The Effect of Purified Compared with Nonpurified Diet on Bone Changes Induced by Hindlimb Suspension of Female Rats

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The purpose of this study was to compare the bone changes induced by unloading in rats fed different diets, because space flight studies use a semipurified diet, whereas space flight simulation studies typically use nonpurified diets. Female Sprague-Dawley rats were fed a purified American Institute of Nutrition (AIN) 93G diet or a standard nonpurified diet and kept ambulatory or subjected to unloading by hindlimb suspension (HLS) for 38 days. Bone mineral content (BMC), mechanical strength, and factors related to the diet that affect bone (i.e., urinary calcium excretion, estradiol, and corticosterone) were measured. Average food intakes (grams per day) differed for diets, but caloric intake (kilocalories per day) and the final body masses of treatment groups were similar. The HLS-induced decrease in femoral BMC was not statistically different for rats fed a nonpurified diet (–8.6%) compared with a purified AIN-93G diet (–11.4%). The HLS-induced decrease in femoral mechanical strength was not statistically different for rats fed a nonpurified diet (–24%) compared with a purified AIN-93G diet (–31%). However, bone lengths were decreased ($P < 0.05$) in rats fed a nonpurified diet compared with a purified diet. Plasma estradiol levels were lower ($P < 0.05$) in the HLS/AIN-93G group but similar in the HLS and ambulatory rats fed a nonpurified diet. Plasma estradiol was related to femoral BMC ($r = 0.85$, $P < 0.01$). Urinary calcium excretion was higher ($P < 0.05$) in rats fed a nonpurified diet than those fed a purified AIN-93G diet, which is consistent with the higher level of calcium in the nonpurified diet. Urinary corticosterone levels were higher ($P < 0.05$) in rats fed a nonpurified diet than rats fed the AIN-93G diet. Although the osteopenia induced by unloading was similar in both diet groups, there were differences in longitudinal bone growth, calcium excretion, plasma estradiol levels, and urinary corticosterone levels. Results indicate that the type of standard diet used is an important factor to consider when measuring bone end points. Exp Biol Med 230:31–39, 2005

Key words: ambulatory; space flight; calcium; corticosterone; estradiol

One of the most striking physiological effects of space flight has been the rapid loss of bone mass and mineral content (1). Due to few opportunities, high cost, and many confounding factors associated with space flight experiments, the ground-based hindlimb suspension (HLS) model was developed. The HLS model is a well-established model for studying the effects of unloading on the musculoskeletal system (2, 3). The HLS model requires that the rat or mouse be suspended by the tail base to produce a 30° head-down position that simulates unloading of the hindlimbs experienced in the reduced gravity environment of space. If ground-based studies are to mimic the alterations observed in bone during space flight, then diets known to have similar effects on bone must be used. Few studies have compared diet effects on bone responses to unloading in ground-based models (4, 5). This has significance because American space flight studies use a semipurified diet to meet space flight requirements of nutrient consistency, high nutrient bioavailability, and flexibility of formulation (4), whereas most ground-based studies used to mimic space flight experiments use commercially available standard nonpurified rodent diets (4).

Nonpurified diets are formulated from natural ingredients and contain multiple sources of nutrients. Bioavailability of nutrients in nonpurified diets may be reduced compared with purified diets due to nutrient interactions and losses during processing (6). Additionally, ingredient differences between purified and nonpurified diets may influence the outcome of bone studies. For example, many commercially available nonpurified rodent diets are high in phytoestrogens, which are plant-derived compounds capable of exerting estrogenic activity (7). Phytoestrogens present in soy have been reported to protect against bone loss in

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ovariectomized rats (8). The HLS of rats induces a decrease in sex steroids that may contribute to osteopenia and bone loss (9). The estrogenic activity associated with phytoestrogens may prevent some of the osteopenic changes in unweighted bones. Another potential mechanism for HLS-induced osteopenia is increased stress hormones (i.e., corticosterone). Phytoestrogen consumption has been reported to enhance stress responses (10). Therefore, it is important to understand the endocrine effects of diet on bone.

Previously, space flight experiments have been short ($\leq$20 days) and consisted of young rats (11, 12). In a short-term (2-week) study, Zerath *et al.* (5) reported no difference ($P < 0.05$) in skeletal growth or bone changes induced by HLS of young adult male rats fed either the semipurified space flight diet or standard nonpurified rodent diet. Current space flight mission durations are increasing, and future experiments propose using animals of different ages and sex. We used young adult female rats to compare the effect of feeding a purified diet compared with a nonpurified diet on bone changes induced by long-term HLS to determine whether the diets can be used interchangeably.

Materials and Methods

All procedures used in this study conformed to the National Research Council (NRC) Guide for the Care and Use of Laboratory Animals (13). The protocol for this study was approved by the Institutional Animal Care and Use Committee at the National Aeronautics and Space Administration (NASA) Ames Research Center.

Animals and Diet. Virgin female Sprague-Dawley rats aged 50 days (n = 36) were received from Simonsen Laboratories (Gilroy, CA). On receipt, animals were individually housed in NASA-designed HLS cages (14) in a room maintained at 22° ± 2°C with a 12:12-hr light:dark cycle (0600 hrs lights-on and 1800 hrs lights-off). Rats were randomly assigned to be fed either the purified ingredient American Institute of Nutrition (AIN) 93G diet (TD 94045; Harlan Teklad, Madison, WI) or a nonpurified ingredient rodent diet (8728C; Harlan Teklad).

The semipurified diet (TD 97071; Harlan Teklad) currently used to feed rodents during space flight was not included in this study, because the nutritional adequacy of the space flight diet for use in long-term studies (>20 days) is not documented (4). The semipurified space flight diet is based on the formulation of the AIN-76 diet that contains some nutrients below current NRC recommended levels (4, 6). The AIN-93G diet conforms to the updated NRC nutritional recommendations for rat growth, maintenance, and reproduction (6, 15). The AIN-93 diets have been determined to be nutritionally adequate for long-term (13-week) animal studies and are widely used (16).

Purified diets are phytoestrogen free, whereas soy-based nonpurified diets contain varying levels of phytoestrogens (7). We selected the certified Harlan Teklad 8728C nonpurified diet that contained dietary phytoestrogens within the range found in other commercially available nonpurified rodent diets (7). Otherwise, the selected nonpurified diet conforms to the NRC nutritional recommendations for rat growth, maintenance, and reproduction (6). Table 1 lists some nutrients and their levels in the purified AIN-93G and nonpurified diets. Both diets were provided in powder form to facilitate measurement of food intake. Assigned diet and water were provided *ad libitum* throughout the study.

Experimental Design. On arrival, female Sprague-Dawley rats (aged 50 days) were immediately assigned to the purified AIN-93G diet or the nonpurified diet and fed for a preexperimental period of 27 days. During the 27 days, estrous cycles of rats were monitored by examination of daily vaginal smears as described in Tou *et al.* (17) to assay for estrogenic effects of diet. Only sexually mature rats (aged 77 days) determined to be cycling normally (4–5 days) were used in the experiment. Two rats in the nonpurified diet group did not meet the criteria for normal estrous cycling due to prolonged estrus and were removed from the study. Rats having normal cycles were assigned (n = 8–10 rats per group) to be kept as ambulatory controls or subjected to HLS according to the procedure described by Morey-Holton and Globus (3). The resulting treatment groups were ambulatory rats fed the purified AIN-93G diet,

Table 1. Nutrient Levels in the Purified American Institute of Nutrition (AIN) 93G Diet and a Standard Nonpurified Diet

Ingredients	AIN-93G diet (TD 94045)	Nonpurified diet (8728C)
Calories (kcal/g)	4.03	3.10
Protein (%)[a]	17.86	24.48
Fat (%)	7.0	4.40
Fiber (%)	4.75	3.69
Total ash (%)	4.17	7.84
Calcium (%)	0.50	1.36
Phosphorus (%)	0.30	1.01
Potassium (%)	0.36	1.04
Sodium (%)	0.10	0.29
Chloride (%)	0.16	0.49
Magnesium (%)	0.05	0.28
Iron (mg/kg)	45.00	352.14
Zinc (mg/kg)	38.00	82.87
Copper (mg/kg)	6.00	24.42
Selenium (mg/kg)	0.18	0.33
Vitamin D (IU/g)	1.00	2.40
Vitamin K (mg/kg)	0.90	4.11
Vitamin A (IU/g)	4.00	12.90
Vitamin E (IU/kg)	75.00	90.18
Vitamin B_{12} (μg/kg)	25.00	51.20
Phytoestrogens (μg/g)	ND	496.00[b]

[a] The protein sources are casein in the AIN-93G diet (TD 94045) and soy protein in the nonpurified diet (8728C). The fiber source in the AIN-93G diet is cellulose. ND, not detectable.
[b] The phytoestrogen level was provided by Dr. C. E. Benton of Harlan Teklad, Madison, WI (personal communication).

ambulatory rats fed the nonpurified diet, HLS rats fed the purified AIN-93G diet, and HLS rats fed the nonpurified diet. Rats were weight matched so that there were no significant differences in initial body weights between diet treatment groups at the start of the experiment (Table 2). An experimental duration of 38 days was selected to examine the long-term effects of unloading on bone. Ambulatory control and HLS animals were maintained in NASA-designed HLS cages (14) located in the same room to provide the same housing conditions and environment. Body mass, food intake, and water consumption were measured twice a week.

Vaginal smears were performed throughout the study, and all rats were handled daily to minimize handling stress. At the end of the 38-day experimental period, rats (aged 115–119 days) were euthanized. To minimize cyclical variations in plasma estradiol measurements, rats were euthanized in the same stage of the estrous cycle (i.e., estrus). Dissections occurred for 4 days to allow rats that were not in estrus to come into estrus. In cases where this was not feasible due to lengthened estrous cycles, animals were excluded from the analysis. Animals were anesthetized with isoflurane, bled by cardiac puncture, decapitated, and then dissected. Dissections started at 0830 hrs and ended at 1100 hrs to minimize diurnal variations in hormone levels.

Bone Analysis. The left femur, tibia, and humerus were collected. The bones were defleshed, with care being taken not to damage the periosteum. Each bone was wrapped in saline-soaked gauze and stored at −20°C until analyzed. For analysis, each bone was brought to room temperature and the bone lengths measured with a caliper.

Mechanical strength was determined in the femur by torsion testing using a technique developed at the NASA Ames Research Center (Moffett Field, CA). Principles are identical to torsion tests reported in the literature (18) except that a metal alloy was used for embedding the bones. For the torsion test, the ends of the bone were embedded in specially designed aluminum cups that contained a metal alloy that is liquid at 56°C and then hardens rapidly at 25°C. The metal cups fit precisely into a torsion instrument. The instrument applies uniform torque along the diaphysis of the bone, rotating the potted bone at $1^{\circ}\ sec^{-1}$ to failure. Fracture occurs at the weakest area of the exposed 10- to 14-mm test segment. The maximum torque was determined in the computer program from the torque-angular deformation data.

The tibia, humerus, and femur bone fragments were dried at 110°C (Oven DK-63; Baxter Scientific Products, Hayward, CA) for 48 hrs to determine dry weights. Bones were then ashed at 600°C in a muffle furnace (Furnatrol I model CP18210; Thermolyne, Dubuque, IA) for 18–24 hrs. Bone mineral content (BMC) was determined by the weight of the bone ash.

Urinary Biochemical Analysis. The NASA-designed HLS cage also functions as a metabolic cage (14). Urine samples were collected for 24 hrs to account for the diurnal variation in hormone levels. Urine volumes and samples were collected at the start of HLS and at the end of the 38-day experiment and changes expressed as Δ the difference in start-end levels. Clean funnels were used for each of the urine collection periods. Decalyne oil (1 ml) was added to the urine collection tubes to prevent evaporation. Collected 24-hr urine samples were centrifuged at 1,500 *g* for 10 mins at 4°C. Following centrifugation, urine samples were aliquoted into fresh tubes and kept frozen at −20°C until assayed. Urinary concentrations of creatinine and calcium were measured using an automated analytical spectrophotometer system (COBAS; Roche Diagnostic Systems, Somerville, NJ).

To monitor stress levels and adaptation, we collected urine samples at the start and end of HLS. Collection of 24-hr urine samples accounts for diurnal variations in corticosterone and bypasses the stress associated with obtaining multiple blood samples. Urinary corticosterone was determined according to Ortiz *et al.* (19). Briefly, aliquots of urine (250 µl) were extracted with 1:1 dichloromethane. Following extraction, urine samples were reconstituted 1:20 using steroid diluent, and urinary corticosterone levels were

Table 2. The Effect of the Purified American Institute of Nutrition (AIN) 93G Diet Compared with a Standard Nonpurified Diet and Hindlimb Unloading on Body Mass and Nutrient Intake[a]

Measurements	AMB/AIN-93G (n = 9)	AMB/NP (n = 9)	HLS/AIN-93G (n = 8)	HLS/NP (n = 10)
Initial body mass (g)	216.5 ± 2.92	214.2 ± 5.04	226.4 ± 3.31	213.5 ± 5.04
Final body mass (g)	247.6 ± 4.13	241.9 ± 7.55	254.7 ± 7.25	248.1 ± 13.40
Body mass gain (g)	31.1 ± 5.21	27.6 ± 6.06	28.3 ± 5.03	34.7 ± 10.46
Food intake (g/d)	15.7 ± 0.45	16.1 ± 0.37	14.9 ± 0.42*	16.9 ± 0.70
Mineral intake (g/d)	0.65 ± 0.07*	1.26 ± 0.12	0.62 ± 0.02*	1.32 ± 0.12
Calcium intake (g/d)	0.08 ± 0.01*	0.22 ± 0.020	0.07 ± 0.01*	0.23 ± 0.038
Phosphorus intake (g/d)	0.05 ± 0.01*	0.16 ± 0.01	0.05 ± 0.01*	0.17 ± 0.03
Ratio of calcium to phosphorus intake	1.6 ± 0.01	1.4 ± 0.01	1.4 ± 0.01	1.3 ± 0.01

[a] Values are the means ± SEM of 8–10 rats per group. AMB/AIN-93G, ambulatory rats fed the purified AIN-93G diet; AMB/NP, ambulatory rats fed the nonpurified diet; HLS/AIN-93G, hindlimb-suspended rats fed the purified AIN-93G diet; HLS/NP, hindlimb-suspended rats fed the nonpurified diet.

*$P < 0.05$ by one-way analysis of variance followed by Tukey test.

determined by the commercially available rat corticosterone ImmunChem double antibody radioimmunoassay kit (ICN Biomedicals, Costa Mesa, CA). Radioisotope counting was performed using a Cobra II Auto-Gamma counter (Packard BioScience, Downers Grove, IL). All samples were measured in duplicate. The intraassay and interassay coefficients of variation for urinary corticosterone were 3.6% and 4.3%, respectively.

Plasma Hormone Analysis. Blood samples were obtained from the rats at dissection. Animals were anesthetized with isoflurane, bled by cardiac puncture, then decapitated. Blood was collected in ice-cold tubes that contained heparin. Collected blood samples were centrifuged at 1,500 *g* for 10 mins at 4°C to separate plasma. Plasma samples were collected and stored at −70°C until assayed for estradiol and corticosterone. A commercially available estradiol Coat-a-Count radioimmunoassay kit (Diagnostic Products Corporation, Los Angeles, CA) was used to measure plasma concentrations of estradiol. The intraassay and interassay coefficients of variation were 6.2% and 7.5%, respectively, for estradiol. A commercially available rat corticosterone ImmunChem double antibody radioimmunoassay kit (ICN Biomedicals) was used to determine plasma corticosterone levels. The intraassay and interassay coefficients of variation were 5.0% and 5.3%, respectively, for plasma corticosterone.

Statistical Analysis. Urinary corticosterone and calcium excretion were calculated as follows: $[\text{urine}]_{\text{calcium or corticosterone}} \times [\text{24-hr volume}]$ and corrected for creatinine. Correlations between bone mass and BMC, plasma estradiol and bone parameters, and plasma or urinary corticosterone and bone parameters were determined by a simple regression of individual animals. Two-way analysis of variance was used to determine differences due to diet, HLS treatment, or diet × HLS treatment. Post hoc multiple comparison tests were performed using the Tukey test. All statistical analyses were performed using StatView statistical software (Abacus Concepts, Berkeley, CA). Results

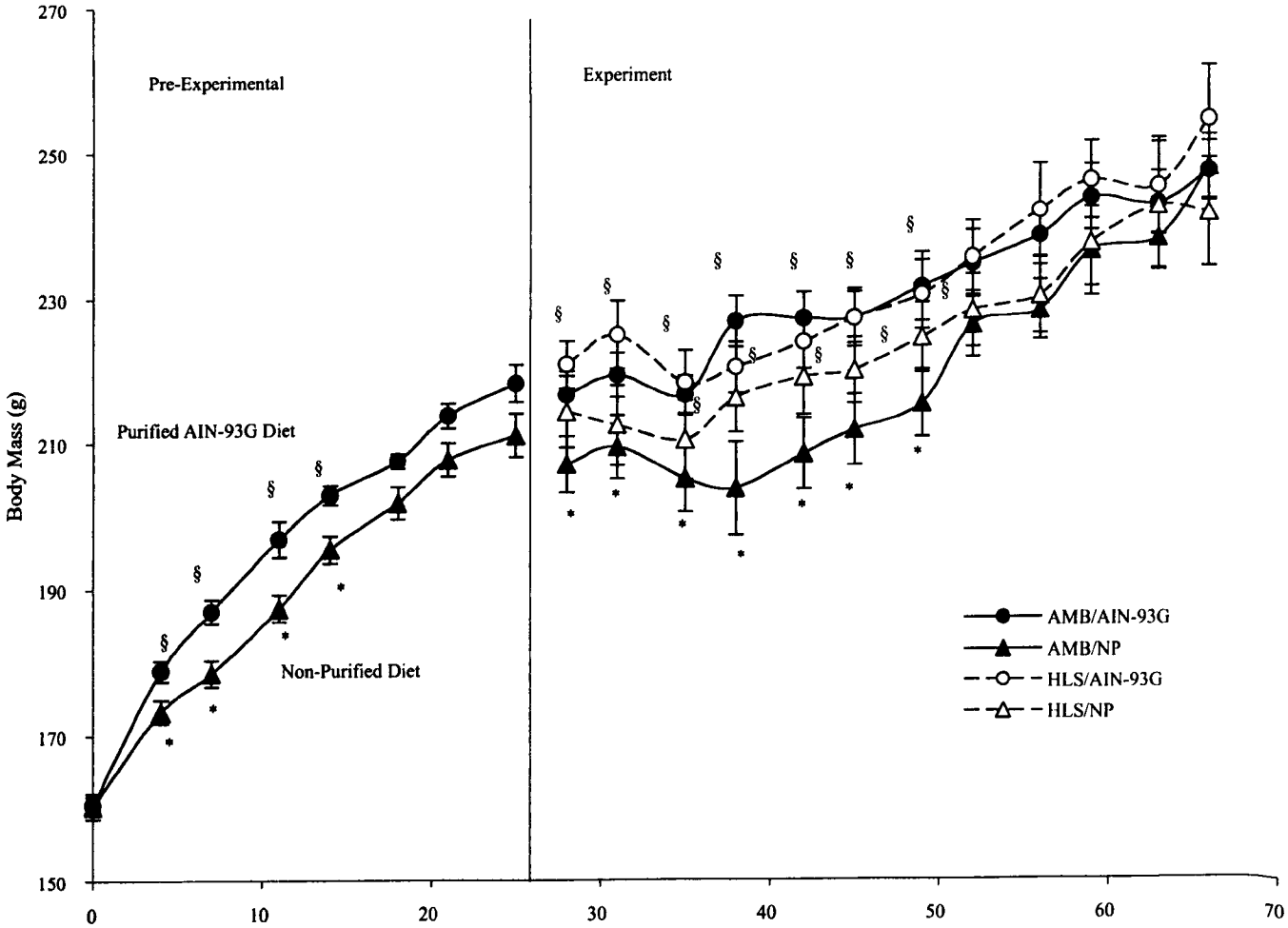

Figure 1. The effect of the purified American Institute of Nutrition (AIN) 93G diet compared with a standard nonpurified diet and hindlimb unloading of female rats on body mass during the 27-day preexperimental period and 38-day experimental period. Values are the means ± SEM of 8–10 rats per group. A two-way analysis of variance indicated a statistically significant diet effect ($P < 0.05$) on body mass. Different symbols indicate statistically significant difference at $P < 0.05$ by the Tukey test. AMB/AIN-93G, ambulatory rats fed the purified AIN-93G diet; AMB/NP, ambulatory rats fed the nonpurified diet; HLS/AIN-93G, hindlimb-suspended rats fed the purified AIN-93G diet; HLS/NP, hindlimb-suspended rats fed the nonpurified diet.

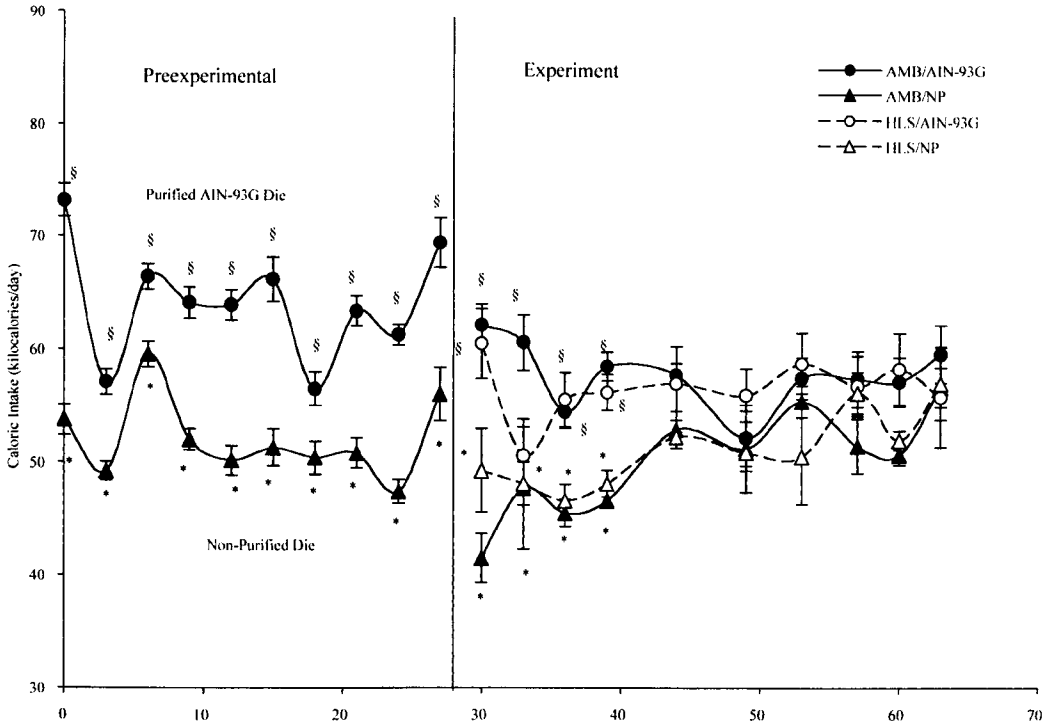

Figure 2. The effect of the purified American Institute of Nutrition (AIN) 93G diet compared with a standard nonpurified diet and hindlimb unloading of female rats on food intake during the 27-day preexperimental period and 38-day experimental period. Values are the means ± SEM of 8–10 rats per group. A two-way analysis of variance indicated a statistically significant diet effect ($P < 0.05$) on caloric intake (kilocalories per day). Different symbols indicate statistically significant difference at $P < 0.05$ by the Tukey test. AMB/AIN-93G, ambulatory rats fed the purified AIN-93G diet; AMB/NP, ambulatory rats fed the nonpurified diet; HLS/AIN-93G, hindlimb-suspended rats fed the purified AIN-93G diet; HLS/NP, hindlimb-suspended rats fed the nonpurified diet.

are expressed as means ± SEM, and differences were considered statistically significant at $P < 0.05$.

Results

Body Mass and Food Consumption. Initial body mass of the rats did not differ (Table 2). During the 27-day preexperimental period of different feeding diets, body mass was higher ($P < 0.05$) in rats fed the AIN-93G diet than rats fed a nonpurified diet (Fig. 1). During the experimental period, body mass of the rats fed the purified AIN-93G diet was significantly higher than the ambulatory animals fed a nonpurified diet, indicating a diet effect ($P < 0.05$). However, toward the end of the experiment, the average body mass of the rats fed the AIN-93G diet was not significantly different from animals fed a nonpurified diet (Fig. 1). Final body mass and body mass gain did not differ between the treatment groups (Table 2).

Average food intake (grams per day) throughout the study was significantly lower in rats fed the AIN-93G diet (Table 2). The HLS rats fed a nonpurified diet ate approximately 12% g/d more than animals fed a purified diet; however, metabolizable energy of the nonpurified diet was lower than the purified diet (Table 1). Adjusted for differences in metabolizable energy and expressed as average caloric intake (kilocalories per day), Figure 2 shows that during the 27-day preexperimental period, caloric intake was less ($P < 0.05$) in rats fed a nonpurified than a purified AIN-93G diet. During the experimental period, caloric intake was less ($P < 0.05$) in rats fed a nonpurified diet than animals fed a purified AIN-93G diet, regardless of treatment (i.e., HLS vs. ambulatory). Toward the end of the experiment, the caloric intake of the rats fed a nonpurified diet was not statistically different from rats fed a purified AIN-93G diet (Fig. 2).

Bone Analysis. *Bone Length.* Diet influenced bone length. Rats fed a nonpurified diet had shorter ($P < 0.05$) femurs and humeri than rats fed a purified AIN-93G diet (Table 3). There was no correlation of femur or humerus length to bone mass, BMC, or femur mechanical strength. In the tibia, there was a trend ($P < 0.06$) of shorter length in rats fed a nonpurified compared with a purified AIN-93 diet (Table 3).

Bone Mass. Dry weights of bones were significantly reduced in HLS rats compared with ambulatory animals. However, there was no decrease in femur dry weight of HLS rats fed a AIN-93G diet compared with animals fed a nonpurified diet (Table 3).

Bone Mineral Content. The BMC of tibia and femur was reduced in HLS rats compared with ambulatory animals, indicating an unloading effect ($P < 0.05$). Femoral BMC was reduced by −8.6% and −11.4% in HLS rats fed a nonpurified diet and in the animals fed the AIN-93G diet,

Table 3. The Effect of Purified American Institute of Nutrition (AIN) 93G Diet Compared with a Standard Nonpurified Diet and Hindlimb Unloading on Bone[a]

Bone measurements	AMB/AIN-93G (n = 9)	AMB/NP (n = 9)	HLS/AIN-93G (n = 8)	HLS/NP (n = 10)
Left femur				
Length (mm)	32.18 ± 0.12†	31.76 ± 0.11*	32.91 ± 0.18†	31.75 ± 0.20*
Dry weight (g)	0.54 ± 0.008†	0.53 ± 0.009†	0.50 ± 0.01*	0.49 ± 0.007*
Bone mineral content (g)	0.35 ± 0.007†	0.35 ± 0.006†	0.31 ± 0.005*	0.32 ± 0.007*
Mechanical strength (N × mm)	312.4 ± 26.0†	261.8 ± 17.3†	215.6 ± 20.1*	199.8 ± 8.5*
Left tibia				
Length (mm)	36.74 ± 0.14†	36.49 ± 0.17	36.52 ± 0.22	36.16 ± 0.21*
Dry weight (g)	0.46 ± 0.003§	0.44 ± 0.01	0.43 ± 0.008†	0.39 ± 0.01*
Bone mineral content (g)	0.29 ± 0.003†	0.28 ± 0.007†	0.25 ± 0.005*	0.24 ± 0.006*
Left humerus				
Length (mm)	25.70 ± 0.16†	25.24 ± 0.15*	25.85 ± 0.22†	24.93 ± 0.16*
Dry weight (g)	0.26 ± 0.003	0.25 ± 0.004	0.26 ± 0.006	0.25 ± 0.007
Bone mineral content (g)	0.17 ± 0.002	0.17 ± 0.003	0.17 ± 0.004	0.16 ± 0.003

[a] Values are the means ± SEM of 8–10 rats per group. Different symbols *, †, and § across rows are significantly different $P < 0.05$ by the Tukey test. A two-way analysis of variance indicated a significant diet effect ($P < 0.05$) on bone length, a significant HLS treatment effect ($P < 0.05$) on bone mineral content, and a significant HLS treatment effect ($P < 0.01$) on femur mechanical strength. AMB/AIN-93G, ambulatory control rats fed purified AIN-93G diet; AMB/NP, ambulatory control rats fed nonpurified diet; HLS/AIN-93G rats, hindlimb-suspended rats fed purified AIN-93G diet; HLS/NP, hindlimb-suspended rats fed nonpurified diet.

respectively. However, the effects of the AIN-93G diet compared with the nonpurified diet on BMC of unweighted bones were not statistically different. The humerus, normally weighted in the HLS model, showed no changes in either dry weight or BMC (Table 3).

Mechanical Strength. Compared with ambulatory controls, mechanical strength of the femur was decreased in HLS rats, indicating an unloading effect ($P < 0.01$). Femoral mechanical strength was decreased by –24% in the rats fed a nonpurified diet and by –31% in the animals fed the AIN-93G diet. The effects of the purified AIN-93G diet compared with the nonpurified diet on mechanical strength of unweighted bones were not statistically different. Mechanical strength of the femur correlated with its dry weight ($r = 0.73$, $P < 0.05$) and BMC ($r = 0.74$, $P < 0.05$) but not length.

Urinary Biochemical Analysis. As a result of differences in urinary creatinine excretion, urinary calcium and corticosterone excretion are presented as 24-hr excretion and referenced to creatinine excretion (Table 4).

Creatinine. Urinary creatinine excretion was higher in rats fed a nonpurified diet compared with rats fed the purified AIN-93G diet, indicating a diet effect ($P < 0.01$) (Table 4). Changes in urinary creatinine determined by Δ the difference in start-end levels during HLS were insignificant.

Calcium. Difference in calcium levels between the diets (Table 1) resulted in rats fed the nonpurified diet having higher dietary calcium consumption than animals fed a purified AIN-93G diet (Table 2). The ratio of dietary calcium to phosphorus for all treatment groups was greater than 1, with no differences among the treatment groups (Table 2). Urinary calcium excretion was higher ($P < 0.05$) in rats fed a nonpurified diet compared with a purified AIN-

Table 4. The Effect of Purified American Institute of Nutrition (AIN) 93G Diet Compared with a Standard Nonpurified Diet and Hindlimb Unloading on Urinary Biochemical Measurements[a]

Measurements	AMB/AIN-93G (n = 9)	AMB/NP (n = 9)	HLS/AIN-93G (n = 8)	HLS/NP (n = 10)
Creatinine excretion (mg/24 hrs)	4.28 ± 0.40*	7.12 ± 0.73†	4.34 ± 0.62*	8.27 ± 1.03†
Δ	Δ –0.13	Δ 0.08	Δ –1.09	Δ 0.84
Urinary Ca excretion (mg/24 hrs)	0.43 ± 0.07*	0.96 ± 0.13†	0.37 ± 0.07*	1.27 ± 0.06†
Δ	Δ –0.13	Δ –0.10	Δ –0.22	Δ –0.28
Ca/creatinine excretion	0.09 ± 0.01*	0.13 ± 0.01	0.07 ± 0.02*	0.23 ± 0.05†
Δ	Δ 0.01	Δ –0.01	Δ 0	Δ 0.07
Urinary CORT excretion (ng/ml)	3.42 ± 1.05*	12.78 ± 2.20†	3.73 ± 0.89*	26.67 ± 4.24§
Δ	Δ –1.0	Δ 1.76	Δ –0.94	Δ 16.50
CORT/creatinine excretion	0.78 ± 0.22*	1.90 ± 0.51†	0.76 ± 0.13*	3.64 ± 0.85§
Δ	Δ –0.15	Δ 0.42	Δ –0.07	Δ 2.35

[a] Values are the means ± SEM of 8–10 rats per group. Different symbols *, †, and § across rows indicate significant difference at $P < 0.05$ by the Tukey test. A two-way analysis of variance indicated a significant diet effect $P < 0.05$ on urinary creatinine, calcium, and corticosterone. Change in urinary biochemical values during 38-day hindlimb suspension was determined by Δ start-end of HLS. AMB/AIN-93G, ambulatory control rats fed purified AIN-93G diet; AMB/NP, ambulatory control rats fed nonpurified diet; HLS/AIN-93G rats, hindlimb-suspended rats fed purified AIN-93G diet; HLS/NP, hindlimb-suspended rats fed nonpurified diet; Ca, calcium; CORT, corticosterone.

93G diet, indicating a diet effect ($P < 0.05$) (Table 4). Changes in urinary calcium determined by Δ the difference in start-end levels during HLS were insignificant.

Corticosterone. Urinary corticosterone results were similar regardless of how values were expressed. Urinary corticosterone excretion was higher ($P < 0.01$) in groups fed a nonpurified diet compared with those fed a AIN-93G diet, indicating a diet effect ($P < 0.05$). Rats subjected to unloading and fed a nonpurified diet showed elevated urinary corticosterone excretion and a large change determined by Δ the difference in start-end urinary corticosterone compared with the animals fed the AIN-93G diet, indicating a significant ($P < 0.05$) diet × HLS effect (Table 4).

Final urinary corticosterone excretion ($r = -0.81$, $P < 0.01$) and urinary corticosterone/creatinine ($r = -0.81$, $P < 0.01$) were inversely correlated to tibia dry weight. There was no relationship between urinary corticosterone excretion and femur or humerus bone parameters (i.e., dry weight, mechanical strength, BMC, or length).

Plasma Hormones Analysis. *Corticosterone.* Plasma corticosterone was inversely correlated to tibia dry weight ($r = -0.75$, $P = 0.03$) and BMC ($r = -0.69$, $P < 0.05$) but not to femur or humerus bone parameters (i.e., dry weight, mechanical strength, BMC, or length). There were no significant differences in adrenal mass, an indicator of chronic stress, among ambulatory rats fed the AIN-93G diet (62 ± 2 mg), ambulatory rats fed the nonpurified diet (58 ± 2 mg), HLS rats fed the AIN-93G diet (60 ± 1 mg), and HLS rats fed the nonpurified diet (61 ± 3 mg).

Estradiol. The HLS rats showed prolonged diestrus, a phase associated with reduced plasma estradiol levels (17). Plasma estradiol levels were lower ($P = 0.05$) in the HLS rats fed the AIN-93G diet (7.2 ± 1.3 pg/ml) compared with the ambulatory rats fed the AIN-93G diet (18.8 ± 3.5 pg/ml) but similar in the HLS rats fed the nonpurified diet (13.0 ± 1.9 pg/ml) and ambulatory rats fed the nonpurified diet (15.2 ± 3.8 pg/ml), indicating a unloading effect ($P = 0.03$). Plasma estradiol was correlated ($r = 0.85$, $P < 0.01$) with femur BMC but not femur dry mass, length, or mechanical strength. There were no correlations between plasma estradiol and bone parameters measured in the tibia and humerus. There was no relationship between plasma estradiol and plasma or urinary corticosterone levels.

Discussion

Despite marked differences in the nutrient composition of purified and nonpurified diets, we found no statistical difference in the osteopenia induced by unloading for 38 days. However, we found decreased longitudinal bone growth in female rats fed a nonpurified diet compared with a purified diet. In contrast, Zerath *et al.* (5) reported no difference in skeletal growth in male rats fed a semipurified space flight diet compared with a standard nonpurified diet. Reduced bone growth was not associated with differences in final body mass (Fig. 1). Similar to Zerath *et al.* (5), we found no significant differences in final body weights between female rats fed the different diets (Fig. 1) despite lower average food intake in rats fed a purified diet (Table 2). Rats fed the AIN-93G diet may have lowered their food intake to adjust for the higher metabolizable energy of this diet (Table 1), and over time this resulted in no differences in average caloric intake between groups fed the purified compared with the nonpurified diet (Fig. 2). The shorter bone lengths involved the femur, an unloaded bone, and the humerus, a loaded bone. This is an indication that unloading was not a factor. As reported by Sibonga *et al.* (20), skeletal unloading generally did not affect longitudinal bone growth in rats, regardless of age, strain, sex, and duration or method of unloading. We speculate that the decrease in bone growth was related to the dietary content of phytoestrogens. Estrogen receptor β (ERβ) has been shown to be a physiological inhibitor of growth in young adult (4-month-old) female mice (21). It has been reported that phytoestrogens have binding specificity for ERβ (22). Phytoestrogens in the nonpurified diet may have acted through the ERβ to reduce skeletal growth in young adult female rats. However, due to various nutrient differences in the diets used in this study (Table 1), we were unable to determine whether phytoestrogens were the nutrient in the nonpurified diet responsible for reduced longitudinal growth.

Studies have reported that HLS of aging (6-month-old) female rats fed a standard nonpurified rodent diet resulted in osteopenia (9, 23). In our study that used young adult (77-day-old) female rats, tibia BMC, dry weight, femoral dry weight, BMC, and mechanical strength were reduced by HLS compared with ambulatory animals (Table 3). A positive correlation ($r = 0.74$, $P = 0.03$) between femur BMC and mechanical strength indicated that osteopenia following unloading increased the susceptibility of bone to fracture. Absence of similar bone changes in the humerus (Table 3), which remained weight bearing in the HLS model, confirmed that osteopenic changes in bones of young female rats were due to unloading. Despite multiple nutrient differences, purified diets compared with nonpurified diets had no measurable difference on the osteopenia induced by unloading.

Still it is important to consider diet; the higher urinary creatinine excretion observed in rats fed a nonpurified diet compared with a purified AIN-93G diet (Table 4) may have been due to higher protein intake. Higher urinary calcium excretion was likely due to the higher calcium content of the nonpurified diet (24). However, Globus *et al.* (25) found that high (2.44%) dietary calcium failed to alter the bone changes induced by HLS, suggesting that the calcium level used in this study may not have been sufficiently high for diet to interfere with BMC changes induced by HLS of rats (Table 1).

Reduced sex steroid levels are another potential mechanism for the development of osteopenia in space flight rats. Space flight male rats have reduced testosterone

levels (26, 27), but no data are available regarding the effects of space flight on estrogen levels in female rats. The HLS studies that used female rats observed no significant changes in plasma estradiol levels in rats (9, 23). In these studies, estrogen findings may have been confounded by feeding nonpurified diets that contained phytoestrogens, not controlling for hormonal fluctuations, and the tendency for aging rats to show large variability in their estrogen levels. In our study, plasma estradiol levels were low ($P = 0.05$) in HLS young adult rats fed a purified AIN-93G diet and euthanized in the same phase of the estrous cycle. The decrease in plasma estradiol levels induced by HLS in the purified but not the nonpurified diet may have occurred because of the dietary phytoestrogens acting as an estrogen agonist. The soy-based nonpurified diet contains phytoestrogen levels reported to exert endocrine changes (7) (Table 1).

Kawano *et al.* (28) reported that ovariectomized female rats subjected to HLS and treated with intramuscular injection of 200 μg/g of estradiol dipropionate prevented reduction in femur mass and calcium content, suggesting that estradiol can protect against osteopenia induced by HLS. In our study, femoral BMC was correlated ($r = 0.85$, $P < 0.01$) to plasma estradiol. In HLS rats, femoral BMC was lower by −8.6% in rats fed a nonpurified diet and by −11.4% in animals fed the AIN-93G diet compared with ambulatory controls. Femur mechanical strength in HLS rats was lower by −24% in rats fed a nonpurified diet and by −31% in the animals fed a AIN-93G diet compared with ambulatory controls (Table 3). The slight reduction in osteopenia in animals fed the nonpurified diet, although not statistically significant, may have been due to the phytoestrogens present in a nonpurified diet exerting estrogenic activity. Other measurements, such as histomorphometry, may be valuable for detecting estrogen and phytoestrogen effects on BMC in unloaded bones.

Another hormonal alteration with potential to affect bone changes induced by unloading is corticosterone, a hormone secreted by the adrenal gland in response to a stressor and known to be a contributing factor in osteoporosis. In our study, an inverse correlation between both urinary corticosterone excretion ($r = -0.79$, $P < 0.01$) and plasma corticosterone ($r = -0.75$, $P = 0.03$) to tibia dry weight suggests that corticosterone may be responsible for the bone changes in the HLS female rats. However, there was no relationship of corticosterone to femur or humerus BMC. The tibia may be more responsive than the other bones to the changes induced by HLS. Lafage-Proust *et al.* (29) examined the effect a 14-day space flight had on various bone sites and found early and major changes in the tibia, less marked and delayed changes in the humerus, and minor changes in the femur.

Halloran *et al.* (30) found no difference in the bone response to unloading in adrenalectomized and steroid-treated male rats. Zerath *et al.* (31) also observed no bone differences between adrenalectomized or steroid-treated male rats with corticosterone implants after a 17-day space flight. The data in male rats indicate that osteopenia induced by unloading was not related to corticosterone. Our data on female rats showed an inverse correlation of corticosterone to tibia BMC. Female rats have a higher and faster plasma corticosterone response to stressors than male rats (32). This is because estrogen plays a role in the stress response as evidenced by increased response to stress in ovariectomized (i.e., estrogen deficient) rats treated with estradiol (33). In male rats, lifelong consumption of high-phytoestrogen diets enhanced the stress response (10). In agreement, in this study female rats subjected to HLS and fed the phytoestrogen-containing nonpurified diet had elevated urinary corticosterone excretion. Absence of chronic stress was indicated by the absence of enlarged adrenal glands. Higher urinary corticosterone excretion in female rats fed phytoestrogen-containing nonpurified diets during the start of HLS may have contributed to the early decrease in growth that manifested as shortened bone length at the end of the study. In male rats, excess corticosterone has been shown to inhibit bone growth (34). Clearly, the role of phytoestrogens and corticosterone on bones requires further study.

In summary, the use of a nonpurified versus purified diet can influence the results of bone studies as indicated by diet-induced effect on longitudinal bone growth and other factors that have a role in bone metabolism, particularly, plasma estradiol and corticosterone. Diet-induced bone effects and nutrient differences did not interfere with the osteopenic changes induced by unloading. It is likely that measurable diet effects on unloaded bones were obscured by the severity of the osteopenia induced by unloading. The role of diet remains an important factor to consider not only in space flight studies of bone but in any experiment that measures bone end points.

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