was important.

CHAPTER III

METHODS

General Plan

This study was a part of a larger one designed to investigate the effect of age, caloric restriction, and level of dietary protein on growth, kidney function, and survival of male rats (75, 76). The present study was undertaken to determine the specific effects of the dietary manipulations and age on the bone composition of the animals. As a part of the design of the original study, weanling male Wistar rats (n = 384) were placed into 1 of 8 dietary groups. Each of the 8 dietary groups (n = 48) was subdivided into 2 subgroups; one subgroup (n = 12) was scheduled to be sacrificed at 1 year of age and the other (n = 36) at 2 years of age. Three groups were fed diets containing either 12, 20, or 28% casein (protein) ad libitum. As reported from the National Research Council's Committee on Animal Nutrition (77), 18-25% protein in the rat diet provides sufficient amounts of the essential amino acids for optimal growth and development. Three other groups were fed diets containing, 18, 30, or 42% casein with a feed intake restricted to two-thirds of that consumed by the ad libitum groups (Table 1). Therefore, each ad libitum group was pair-fed with a restricted group that consumed the same absolute amount of protein but only two-thirds of the calories. In addition, two groups were initially fed diets containing 20% casein; then the casein level was either gradually (0.8%/month) decreased to 12% or increased to 28% by 1 year of age

TABLE 1

Composition of a Typical Diet
(20% Casein)

Dietary Component	Percent of Diet
Casein[1,2]	20.0
Cornstarch	29.0
Sucrose	29.0
Alphacel[1]	9.0
Crisco vegetable shortening	6.0
Salt mix[1,3]	3.0
Wesson vegetable oil	2.0
Vitamin mix[1,4]	2.0

[1]Nutritional Biochemical Corporation, Cleveland, Ohio 44128.

[2]Determined by the Kjeldahl method to contain 91.5% protein.

[3]Formulated to supply the following amounts of minerals (g/kg salt mixture): $CaCO_3$, 54.3.0; $MgCO_3$, 25.0; $MgSO_4$, 16.0; NaCl, 69.0; KCL, 112.0; KH_2PO_4, 212.0; $FePO_4 \cdot 4H_2O$, 20.5; KI, 0.08; $MnSO_4$, 0.35; NaF, 1.00; $Al_2(SO_4)K_2SO_4$, 0.17; and $CuSO_4$, 0.90.

[4]Vitamin Diet Fortification Mixture formulated to supply the following amounts of vitamins (g/kg vitamin mix): vitamin A, 4.5; vitamin D, 0.25; thiamin hydrochloride, 1.0; riboflavin, 1.0; niacin, 4.5; p-amino-benzoic acid, 5.0; calcium pantothenate, 3.0; pyridoxine hydrochloride, 1.0; ascorbic acid, 45.0; inositol, 5.0; choline chloride, 75.0; menadione, 2.25; biotin, 0.02; folic acid, 0.09; vitamin B_{12}, 0.00135; and alpha-tocopherol, 5.0. Sufficient dextrose was added to make 1 kg.

and maintained at those levels until the animals were sacrificed. Equal amounts of the carbohydrate components were manipulated so that the ad libitum diets remained isocaloric as the protein levels were changed; this was also the procedure for the restricted diets. At sacrifice, the carcasses were stored in a freezer maintained at 0°C until bone analyses could be performed. As shown in Table 2, the number of animals alive and available for analysis in each experimental group was variable. Femur composition was analyzed by the methods of Barzel (50) and Whiting and Draper (60); the analyses included length, wet weight, dry weight, fat-free dry weight, fat and ash.

Length and Wet Weight

The right femur was removed from the rat carcass, cleaned of adherent soft tissue, and measured for length using a micrometer. Then, the sample femur was placed on a preweighed aluminum foil square, weighed and stored in the refrigerator at 4°C until further analyses could be made.

Dry Weight

For determination of dry weight, the femur was dried at 105°C for 24 hours on the aluminum foil to evaporate any remaining water, cooled in a dessicator to room temperature, and then reweighed.

Fat Content

To extract the fat, each femur was wrapped in cotton gauze with a small piece of cardboard which had an identification number marked

TABLE 2

Number of Animals Sacrificed by Age and Dietary Treatment[1]

Dietary Treatment and Level of Protein	Age at Sacrifice	
	Young (1 yr)	Old (2 yr)
Ad Libitum		
Constant		
12%	8	5
20%	9	5
28%	8	7
Changing[2]		
20 → 12%	9	5
20 → 28%	12	5
Restricted[3]		
18%	9	5
30%	10	8
42%	11	7

[1]There were initially 12 animals in each group to be sacrificed at 1 year of age and 36 in each group to be sacrificed at 2 years of age (n = 384 initially) but some of the animals died before the time of sacrifice.

[2]Changing-protein level groups were initially fed diets containing 20% casein then the casein level was either gradually (0.8%/month) decreased to 12% or increased to 28% by 1 year of age and maintained at those levels until sacrifice.

[3]Restricted-fed rats were fed one-third less calories but the same amount of protein as their ad libitum-fed controls.

on it with a lead pencil. Eight femurs were extracted in a Soxhlet unit at a time. About 125 ml of ethyl ether were used as the extraction solvent, and the extraction time was 24 hours. After the extraction, the femurs were unwrapped, immersed in acetone to remove any remaining diethyl ether, and air dried overnight. Then, they were dried again on aluminum foil at 105°C for 6 hours and cooled in a dessicator to room temperature. Each femur was weighed, and the fat content was determined by the difference between the dry weight and the fat-extracted dry weight.

Ash Content

For determination of femur ash, each femur was placed in a preweighed labeled crucible and ashed in a muffle furnace at 550°C for 24 hours. After the ashing process, each ash-containing crucible was allowed to cool to room temperature in a dessicator and then weighed. The ash content was determined by the difference between the crucible-ash weight and the crucible weight alone.

Calculations

The equations used to calculate the percentages of fat, water, and ash of the femurs were as follows:

$$\text{Percent Water} = \frac{(\text{Wet Bone Wt.} - \text{Dry Bone Wt.}) \times 100}{\text{Wet Bone Wt.}}$$

$$\text{Percent Fat} = \frac{\text{Fat Wt. of Bone} \times 100}{\text{Dry Bone Wt.}}$$

$$\text{Percent Ash} = \frac{\text{Ash Wt. of Bone} \times 100}{\text{Fat-Free Dry Bone Wt.}}$$

<u>Statistical Analysis</u>

The relationships among age, caloric restriction, and level of dietary protein were analyzed by procedures available in GLM (general linear models) of SAS (Statistical Analysis System), a computer software package (78). Analysis of variance was used to test the overall differences among dietary treatments and age (79). Specific differences among group means for the effect of calories (constant ad libitum vs restricted), protein (constant vs changing ad libitum, 20 → 12% vs 20 → 28%, and linear and/or quadratic for constant ad libitum vs restricted) and the interaction of calories and protein (linear and/or quadratic interaction for constant ad libitum vs restricted) were tested with orthogonal contrasts (80). The group means and standard errors were obtained using the least-squares means option in GLM. In the contrast statements, both ages were combined when there was no significant diet x age interaction, and the results were considered statistically significant when the probability level was $p < 0.05$.

CHAPTER IV

RESULTS

The effects of caloric restriction, level of dietary protein, and age on femur length of the ad libitum- and restricted-fed rats are shown in Table 3. In the statistical analysis, both ages were combined when there was no significant diet by age interaction as was the case with femur length. The femurs of the ad libitum-fed group were significantly longer than those of the restricted-fed group ($p < 0.0001$). The level of protein alone or the level of protein by level of caloric restriction had no significant linear or quadratic relationship to femur length. The femur length of the constant protein ad libitum-fed group was not significantly different from that of the changing protein ad libitum-fed group. Of the changing ad libitum-fed groups, the 20 → 28% protein group had significantly longer femurs than the 20 → 12% protein group ($p < 0.001$). The mean femur length of old animals was significantly greater ($p < 0.001$) than that of young animals.

The mean wet femur weight of the ad libitum-fed group was significantly greater than the restricted-fed group ($p < 0.0001$, Table 4). The level of protein influenced the wet femur weight in both a linear ($p < 0.02$) and quadratic ($p < 0.02$) fashion. When the level of protein by caloric level interaction was determined for the ad libitum-fed and restricted-fed groups, the relationship to wet femur weight was quadratic ($p < 0.004$). No significant difference was found between the

TABLE 3

Effect of Caloric Restriction, Level of Dietary Protein and Age on Mean Femur Length of Ad Libitum- and Restricted-Fed Rats

Dietary Treatment and Protein Level	Femur Length		
	Young (1 yr)	Old (2 yr)	Both Ages
	cm	cm	cm
Ad Libitum			
Constant			
12%	4.16	4.18	4.17 ± 0.02[1]
20%	4.16	4.20	4.18 ± 0.02
28%	4.16	4.21	4.19 ± 0.02
Changing[2]			
20 → 12%	4.07	4.18	4.12 ± 0.02
20 → 28%	4.18	4.31	4.24 ± 0.02
Restricted[3]			
18%	4.12	4.16	4.14 ± 0.02
30%	4.12	4.14	4.13 ± 0.02
42%	4.11	4.14	4.13 ± 0.02
Mean ± SEM for column	4.14 ± 0.01	4.19 ± 0.01[4]	

Contrast Statements--P Values[5]

Ad Libitum vs Restricted	< 0.0001[6]
Protein Intake[7]	
Linear	NS[8]
Quadratic	NS
Protein Intake x Caloric Level[9]	
Linear	NS
Quadratic	NS
Constant Ad Libitum vs Changing Ad Libitum	NS
20 → 12% vs 20 → 28%	< 0.001

TABLE 3 (Continued)

[1]LS Mean ± SEM.

[2]Changing-protein level groups were initially fed diets containing 20% casein then the casein level was either gradually (0.8%/month) decreased to 12% or increased to 28% by 1 year of age and maintained at those levels until sacrifice.

[3]Restricted-fed rats were fed one-third less calories but the same amount of protein as their ad libitum-fed controls.

[4]Values of old animals were significantly greater ($p < 0.001$) than those of young animals.

[5]Both ages were combined; there was no significant diet by age interaction.

[6]Mean ± SEM for Ad Libitum = 4.18 ± 0.01; Mean ± SEM for Restricted = 4.13 ± 0.01.

[7]Constant ad libitum and restricted groups with same protein intake were combined; ad libitum groups with changing protein levels not included.

[8]$P > 0.05$ designated as NS (nonsignificant).

[9]Does not include ad libitum-fed rats consuming diets with changing levels of protein.

TABLE 4

Effect of Caloric Restriction, Level of Dietary Protein and Age on Mean Wet Femur Weight of Ad Libitum- and Restricted-Fed Rats

Dietary Treatment and Protein Level	Wet Femur Weight		
	Young (1 yr)	Old (2 yr)	Both Ages
	g	g	g
Ad Libitum			
Constant			
12%	1.242	1.420	1.331 ± 0.039[1]
20%	1.199	1.288	1.244 ± 0.038
28%	1.310	1.568	1.439 ± 0.035
Changing[2]			
20 → 12%	1.261	1.568	1.414 ± 0.038
20 → 28%	1.366	1.590	1.478 ± 0.036
Restricted[3]			
18%	1.095	1.163	1.129 ± 0.037
30%	1.118	1.216	1.167 ± 0.032
42%	1.128	1.220	1.174 ± 0.032
Mean ± SEM for column	1.215 ± 0.015	1.379 ± 0.020[4]	
Contrast Statements--P Values[5]			
Ad Libitum vs Restricted			< 0.0001[6]
Protein Intake[7]			
Linear			< 0.02
Quadratic			< 0.02
Protein Intake x Caloric Level[8]			
Linear			NS[9]
Quadratic			< 0.004
Constant Ad Libitum vs Changing Ad Libitum			NS
20 → 12% vs 20 → 28%			< 0.001

TABLE 4 (Continued)

[1] LS Mean ± SEM.

[2] Changing-protein level groups were initially fed diets containing 20% casein then the casein level was either gradually (0.8%/month) decreased to 12% or increased to 28% by 1 year of age and maintained at those levels until sacrifice.

[3] Restricted-fed rats were fed one-third less calories but the same amount of protein as their ad libitum-fed controls.

[4] Values of old animals were significantly greater ($p < 0.001$) than those of young animals.

[5] Both ages were combined; there was no significant diet by age interaction.

[6] Mean ± SEM for Ad libitum = 1.32 ± 0.02; Mean ± SEM for Restricted = 1.15 ± 0.02.

[7] Constant ad libitum and restricted groups with same protein intake were combined; ad libitum groups with changing protein levels not included.

[8] Does not include ad libitum-fed rats consuming diets with changing levels of protein.

[9] $P > 0.05$ designated as NS (nonsignificant).

wet femur weight of the constant ad libitum-fed group and the changing ad libitum-fed group. In the changing ad libitum-fed groups, the 20 → 28% protein group had a wet femur weight significantly greater than that of the 20 → 12% protein group ($p < 0.001$). In addition, the mean femur weight of the old animals was significantly greater ($p < 0.001$) than that of the young animals.

The mean dry femur weight of the ad libitum-fed animals was significantly greater than the mean dry femur weight of the restricted-fed animals ($p < 0.0001$, Table 5). In the ad libitum-fed and restricted-fed groups, there were both linear ($p < 0.05$) and quadratic ($p < 0.02$) relationships between the level of dietary protein and dry femur weight. When the level of protein x caloric intake interaction was considered, the relationship to dry femur weight was quadratic ($p < 0.05$). There was no significant difference in dry femur weight between the constant ad libitum-fed group and the changing ad libitum-fed group. Furthermore, no significant difference in dry femur weight was observed between the animals fed 20 → 12% protein and those fed 20 → 28% protein. The mean dry femur weight of the old rats was significantly greater ($p < 0.0001$) than that of the young rats.

The fat-free dry femur weight of ad libitum-fed rats was greater than that of restricted-fed rats ($p < 0.0001$, Table 6). When the ad libitum- and restricted-fed groups with the same protein intake were combined, protein level affected fat-free dry femur weight quadratically ($p < 0.005$). Also, a quadratic relationship was found between the fat-free dry femur weight and the level of protein x caloric intake

TABLE 5

Effect of Caloric Restriction, Level of Dietary Protein and Age on Mean Dry Femur Weight of Ad Libitum- and Restricted-Fed Rats

Dietary Treatment and Protein Level	Dry Femur Weight		
	Young (1 yr)	Old (2 yr)	Both Ages
	g	g	g
Ad Libitum			
Constant			
12%	0.900	1.015	0.958 ± 0.028[1]
20%	0.868	0.938	0.903 ± 0.026
28%	0.932	1.102	1.017 ± 0.025
Changing[2]			
20 → 12%	0.889	1.085	0.987 ± 0.027
20 → 28%	0.969	1.076	1.022 ± 0.026
Restricted[3]			
18%	0.772	0.820	0.796 ± 0.026
30%	0.796	0.806	0.801 ± 0.023
42%	0.796	0.858	0.827 ± 0.023
Mean ± SEM for column	0.865 ± 0.011	0.962 ± 0.014[4]	
Contrast Statements--P Values[5]			
Ad Libitum vs Restricted			< 0.0001[6]
Protein Intake[7]			
Linear			< 0.05
Quadratic			< 0.02
Protein Intake x Caloric Level[8]			
Linear			NS[9]
Quadratic			< 0.05
Constant Ad Libitum vs Changing Ad Libitum			NS
20 → 12% vs 20 → 28%			NS

TABLE 5 (Continued)

[1]LS Mean ± SEM.

[2]Changing-protein level groups were initially fed diets containing 20% casein then the casein level was either gradually (0.8%/month) decreased to 12% or increased to 28% by 1 year of age and maintained at those levels until sacrifice.

[3]Restricted-fed rats were fed one-third less calories but the same amount of protein as their ad libitum-fed controls.

[4]Values of old animals were significantly greater ($p < 0.0001$) than those of young animals.

[5]Both ages were combined; there was no significant diet by age interaction.

[6]Mean ± SEM for Ad Libitum = 0.949 ± 0.014; Mean ± SEM for Restricted = 0.802 ± 0.013.

[7]Constant ad libitum and restricted groups with same protein intake were combined; ad libitum groups with changing protein levels not included.

[8]Does not include ad libitum-fed rats consuming diets with changing levels of protein.

[9]$P > 0.05$ designated as NS (nonsignificant).

TABLE 6

Effect of Caloric Restriction, Level of Dietary Protein and Age on Mean Fat-Free Dry Femur Weight of Ad Libitum- and Restricted-Fed Rats

Dietary Treatment and Protein Level	Fat-Free Dry Femur Weight		
	Young (1 yr)	Old (2 yr)	Both Ages
	g	g	g
Ad Libitum			
Constant			
12%	0.826	0.921	0.873 ± 0.026[1]
20%	0.799	0.793	0.796 ± 0.025
28%	0.840	0.972	0.906 ± 0.023
Changing[2]			
20 → 12%	0.828	0.999	0.913 ± 0.025
20 → 28%	0.880	0.932	0.906 ± 0.024
Restricted[3]			
18%	0.703	0.719	0.711 ± 0.024
30%	0.700	0.739	0.719 ± 0.021
42%	0.732	0.750	0.741 ± 0.021
Mean ± SEM for column	0.788 ± 0.010	0.853 ± 0.014[4]	
Contrast Statements--P Values[5]			
Ad Libitum vs Restricted			< 0.0001[6]
Protein Intake[7]			
Linear			NS[8]
Quadratic			< 0.005
Protein Intake x Caloric Level[9]			
Linear			NS
Quadratic			< 0.01
Constant Ad Libitum vs Changing Ad Libitum			NS
20 → 12% vs 20 → 28%			NS

TABLE 6 (Continued)

[1]LS Mean ± SEM.

[2]Changing-protein level groups were initially fed diets containing 20% casein then the casein level was either gradually (0.8%/month) decreased to 12% or increased to 28% by 1 year of age and maintained at those levels until sacrifice.

[3]Restricted-fed rats were fed one-third less calories but the same amount of protein as their ad libitum-fed controls.

[4]Values of old animals were significantly greater ($p < 0.0003$) than those of young animals.

[5]Both ages were combined; there was no significant diet by age interaction.

[6]Mean ± SEM for Ad Libitum = 0.854 ± 0.012; Mean ± SEM for Restricted = 0.721 ± 0.011.

[7]Constant ad libitum and restricted groups with same protein intake were combined; ad libitum groups with changing protein levels not included.

[8]$P > 0.05$ designated as NS (nonsignificant).

[9]Does not include ad libitum-fed rats consuming diets with changing levels of protein.

interaction in the combined ad libitum- and restricted-fed groups ($p < 0.01$). No significant differenct was found between the fat-free dry femur weight of the constant and the changing ad libitum-fed rats. There was no significant difference between the 20 → 12% and the 20 → 28% protein ad libitum-fed groups. Fat-free dry femur weight was greater ($p < 0.0003$) in old rats than in young rats.

The femur ash weight of the constant ad libitum-fed group was significantly greater than that of the restricted-fed group ($p < 0.0001$, Table 7). There was a quadratic effect of the level of dietary protein on ash weight ($p < 0.01$). Furthermore, a quadratic relationship was found between ash weight and the interaction of the level of protein x caloric intake in the ad libitum- and restricted-fed groups ($p < 0.006$). The ash weight of the constant ad libitum-fed group was significantly less ($p < 0.01$) than that of the changing ad libitum-fed group. In the changing ad libitum-fed groups, there was no significant difference in the ash weight of the 20 → 12% protein group and the 20 → 28% protein group. The mean femur ash weight was significantly greater ($p < 0.006$) in old animals than in the young ones.

The femurs of the ad libitum-fed rats contained a lesser percent water than the restricted-fed rats ($p < 0.0001$, Table 8). In addition, neither the linear nor the quadratic relationship between the level of dietary protein and percent femur water was significant; however, the interaction of protein x caloric intake did show a quadratic relationship ($p < 0.005$). The constant ad libitum-fed group contained less percent water than the changing ad libitum-fed group ($p < 0.02$). There

TABLE 7

Effect of Caloric Restriction, Level of Dietary Protein and Age on Mean Femur Ash Weight of Ad Libitum- and Restricted-Fed Rats

Dietary Treatment and Protein Level	Ash Weight		
	Young (1 yr)	Old (2 yr)	Both Ages
	g	g	g
Ad Libitum			
Constant			
12%	0.570	0.614	0.592 ± 0.020[1]
20%	0.531	0.533	0.532 ± 0.019
28%	0.582	0.672	0.627 ± 0.018
Changing[2]			
20 → 12%	0.602	0.705	0.654 ± 0.020
20 → 28%	0.599	0.646	0.623 ± 0.019
Restricted[3]			
18%	0.477	0.495	0.486 ± 0.019
30%	0.507	0.492	0.499 ± 0.017
42%	0.491	0.514	0.503 ± 0.017
Mean ± SEM for column	0.545 ± 0.008	0.584 ± 0.010[4]	
Contrast Statements--P Values[5]			
Ad Libitum vs Restricted			< 0.0001[6]
Protein Intake[7]			
Linear			NS[8]
Quadratic			< 0.01
Protein Intake x Caloric Level[9]			
Linear			NS
Quadratic			< 0.006
Constant Ad Libitum vs Changing Ad Libitum			< 0.01[10]
20 → 12% vs 20 → 28%			NS

TABLE 7 (Continued)

[1]LS Mean ± SEM.

[2]Changing-protein level groups were initially fed diets containing 20% casein then the casein level was either gradually (0.8%/month) decreased to 12% or increased to 28% by 1 year of age and maintained at those levels until sacrifice.

[3]Restricted-fed rats were fed one-third less calories but the same amount of protein as their ad libitum-fed controls.

[4]Values of old animals were significantly greater ($p < 0.006$) than those of young animals.

[5]Both ages were combined; there was no significant diet by age interaction.

[6]Mean ± SEM for Ad Libitum = 0.581 ± 0.010; Mean ± SEM for Restricted = 0.494 ± 0.009.

[7]Constant ad libitum and restricted groups with same protein intake were combined; ad libitum groups with changing protein levels not included.

[8]$P > 0.05$ designated as NS (nonsignificant).

[9]Does not include ad libitum-fed rats consuming diets with changing levels of protein.

[10]Mean ± SEM for Constant Ad Libitum = 0.581 ± 0.010; Mean ± SEM for Changing Ad Libitum = 0.637 ± 0.014.

TABLE 8

Effect of Caloric Restriction, Level of Dietary Protein and Age on Mean Percent Water in Femurs of Ad Libitum- and Restricted-Fed Rats

Dietary Treatment and Protein Level	Percent Water		
	Young (1 yr)	Old (2 yr)	Both Ages
	%	%	%
Ad Libitum			
Constant			
12%	27.44	28.62	28.03 ± 0.79[1]
20%	27.71	27.23	27.47 ± 0.76
28%	28.76	29.58	29.17 ± 0.72
Changing[2]			
20 → 12%	29.45	30.41	29.93 ± 0.77
20 → 28%	29.07	32.16	30.61 ± 0.74
Restricted[3]			
18%	29.51	29.52	29.52 ± 0.75
30%	28.73	33.60	31.16 ± 0.66
42%	29.45	29.68	29.56 ± 0.66
Mean ± SEM for column	28.77 ± 0.31	30.10 ± 0.41[4]	

Contrast Statements--P Values[5]

Ad Libitum vs Restricted	< 0.0001[6]
Protein Intake[7]	
Linear	NS[8]
Quadratic	NS
Protein Intake x Caloric Level[9]	
Linear	NS
Quadratic	< 0.005
Constant Ad Libitum vs Changing Ad Libitum	< 0.02[10]
20 → 12% vs 20 → 28%	NS

TABLE 8 (Continued)

[1]LS Mean ± SEM.

[2]Changing-protein level groups were initially fed diets containing 20% casein then the casein level was either gradually (0.8%/month) decreased to 12% or increased to 28% by 1 year of age and maintained at those levels until sacrifice.

[3]Restricted-fed rats were fed one-third less calories but the same amount of protein as their ad libitum-fed controls.

[4]Values of old animals were significantly greater ($p < 0.006$) than those of young animals.

[5]Both ages were combined; there was no significant diet by age interaction.

[6]Mean ± SEM for Ad Libitum = 28.20 ± 0.35; Mean ± SEM for Restricted = 29.98 ± 0.32.

[7]Constant ad libitum and restricted groups with same protein intake were combined; ad libitum groups with changing protein levels not included.

[8]$P > 0.05$ designated as NS (nonsignificant).

[9]Does not include ad libitum-fed rats consuming diets with changing levels of protein.

[10]Mean ± SEM for Constant Ad Libitum = 28.20 ± 0.35; Mean ± SEM for Changing Ad Libitum = 30.31 ± 0.52.

was no significant difference in percent water between the 20 → 12% protein group and the 20 → 28% protein group. The percent water in femurs of old rats was greater ($p < 0.006$) than in the young rats.

Since there was a diet by age interaction ($p < 0.0002$) for the percent fat in the femurs, significance was tested at each age (Table 9). There was a significant relationship between the percent fat and the interaction of the level of protein x caloric intake where a quadratic effect was noted for both the young ($p < 0.005$) and the old ($p < 0.0001$) animals. In the old animals, there was a greater percent fat in femurs of the 20 → 28% group than in the 20 → 12% group ($p < 0.003$). No other significant differences were observed between the dietary treatments at each age and percent femur fat.

When ash was expressed on a percentage basis (Table 10), there were no statistically ($p < 0.05$) significant differences observed. Significance was approached in the comparison of the constant and changing protein ad libitum-fed groups where the percent ash was slightly less ($p < 0.06$) in the constant group than in the changing one. Significance was also approached within the changing groups where the percent ash of the 20 → 28% protein group was slightly less ($p < 0.06$) than that of the 20 → 12% group. No significant age differences were observed in femur percent ash values.

TABLE 9

Effect of Caloric Restriction, Level of Dietary Protein and Age on Mean Percent Fat in Femurs of Ad Libitum- and Restricted-Fed Rats

Dietary Treatment and Protein Level	Percent Fat	
	Young (1 yr)	Old (2 yr)
	%	%
Ad Libitum		
Constant		
12%	8.30 ± 1.05[1]	9.12 ± 1.33
20%	7.97 ± 0.94	15.11 ± 1.33
28%	9.90 ± 1.05	11.78 ± 1.12
Changing[2]		
20 12%	7.03 ± 0.99	7.77 ± 1.33
20 28%	9.11 ± 0.86	13.48 ± 1.33
Restricted[3]		
18%	8.80 ± 0.90	12.28 ± 1.33
30%	12.03 ± 0.94	8.52 ± 1.05
42%	8.00 ± 0.86	12.56 ± 1.12
Contrast Statements--P Values[4]		
Ad Libitum vs Restricted	NS[5]	NS
Protein Intake[6]		
Linear	NS	NS
Quadratic	NS	NS
Protein Intake x Caloric Level[7]		
Linear	NS	NS
Quadratic	< 0.005	< 0.0001
Constant Ad Libitum vs Changing Ad Libitum	NS	NS
20 → 12% vs 20 → 28%	NS	< 0.003

TABLE 9 (Continued)

[1]LS Mean ± SEM.

[2]Changing-protein level groups were initially fed diets containing 20% casein then the casein level was either gradually (0.8%/month) decreased to 12% or increased to 28% by 1 year of age and maintained at those levels until sacrifice.

[3]Restricted-fed rats were fed one-third less calories but the same amount of protein as their ad libitum-fed controls.

[4]Values were given at each age because there was a diet by age interaction ($p < 0.0002$).

[5]$P > 0.05$ designated as NS (nonsignificant).

[6]Constant ad libitum and restricted groups with same protein intake were combined; ad libitum groups with changing protein levels not included.

[7]Does not include ad libitum-fed rats consuming diets with changing levels of protein.

TABLE 10

Effect of Caloric Restriction, Level of Dietary Protein and Age on Mean Percent Ash in Femurs of Ad Libitum- and Restricted-Fed Rats

Dietary Treatment and Protein Level	Percent Ash		
	Young (1 yr)	Old (2 yr)	Both Ages
	%	%	%
Ad Libitum			
Constant			
12%	68.93	66.62	67.77 ± 1.65[1]
20%	66.44	67.12	66.78 ± 1.58
28%	69.54	68.90	69.22 ± 1.50
Changing[2]			
20 → 12%	73.79	70.50	72.15 ± 1.61
20 → 28%	68.28	69.31	68.80 ± 1.54
Restricted[3]			
18%	67.93	69.04	68.48 ± 1.56
30%	72.44	65.55	69.00 ± 1.43
42%	67.12	68.80	67.96 ± 1.38
Mean ± SEM for column	69.31 ± 0.65	68.23 ± 0.86	

Contrast Statements--P Values[4]

Ad Libitum vs Restricted	NS[5]
Protein Intake[6]	
Linear	NS
Quadratic	NS
Protein Intake x Caloric Level[7]	
Linear	NS
Quadratic	NS
Constant Ad Libitum vs Changing Ad Libitum	NS
20 → 12% vs 20 → 28%	NS

TABLE 10 (Continued)

[1]LS Mean ± SEM.

[2]Changing-protein level groups were initially fed diets containing 20% casein then the casein level was either gradually (0.8%/month) decreased to 12% or increased to 28% by 1 year of age and maintained at those levels until sacrifice.

[3]Restricted-fed rats were fed one-third less calories but the same amount of protein as their ad libitum-fed controls.

[4]Both ages were combined when there was no significant diet by age interaction.

[5]$P > 0.05$ designated as NS (nonsignificant).

[6]Constant ad libitum and restricted groups with same protein intake were combined; ad libitum groups with changing protein levels not included.

[7]Does not include ad libitum-fed rats consuming diets with changing levels of protein.

CHAPTER V

DISCUSSION

In another phase of this study (75, 76), it was shown that the caloric restriction imposed, as described in Chapter III (Methods), caused a decreased mature body weight. Furthermore, caloric restriction was associated with an improvement in kidney function and an increase in survival at 2 years of age. Protein restriction was related to decreased growth rate, improved renal function, and a diminished survival rate. The investigators concluded that even though high levels of dietary protein adversely affected kidney function, diets containing increasing amounts of protein must be beneficial to the overall physiology of the rats and thereby increase survival.

Since the restricted-fed animals were smaller in size (75, 76), it was expected that their femurs would, in general, be smaller than those of the ad libitum-fed animals. The caloric level had a highly significant effect on femur length; however, the data were not distributed in either a linear or quadratic fashion as the level of dietary protein increased. This was in agreement with a study by Calvo et al. (9) in which they found no differences in the femurs and mandibles of rats fed high protein diets for 20 weeks and those of the control group. Furthermore, Dekanic (64) showed that in a chronic state of acidosis, which could occur with long-term ingestion (8 months) of high levels of protein, the femurs were similar in length to the controls

even though they were deficient in the mineral component. Also, Whiting and Draper (51) observed no significant difference in the mean femur length of animals fed a high protein diet for 10 months and that of those fed a control diet. In the present study when the diets were tested to see if a pattern to the femur length data existed, there was no linear or quadratic relationship to protein, nor was there an interaction of protein level by caloric intake. The major difference between the present study and those cited concerning femur length was the much greater duration of the experimental period in this investigation resulting in data from a 2-year period versus 20 weeks (9), 8 months (64), or 10 months (51), respectively.

Changing the level of protein over time was a concept introduced in this study. There was no significant difference between the constant protein ad libitum-fed group and the changing protein ad libitum-fed group which indicated that inducing a dietary protein change during the first year of life in the rats was not detrimental to femur length. However, within the changing groups, it was found that gradually increasing the amount of protein was more beneficial to the lengthening of the femur than decreasing the amount of dietary protein. The mean femur length of the old animals being significantly greater than the young animals is consistent with the findings of Newell and Beauchene (63). Apparently the long bones of the rat continue to grow after sexual maturity (81) and even during the second year of life.

As was expected, the femurs of the ad libitum-fed rats were heavier on a wet bone weight basis than those of the restricted-fed

ones. This was in agreement with the findings that femur length was significantly greater in the ad libitum-fed animals. It may have been that the protein was beneficial in depositing ash only if enough calories were present in the diet; consequently in the restricted-fed animals, the process could have been calorie-limited. It appeared the level of protein influenced the mean wet femur weight in both a linear and a quadratic fashion. Presently, neither the author nor the literature can give justification for these results; further investigation is needed with a larger sample size or more than three levels of protein. When the level of protein by level of caloric restriction was determined for the ad libitum-fed and restricted-fed groups, the relationship to wet femur weight was quadratic.

The femurs of the constant ad libitum-fed groups were not significantly heavier on a wet bone weight basis than those of the changing ad libitum-fed groups. These data may lead to the conclusion that if the caloric intake is not restricted and the level of protein is similar over time, it makes no difference whether the protein level is constant or changing. Within the changing ad libitum-fed groups, the 20 → 28% protein group had a mean wet femur weight that was greater than that of the 20 → 12% protein group. This finding is in general agreement with the constant protein groups where the femurs from the group with the highest level of protein were heavier on a wet bone weight basis than the femurs from the lowest protein group. Furthermore, old animals had femurs that were significantly heavier than young animals which may have resulted from a continuing of some bone growth

and development after maturity. Since the mean femur length of old animals was significantly greater than that of young animals, it was expected that they also would be heavier.

Hammet (82) suggested that less water corresponded to a greater amount of ash indicating an increase in bone mineralization. Thus, the lesser percent water in the femurs of the ad libitum-fed as compared to the restricted-fed animals would be consistent with greater amounts of ash in the ad libitum-fed animals; on the contrary, no significant difference was observed when ash was expressed on a percentage basis. When the effect of the level of dietary protein was tested, no linear nor quadratic relationship was observed for percent water. However, when protein intake by caloric level was studied, the ad libitum-fed group appeared to respond differently than the restricted-fed group. In the femurs of the ad libitum-fed rats, there was a tendency for the 20% protein group to have a lesser mean percent water than either the 12% or the 28% protein group; however, in the restricted-fed rats, the trend for the 30% protein group was to have a greater mean percent water than the other two groups. Further investigation is needed to explain the interaction between the level of dietary protein and caloric intake.

In comparing a constant level of protein to changing levels over time, it was found that the mean percent water in femurs of the constant group was significantly less. Either increasing or decreasing the level of dietary protein during the first year of life may have stressed the rats resulting in decreased mineralization and an increased

percent water in the femurs. Although there was a significant difference in the percent water between the constant and changing protein ad libitum-fed groups, there was no difference induced by gradually increasing or decreasing the level of protein. In the present study, the mean percent water was greater in old animals than in young ones suggesting a decrease in mineralization in the old animals; however, mean percent ash did not differ significantly between the two groups. This finding was not in agreement with a human study by Mueller et al. (83) in which water content decreased as mineralization increased. On the contrary, Vogt and Tønsager(84) found only a decrease in bone water content with increasing age.

In an investigation by Nichols and Nichols (85) on the effect of acidosis on the mineral content of bone, an increase in bone fat with age was observed. Newell and Beauchene (63) also found a significantly greater fat content in the tibias of old rats than that in the young ones; they reported, however, that acid stress did not significantly affect the tibia analysis in either age group. In the present study, the percent fat was presented at each age because of the diet by age interaction; for example, different diets caused different effects depending on the age of the rats. The diet by age interaction for percent fat was an effect noted in the present study but not found in the literature; neither Nichols and Nichols (85) nor Newell and Beauchene (63) researched the possibility of a diet by age interaction. In both the young and old animals, there was no significant difference in percent fat between the ad libitum-fed and restricted-fed

groups; furthermore, no significant differences were noted among the levels of dietary protein. Therefore, neither caloric intake nor level of protein alone had any significant effect on percent femur fat.

However, a quadratic effect was observed between percent fat and the protein intake by caloric level, and there was a diet by age interaction. In the young ad libitum-fed animals, the 20% protein group had femurs containing a lesser percent fat than either the 12% or 28% group; but in the young restricted-fed groups, the 30% protein group had femurs containing a greater percent fat than either the 18% of 42% group. On the contrary, the 20% protein ad libitum-fed group in the old rats had a greater percent fat than the 12% or 28% group while the 30% protein group of the restricted-fed rats had femurs with a lesser percent fat than the other two groups. A rational explanation for these results cannot be offered by the author or found in the literature.

Within the changing groups of the old rats, the 20 → 28% protein group had a greater percent femur fat than the 20 → 12% protein group. The higher level of dietary protein in the 20 → 28% group may have been responsible for increased femur size and possibly a larger marrow cavity containing a greater amount of fat.

Bone ash, expressed either on an absolute basis or as a percentage, has been used as an indirect measurement of bone mineralization and density (64). When the ad libitum-fed and restricted-fed groups were compared, the femurs of the ad libitum-fed group contained a greater amount of absolute ash than those of the restricted-fed group. On the

contrary to the present long-term study (2-year experimental period), Shires et al. (13) found no significant difference in the tibia ash weight in semistarved rats versus the controls but suggested the results may have been influenced by the short duration of the caloric deprivation (48 days). When the ash was expressed as a percentage, there was no significant difference between the two groups. As the level of protein increased, a quadratic distribution was observed in the absolute ash weight. However, no significant linear or quadratic relationship was seen when the ash was expressed on a percentage basis. That larger femurs (absolute ash not percent ash) were found in ad libitum-fed animals is consistent with the results of Allen and Hall (52); they concluded that differences in bone development between two groups of rats fed different levels of protein were explained by the larger body weights of the animals fed high protein diets. Other investigators studying the effects of high protein diets on bone composition noted no changes that would indicate increased bone loss related to the increased level of dietary protein (9, 10). When the effect of protein deficiency on bone quality was examined by Jha et al. (38) and LeRoith and Pimstone (86), there was no significant difference in the percent ash between the controls and that of rats fed less dietary protein which was in agreement with the findings of the present study. Changing the level of protein throughout the first year of life appeared to have a positive effect on bone mineralization because the absolute ash weight was significantly greater in the changing ad libitum-fed animals than in the constant ones and percent ash also approached

significance. Within the changing groups, no significant difference in mean absolute ash weight was observed between the 20 → 12% protein group and the 20 → 28% protein group; furthermore, when ash was expressed as a percentage of fat-free dry weight, the 20 → 12% protein group did not contain a greater percent femur ash but significance was approached. In regard to the relationship of age and the mineral component of bone, the absolute ash weight of the old animals was significantly greater than that of the young animals but the percent ash was not different. Since the old rats had larger bones than the young ones, a greater amount of ash by weight was expected. Furthermore, the percent ash remained fairly constant from birth to old age which is in agreement with a study by Mueller et al. (83) on age-related changes in bone density and composition.

In agreement with the present study, Allen and Hall (52) suggested that there was no significant difference in bone mineralization between their control (18% casein) and their experimental group (36% casein) after a 32-day consumption period. Furthermore, LeRoith and Pimstone (86) found that some bone growth continued to occur in protein-deprived rats even when there was no weight gain. Also in agreement with the present study, Matkovic et al. (73) concluded from a study on bone status of humans that nutrition was important in determining bone mass in young adults but seemed to have little effect on age-related bone loss. Therefore, the present investigator hypothesizes that ad libitum-feeding, especially during growth and development, and the resultant high body weights (76), may have been responsible for

the development of femurs that were longer, heavier on a wet bone weight basis, and contained greater amounts of ash but exhibited no difference in the percent ash; furthermore, the highest level of dietary protein may also have been partially responsible for femurs that were heavier (wet weight) than those of animals fed lower levels. On the contrary, changing the level of protein over time was neither detrimental nor beneficial to the femur length and wet weight.

CHAPTER VI

SUMMARY

Three groups of rats were fed diets containing 12, 20, or 28% casein (protein) ad libitum. Restricted-fed rats were fed one-third less calories than ad libitum-fed rats with casein levels of 18, 30, or 42%. Two other groups of rats were fed ad libitum diets which were gradually (0.8%/month) increased or decreased in casein content, i.e., from 20% at 1 month of age to either 28 or 12% at 1 year of age. Groups of animals on the diets were sacrificed at either 1 or 2 years of age. Femur components, expressed in terms of either absolute amounts or on percentage bases, were calculated in young and old rats. Wet length and weight, dry weight, fat-free dry weight, and ash weight of femurs were measured to note if any differences occurred due to caloric restriction, level of dietary protein and/or age-related changes. Furthermore, the percentage compositions of water, fat, and ash were calculated so that the size of the individual bones would not be a factor.

The individual femur components differed to varying degrees according to which protein level, caloric intake, and age group combinations were compared statistically. Only percent fat had to be presented at each age because there was a diet by age interaction.

The femurs of the ad libitum-fed groups were longer, heavier on a wet bone weight basis, and contained greater amounts of ash than those of the calorie-restricted groups. The highest level of dietary

protein was also associated with increases in the wet femur weight and amount of absolute ash. Furthermore, restricted-feeding was associated with an increased percentage of water. Percent ash was not significantly affected by caloric intake or protein level.

Length, wet weight, and percent fat of the femurs were similar in ad libitum-fed groups regardless of whether the protein was constant or changing, but the percent water and percent ash were less in the constant group. Within the changing group, the 20 → 12% protein group had femurs which were shorter and weighed less (wet weight) than the 20 → 28% group, but the percent water was not significantly different. Also, in the old animals, the 20 → 12% protein group had femurs which contained less percent fat than the 20 → 28% group.

The femurs of old animals regardless of dietary treatment were longer, heavier, contained greater absolute amounts of ash, and greater percent water than those of the young ones. When ash was expressed as a percentage of dry weight, no age-associated differences were noted.

It can be concluded from this study that reduced caloric and/or protein intake resulted in decreased bone mass. However, percent fat and percent ash of the femur, in general, were not significantly affected by caloric or protein intake. Femurs of old rats were larger than those of young animals, but again percent fat and percent ash were not affected by age. Only percent water in the femur was affected by caloric intake and age; however, the changes in percent water may have occurred during the less than optimum storage conditions previous to this phase of the larger study.

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LITERATURE CITED

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