Nutrient Requirements and Interactions

Development and Testing of the AIN-93 Purified Diets for Rodents: Results on Growth, Kidney Calcification and Bone Mineralization in Rats and Mice

PHILIP G. REEVES, KERRY L. ROSSOW AND JAMES LINDLAUF

United States Department of Agriculture, Agricultural Research Service, Grand Forks Human Nutrition Research Center, Grand Forks, ND 58202-9034

ABSTRACT Because of nutritional and technical problems the AIN-76A rodent diet was revised. One of the new formulations was designated AIN-93G and was suggested for use during growth, pregnancy and lactation studies. Some major differences in this new formulation compared with the AIN-76A diet are as follows: 7 g soybean oil was substituted for 5 g corn oil/100 g diet to increase the amount of linolenic acid; the amounts of vitamins E and K were increased; cornstarch was substituted for most of the sucrose; the amount of phosphorus was reduced to eliminate the problem of nephrocalcinosis in female rats; L-cystine was substituted for DL-methionine; and the manganese concentration was reduced. Various developmental modifications of the AIN-93G diet were fed to weanling rats and mice to determine effects on growth and tissue mineralization. After rats were fed the developmental version of AIN-93G for 16 wk, body weights in both male and female rats were not different from those of rats fed a cereal-based nonpurified diet. After 13 wk, male mice fed this diet weighed 13% more than those fed the nonpurified diet. Body weights of female mice were not affected. The new diet formulation prevented kidney calcification in female rats and mice during 16 wk of feeding. J. Nutr. 123: 1923-1931, 1993.

INDEXING KEY WORDS:

- growth
- L-cystine
- 17β-estradiol
- nephrocalcinosis
- rats
- mice

Since their inception, there have been many problems with the AIN-76 and AIN-76A rodent diets, the major one being their propensity to cause kidney calcification in female rats [Shah et al. 1986]. In 1989, a workshop was sponsored by the American Institute of Nutrition (AIN) to determine if sufficient evidence had accumulated to warrant modification of the AIN-76A diet. Many suggestions were made for improving the diet, and a summary of the workshop proceedings was published in The Journal of Nutrition [Reeves 1989].

Over the past four years, my laboratory has been modifying the composition of the AIN-76A diet and testing the results. The outcome was a new diet, AIN-93, with two different formulations: AIN-93G for growth, pregnancy and lactation, and AIN-93M for maintenance of adult rodents. Major differences in the formulations of AIN-93G and AIN-76A were as follows: 7 g soybean oil/100 g diet was substituted for 5 g corn oil/100 g diet to increase the amount of linolenic acid; the amounts of vitamin E and K were increased; cornstarch was substituted for most of the sucrose; the amount of phosphorus was reduced to help eliminate the problem of kidney calcification in female rats; L-cystine was substituted for DL-methionine and the manganese concentration was reduced. See AIN [1993] for details of the formulation of these diets and the rationale for making changes.

During the development of AIN-93G, formulations were devised to test combinations of ingredients that would solve both nutritional and technical problems, and yield a diet that could be used over a wide range of applications. This report describes results on growth, kidney calcification and bone mineralization in weanling male and female rats and mice fed various formulations that ultimately led to the final recommended version (AIN 1993).

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1A portion of this work was presented at the Annual Meeting of the Federation of American Societies for Experimental Biology, April 5-9, 1992, Anaheim, CA [Reeves, P. G. [1992] Reformulation of the AIN-76 mineral mix to prevent kidney calcification in female rats. FASEB J. 6: A1651 [abs. 4188]].

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This report contains two studies and deals with developmental modifications of AIN-93G only. Each study consisted of two different experiments. Some experiments were done simultaneously and used the same controls. To avoid confusion, we have duplicated data for control groups in some tables and figures. All studies were approved by the USDA, ARS, Grand Forks Human Nutrition Research Center Animal Use Committee and were in accordance with the guidelines of the National Institutes of Health on the experimental use of laboratory animals [NRC 1985].

**MATERIALS AND METHODS**

Study A: Growth and kidney calcification and bone mineralization in male and female rats and mice fed a developmental formulation of the AIN-93G diet. Experiment A1. Male and female Sprague-Dawley rats and Swiss-Webster mice were purchased from Sasco (Lincoln, NE) at 3 wk of age. Each sex of each species was divided into two groups of equal weight, and housed by sex and species in separate rooms. One group in each of the sex and species groups was fed a developmental formulation (DF) of the AIN-93G diet. This diet contained 7.5% soybean oil instead of 7.0%, 100 g/kg dextrinized starch instead of 132 g/kg, 25 mg Si/kg diet as silicon dioxide instead of 5 mg/kg as sodium silicate, and 5 mg tertiary-butyldihydroquinone/kg diet instead of 14. It also contained an ultratrace element mix that differed from the final version (AIN 1993) and supplied the following (mg/kg diet): chromium, 1.0; fluoride, 1.0; nickel, 0.5; boron, 0.5; lithium, 0.5; tin, 0.2; arsenic, 0.2; vanadium, 0.2. To avoid confusion between the final version of AIN-93G and the developmental version, the diet used in this experiment is designated AIN-DF1. The remaining groups were fed Certified Rodent Diet-5002 (CRD-5002; PMI Feeds, Richmond, IN). The nutrient composition of this diet can be found in PMI [1992]. All diets were in pelleted form and supplied gratis by Dyets, Inc. (Bethlehem, PA). Diets were kept frozen until used.

Animals were housed in stainless steel hanging cages with wire-mesh floors in temperature- [27 ± 1°C] and humidity-controlled (50%) rooms, two animals per cage. The animals had free access to food and deionized water throughout the study. Animals were weighed each week for 13–17 wk. At the end of this period, rats were anesthetized with pentobarbital sodium, without first withholding food, and killed by exsanguination from the abdominal aorta. Mice were killed by decapitation following cervical dislocation. Kidneys were removed for analysis of calcium contents. An unfortunate mechanical problem prevented us from carrying the group of male mice to completion.

Originally, arsenic was recommended for inclusion in the ultratrace element mix of the new diet. Rats are unique among laboratory animals with regard to arsenic metabolism. When present in the diet, it accumulates in their red blood cells. Arsenic is a natural component in most cereal-based diets such as the PMI rodent diets (PMI Feeds); therefore, we determined the arsenic concentration in whole blood of rats that consumed these diets.

Experiment A2. In a preliminary experiment we learned that a molar ratio of Ca:P in this type of purified diet must be >1 to prevent kidney calcification in female rats. Thus, the use of calcium at 0.5% of the diet required phosphorus to be lower than in AIN-76A to achieve an appropriate Ca:P molar ratio. Because of concerns about adequate bone mineralization in rats fed diets with the lower phosphorus concentration, an experiment was conducted to compare the effects of different dietary concentrations of calcium and phosphorus on bone mineralization.

The design for this experiment was similar to that for Experiment A1 except that two other diet groups were added, one with low calcium and phosphorus (0.33% Ca, 0.20% P) and one with high calcium and phosphorus (0.67% Ca, 0.40% P). The third group was the same as that in Experiment A1 [0.50% Ca, 0.30% P]. All diets had a molar ratio of Ca:P of 1.3. The addition of phosphorus to these diets took into account the endogenous phosphorus content of the casein used as the protein source. The results obtained from animals fed these purified diets were compared with those from animals fed CRD-5002 (PMI Feeds; 0.89% Ca, 0.79% P), which has a Ca:P molar ratio of 0.87 (LabDiet 1992). All diets including CRD-5002 were supplied gratis by Dyets, Inc. After 16–17 wk of feeding the diets to rats and mice, tibiae from male and female rats and femora from female mice were taken for the determination of calcium, phosphorus and magnesium.

Study B. Effects of dietary and physiological factors on growth and kidney calcification in rats. Experiment B. Because the substitution of L-cysteine for DL-methionine was a major change from AIN-76A, we determined its effect on growth. Because preliminary studies had shown that the addition of L-cysteine might have an effect on kidney calcification in female rats fed the AIN-76A formulation, we reinvestigated this phenomenon.

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4 Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

5 Abbreviations used: AIN-76A95, AIN-76A diet with 0.3% L-cystine substituted for 0.3% DL-methionine, CRD-5002, Certified Rodent Diet-5002 (PMI Feeds, Richmond, IN); DF, developmental formulation; LRD-5001, Laboratory Rodent Diet (PMI Feeds).
Fourteen weaning Sprague-Dawley rats (Sasco) of each sex were divided into four groups of 10 rats each. One group in each sex group was fed AIN-76A [Teklad Premier, Madison, WI], one group was fed AIN-76A with 0.3% L-cystine substituted for 0.3% DL-methionine [AIN-76ACy3] and another group was fed a diet similar to AIN-DFI, except that this diet, designated AIN-DF2, contained 10% fat, 4% as corn oil and 6% as soybean oil. The AIN-DF2 diet was prepared by personnel in the animal diet kitchen of our research facility. A fourth group was fed Laboratory Rodent Diet 5001 [PMI Feeds, PMI 1992].

Animal husbandry was similar to that in Experiment A1 except that animals were housed individually. Rats were weighed weekly, and after 10 wk on experiment, they were anesthetized (without first withholding food), and the kidneys from each animal were removed for analysis of calcium content. Epididymal fat pads from males and perirenal fat pads from females were removed and weighed immediately to estimate the relative amount of body fat. Livers were collected and weighed.

**Experiment B2.** Because only female rats accumulate large amounts of calcium in their kidneys when fed a purified diet with Ca:P molar ratios of <1.0, we were interested in whether this phenomenon was hormonally related. We determined the effect of ovariectomy, with and without estrogen replacement, on kidney calcification in rats fed the AIN-76A diet.

Thirty female rats, 3 wk of age, were divided into three groups of 10 rats each and 20 were ovariec
tomized. All rats were fed AIN-76A. At 6 wk of age, one group of ovariectomized rats received a subcutaneous implant containing 0.72 mg 17β-estradiol per pellet with a release rate of 12 μg/d [Innoven Research, Toledo, OH]. The other ovariectomized group received a placebo pellet. Animal husbandry was the same as in Experiment B1. Rats were weighed weekly, and after a total of 10 wk on experiment, all rats were anesthetized (without withholding food) with pentobarbital sodium. Blood was drawn from the abdominal aorta and serum collected for the determination of estrogen concentration [RIA Kit, Abcoated Tubes, 238-102, ICN Biomedical, Costa Mesa, CA]. The kidneys were removed and analyzed for calcium content. Perirenal fat pads were removed and weighed immediately to estimate the relative amount of body fat. Livers were collected and weighed.

**Mineral analysis.** Bones and kidneys were lyophilized to constant weight and ashed in a muffle furnace at 500°C for 24 h. The ash was dissolved in 1.0 mol/L HCl and the concentrations of calcium, phosphorus and magnesium were determined by inductively coupled argon plasma spectrometry. For arsenic determinations, 0.5 mL of whole blood was digested in 2 mL of concentrated nitric acid saturated with MgSO4 and slowly brought to complete dryness. The dried samples were charred at 200°C on a hot plate and then ashed overnight in a muffle furnace at 600°C. Arsenic was determined by hydride generation and atomic absorption spectrometry. To ensure quality control for mineral samples, certified standards (Bovine liver, 1577b, U.S. National Institute of Standards and Technology, Gaithersburg, MD) were assayed with each batch. Values obtained were within the range specified for each mineral standard.

**Statistical analyses.** Depending on the experimental design, data were treated with a one or two-way ANOVA. Significant differences were determined by single degree of freedom contrasts. If an interaction occurred in the two-way ANOVA, differences between group means were determined by a step-down, multiple stage F test [Einot and Gabriel 1975, Ryan 1960, Welsch 1977]. Because it was not possible to satisfy the condition of equal variance for kidney calcium by data transformation for either Experiment B1 or B2, we used the Kruskal-Wallis rank test [Kruskal and Wallis 1952] and the t statistic to determine differences between means. Although by convention, the pooled error mean square is used instead of SEM in data tables, we chose to use the SEM instead of the error mean square because it gives more immediate information about variability in individual groups. For all comparisons, a difference with P ≤ 0.05 was considered significant. The Crunch-4 computer program [Crunch Software, Oakland, CA] was used for all statistical analyses.

**RESULTS AND DISCUSSION**

**Experiment A1.** Figure 1 shows the effects of feeding diets AIN-DF1 and CRD-5002 on growth in male and female rats. Although, by the end of the experiment, male rats fed the AIN-DF1 diet weighed ~6% more than those fed the nonpurified diet, the difference was not significant (P < 0.09). Female rats fed this diet also tended (P < 0.1) to be heavier than those fed CRD-5002, but the difference was not signif

**Figure 2** shows the effects of the diets on growth of mice. The general effects were different from those in rats. A two-way ANOVA showed a significant effect of diet at wk 2 (P < 0.006) and three (P < 0.009), when mice fed CRD-5002 weighed less than those fed the purified diet. At the end of wk 13 an interaction revealed that male mice fed CRD-5002 weighed ~4 g less (P < 0.04) than those fed the AIN-DF1. There was not a significant difference between diets for female mice at this period.

**Table 1** shows the concentration of calcium in kidneys of rats and mice that consumed these diets. A two-way ANOVA was used to analyze the data for rats. A significant interaction (P < 0.003) was calculated between sex and diet. When individual means were compared by using the step-down, multiple
TABLE 1
Calcium concentration in kidneys of male and female rats and female mice fed Certified Rodent Diet-5002 (CRD-5002) and AIN developmental formulation-1 (AIN-DF1) diets (Experiment A1)\(^1\)

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th></th>
<th>Female</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CRD-5002</td>
<td>AIN-DF1</td>
<td>CRD-5002</td>
<td>AIN-DF1</td>
</tr>
<tr>
<td>Ca, mmol/kg</td>
<td>7.58 ± 0.26(^a)</td>
<td>8.61 ± 0.18(^b)</td>
<td>7.90 ± 0.25(^a)</td>
<td>7.53 ± 0.13(^a)</td>
</tr>
</tbody>
</table>

\(^1\)Values are means ± SEM of 10 replicates per group and are expressed on the basis of dry tissue.

\(^2\)A two-way ANOVA showed a significant \(P < 0.003\) interaction between sex and diet. Superscripts are used to show significance derived from pairwise comparisons of means. Values with different superscript letters are significantly different at \(P \leq 0.05\).

\(^3\)There was no significant difference between diets for mice.

stage F test, it was revealed that male rats fed AIN-DF1 had ~14% more \(P < 0.003\) calcium in their kidneys than males and females fed CRD-5002 or females fed AIN-DF1. Because we did not compare these values with those from male rats fed other formulations of purified diet, we have no evidence that the higher value is not normal for male rats fed the new diet. Kidney calcium concentration in female mice was not affected by diet. After 13 wk on experiment, a mechanical problem occurred in the animal room, and the portion of this study with male mice was disrupted. Data for kidney calcium concentrations in these animals are not available.

The initial suggestion by the participants at the AIN Diet Workshop was to maintain dietary calcium at 0.5% and lower phosphorus to 0.4% in the new purified diet (Reeves 1989). Preliminary studies (data not shown) showed that lowering the phosphorus concentration to 0.4% with a Ca:P molar ratio of 0.97 did not eliminate kidney calcification in female rats. In the present study, however, kidney calcium concentrations in female rats and mice fed a purified diet with a Ca:P molar ratio of 1.3 were normal.

Although the Ca:P molar ratio in the CRD-5002 diet was only 0.87, there was no excess calcium accumulation in kidneys of female rats. Other factors are known to affect kidney calcification. Shah and Belonje (1983) showed that female rats fed purified diets with 450 mg magnesium/kg and no fluoride had 100 times more calcium in their kidneys than similar rats fed diets with 2700 mg Mg/kg and 50 mg F/kg. The CRD-5002 diet used in the present experiment contained more than 1800 mg Mg/kg. This is more than three times the amount of magnesium found in purified diets and might be one of the ameliorating factors for kidney calcification in rats fed the nonpurified diet. In addition, ~50% of the phosphorus content of nonpurified cereal-based diets, such as the CRD-5002, is in the form of inositol hexaphosphate (phytate) (PMI 1992). The availability of this phosphorus is relatively low compared with that of nonphytate phosphorus (Moore et al. 1984, Taylor 1980). Therefore, the molar ratio of utilizable calcium and phosphorus in the nonpurified diet might have been closer to 1.7 than 0.87. The increased ratio also would help reduce the incidence of kidney calcification in female rats fed this diet.

**FIGURE 1** (Experiment A1) Growth of male and female rats fed diets AIN developmental formulation 1 (AIN-DF1) and Certified Rodent Diet-5002 (CRD-5002; PMI Feeds, Richmond, IN). Data are means ± SEM of 10 rats per group. A two-way ANOVA showed no differences caused by diet, but a significant \(P < 0.0001\) effect with regard to sex.
At the AIN-Diet Workshop, it was suggested that ultratrace elements be added to the diet to provide those elements for which there is limited evidence of essentiality, and to help eliminate the variations in concentrations that might be found among different diet preparations in different laboratories and among suppliers. Arsenic was suggested as one of these elements. Both diets used in this experiment contained similar amounts of arsenic: 0.20 mg/kg in AIN-DF1 and 0.23 in CRD-5002. Because arsenic accumulates in the erythrocytes of rats but not other species (Hunter et al. 1942, Vaheri 1983), we determined arsenic concentration of whole blood of the rats fed these two diets. Table 2 shows the arsenic concentration of whole blood. There were no significant differences between diet groups. However, female rats had significantly $[P < 0.001]$ more arsenic in their blood than males.

A diet similar in composition to AIN-76A, but with no added arsenic, was found to contain $-5 \mu g$ As/kg. Arsenic concentration of whole blood in rats fed this diet was $-0.67 \mu mol/L$. The possible effects of the higher concentrations of arsenic on red cell function were not determined in this study; however, Fuentes et al. (1981) showed a possible effect of arsenic on oxygen-carrying capacity of red cells of rats. They demonstrated an inverse relationship $[r = -0.87]$ between the concentration of arsenic in red cells and the animal's oxygen consumption. When arsenic concentration increased from $0.13$ to $1.3 \text{ mmol/L}$ of red cells ($0.06$ to $0.61 \text{ mmol/L}$ whole blood), the consumption of oxygen decreased $-10\%$, and $-30\%$ at $4.0 \text{ mmol As/L}$. Because the $10\%$ difference occurred at concentrations seven times that found in rats fed $0.2 \text{ mg As/kg diet}$, an effect on oxygen consumption of rats in the present experiment most likely would not be demonstrable. Although rats fed low arsenic diets performed well, uncertainty about the effects of elevated arsenic in red cells suggests prudence in adding arsenic to the diet.

**Experiment A2.** This experiment was performed to determine the effects of feeding various dietary concentrations of calcium and phosphorus on bone mineral content of rats and mice, while maintaining a Ca:P molar ratio of 1.3. Table 3 shows the calcium, phosphorus and magnesium concentrations in tibiae of male and female rats. A two-way ANOVA of the calcium data showed a significant sex effect $[P < 0.0004]$ in which tibia calcium was higher in female

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**TABLE 2**

Comparison of arsenic concentrations in whole blood of male and female rats fed Certified Rodent Diet-5002 (CRD-5002) and AIN developmental formulation-1 (AIN-DF1) diets (Experiment A1)

<table>
<thead>
<tr>
<th></th>
<th>CRD-5002</th>
<th>AIN-DF1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>65.6 ± 2.8</td>
<td>65.4 ± 1.6</td>
</tr>
<tr>
<td>Female</td>
<td>89.9 ± 4.7</td>
<td>86.0 ± 3.5</td>
</tr>
</tbody>
</table>

$^1$Values are means ± SEM of 10 replicates per group. A two-way ANOVA showed no differences caused by diet. There was a significant $[P < 0.001]$ difference between the sexes.

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**FIGURE 2** (Experiment A1) Growth of male and female mice fed diets AIN developmental formulation 1 (AIN-DF1) and Certified Rodent Diet-5002 (CRD-5002, PMI Feeds, Richmond, IN). Data are means ± SEM of 10 mice per group. A two-way ANOVA at 2 and 3 wk of the study showed significant effects: Sex, $P < 0.0008$; Diet, $P < 0.009$. At 13 wk, a significant $[P < 0.04]$ interaction between sex and diet was calculated. A stepdown, multiple stage $F$ test showed that male mice fed the AIN-DF1 diet weighed significantly $[P < 0.03]$ more than those fed CRD-5002. Letters on the graph at wk 13 indicate differences between groups. Groups with different letters are significantly different from each other.
### TABLE 3

Calcium, phosphorus and magnesium in tibiae of rats and femora of mice fed Certified Rodent Diet (CRD-5002) and AIN developmental formulation-1 (AIN-DF1) with various concentrations of dietary calcium and phosphorus (Experiment A2)\(^1,2\)

<table>
<thead>
<tr>
<th>Mineral</th>
<th>Male</th>
<th></th>
<th></th>
<th>Female</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CRD-5002</td>
<td>LCA-P</td>
<td>MCA-P</td>
<td>HCA-P</td>
<td>CRD-5002</td>
<td>LCA-P</td>
</tr>
<tr>
<td>Ca, mol/kg</td>
<td>5.58 ± 0.07</td>
<td>5.17 ± 0.09</td>
<td>5.34 ± 0.11</td>
<td>5.39 ± 0.1</td>
<td>5.76 ± 0.06</td>
<td>5.54 ± 0.08</td>
</tr>
<tr>
<td>P, mol/kg</td>
<td>3.49 ± 0.08</td>
<td>3.31 ± 0.10</td>
<td>3.37 ± 0.08</td>
<td>3.41 ± 0.07</td>
<td>3.40 ± 0.06</td>
<td>3.38 ± 0.08</td>
</tr>
<tr>
<td>Mg, mmol/kg</td>
<td>166 ± 3</td>
<td>141 ± 3</td>
<td>149 ± 3</td>
<td>147 ± 3</td>
<td>168 ± 2</td>
<td>156 ± 3</td>
</tr>
</tbody>
</table>

\(^1\)Values are means ± SEM of 10 replicates per group and are expressed on the basis of dry tissue.
\(^2\)Abbreviation used: HCA-P, high Ca and P diet (0.67% Ca, 0.40% P); LCA-P, low Ca and P diet (0.33% Ca and 0.20% P); MCA-P, medium Ca or P diet (0.50% Ca, 0.30% P).
\(^3\)A two-way ANOVA gave the following P values for main effects and planned contrasts: Ca, Sex, P < 0.0004; Diet, P < 0.004; CRD-5002 vs. all other diets, P < 0.001; LCA-P vs. MCA-P and HCA-P, P > 0.15; MCA-P vs. HCA-P, P > 0.55; P, no significant differences, Mg, Sex, P < 0.002, Diet, P < 0.0001; CRD-5002 vs. all other diets, P < 0.0001; LCA-P vs. MCA-P and HCA-P, P > 0.37; MCA-P vs. HCA-P, P > 0.62.
\(^4\)A one-way ANOVA showed no significant differences for calcium and phosphorus. The only significant difference for magnesium was CRD-5002 vs. all other diets, P < 0.0004.

### TABLE 4

Body, fat pad and liver weights in rats fed Laboratory Rodent Diet-5001 (LRD-5001), AIN-76A, AIN-76A with L-cystine substituted for D-methionine (AIN-76A\(^{Lys}\)), and AIN developmental formulation-2 (AIN-DF2) diets (Experiment B)\(^3\)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Male rats</th>
<th>Female rats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LRD-5001</td>
<td>AIN-76A</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>319 ± 12</td>
<td>341 ± 9</td>
</tr>
<tr>
<td>Fat pad, g/100 g body wt</td>
<td>0.89 ± 0.07</td>
<td>1.20 ± 0.08</td>
</tr>
<tr>
<td>Liver, g/100 g body wt</td>
<td>2.77 ± 0.12</td>
<td>2.61 ± 0.08</td>
</tr>
</tbody>
</table>

\(^1\)Values are means ± SEM of 10 rats per group.
\(^2\)A two-way ANOVA of body weights and liver weights gave the following P values for main effects and planned contrasts: Body wt., Sex, P < 0.0001; Diet, P < 0.0006; LRD-5001 vs. all other diets, P < 0.064; AIN-76A vs. AIN-76A\(^{Lys}\) and AIN-DF2, P > 0.19; AIN-76A\(^{Lys}\) vs. AIN-DF2, P < 0.0005; Liver, Sex, P > 0.10; Diet, P < 0.05; LRD-5001 vs. all other diets, P < 0.0073; AIN-76A vs. AIN-76A\(^{Lys}\) and AIN-DF2, P > 0.51; AIN-76A\(^{Lys}\) vs. AIN-DF2, P > 0.63. A one-way ANOVA for fat pad weights gave the following P values for main effects and planned contrasts: Epididymal fat pads, Diet, P < 0.0002; LRD-5001 vs. all other diets, P < 0.0003; AIN-76A vs. AIN-76A\(^{Lys}\) and AIN-DF2, P > 0.80; AIN-76A\(^{Lys}\) vs. AIN-DF2, P < 0.009. Perirenal fat pads, Diet, P < 0.01; LRD-5001 vs. all other diets, P < 0.0026; AIN-76A vs. AIN-76A\(^{Lys}\) and AIN-DF2, P > 0.17; AIN-76A\(^{Lys}\) vs. AIN-DF2, P > 0.30.
\(^3\)Epididymal fat pads in male and perirenal fat pads in female rats.
\(^4\)Tissues were weighed immediately upon removal from the animals.
rats than male rats. A significant diet effect \( P < 0.004 \) was also observed. By a single degree of freedom comparison, tibia calcium in rats fed the nonpurified diet was significantly \( P < 0.001 \) higher than in rats fed purified diets. There was no significant difference for low Ca and P diets vs. those with medium and high P. Tibia phosphorus concentration was not affected by either sex or diet. As a result of these findings, we decided to retain the dietary calcium concentration at 0.5% and phosphorus at 0.3% in the final version of AIN-93 [AIN 1993].

There were significant effects of sex and diet on bone magnesium in rats. The ANOVA showed a significant sex effect \( P < 0.0015 \) in which tibia magnesium concentration was higher in female rats than male rats. A significant diet effect \( P < 0.0001 \) was also calculated. By a single degree of freedom comparison, tibia magnesium in rats fed the nonpurified diet was significantly \( P < 0.0001 \) higher than in rats fed purified diets. There was no significant difference for low Ca and P diets vs. those with medium and high Ca and P. The higher magnesium concentration in tibiae of rats fed the cereal-based diet was probably caused by the higher magnesium concentration of this diet.

Table 3 shows data for female mice as well. A one-way ANOVA showed that the only significant effect among diets was a higher concentration \( P < 0.0004 \) of magnesium in the femora of mice fed CRD-5002 than in those fed the other diets.

Data are not shown, but a two-way ANOVA of the kidney calcium concentrations showed no effect of diet among groups for rats, but an interaction between sex and diet was calculated. Kidney calcium concentrations in male rats fed the purified diets were consistently higher \(-12\%\) than in female rats \( P < 0.03 \). However, this difference between sexes was not seen when they were fed the nonpurified diet.

**Experiment B1.** This experiment was done to determine the effects of substituting dietary L-cystine for DL-methionine on growth of rats. Table 4 shows the final body weights as they were affected by the various diets. A two-way ANOVA showed significant \( P < 0.001 \) effects caused by both sex and diet. Single degree of freedom contrasts showed that the final weight of rats fed the nonpurified diet tended to be \( P < 0.06 \) less than that of rats fed the AIN-76 formulation. When rats fed the diet containing DL-methionine [AIN-76A] were compared with those fed diets containing L-cystine [AIN-76ACys], there was no significant effect on body weight. However, rats fed the AIN-DF2 formulation were heavier \( P < 0.0005 \) than those fed AIN-76ACys.

Because AIN-DF2 contained 10% fat whereas the other diets contained only 5%, part of the difference in final body weights could have been caused by a difference in the amount of body fat. We used the weights of epididymal fat pads in males and perirenal fat pads in females, expressed relative to body weight, to estimate the relative amount of body fat. A one-way ANOVA with single degree of freedom comparisons showed that epididymal fat pads of male rats fed the nonpurified diet weighed \(-30\%\) less \( P < 0.0003 \) than those of rats fed the purified diets [Table 4]. The weights of fat pads in male rats fed AIN-76ACys were significantly less \( P < 0.009 \) than in those fed AIN-DF2.

Similar to those of males, perirenal fat pads of female rats fed the nonpurified diets weighed \(-50\%\) \( P < 0.003 \) less than those of rats fed the purified formulations. However, in females, there was not a significant difference between the two formulations containing L-cystine instead of DL-methionine. These data, then, suggest that a large portion of the difference in body weight between rats fed nonpurified and purified diets was caused by the difference in body fat. In a similar experiment [data not shown] in which males and females were fed a purified diet similar to AIN-DF2 and a nonpurified diet for 6 mo, whole-body composition determinations showed that the difference in body fat between males fed the two diets was 22.4 ± 1.2 vs. 13.8 ± 1.5 g/100 g body wt, and for females, 24.6 ± 2.7 vs. 18.6 ± 2.9 g/100 g body wt (means ± SEM, five rats per mean). The difference between diets for males was significant \( P < 0.04 \), but for females, it was not.

One of the outcomes of this experiment was an apparent effect of dietary L-cystine on kidney calcification in female rats fed AIN-76A. **Figure 3** shows the effects of diet on the concentration of kidney calcium for male and female rats. Because of large variability observed in two of the groups, equal variances could not be achieved by data transformation. Therefore, a Kruskal-Wallis test [Kruskal and Wallis 1952] was used to determine differences between group means for each sex. The \( P \) value for this test was <0.0001. Pair-wise comparisons of the ranks using the \( t \) statistic revealed that although the mean kidney calcium in female rats fed AIN-76A Cys was only one-fourth that of rats fed the original AIN-76A formulation, the difference of the ranks was not significant. Consequently, these data only weakly suggest that the substitution of L-cystine for DL-methionine in the AIN-76A diet might aid in the prevention of kidney calcification in female rats. The AIN-DF2 formulation, on the other hand, prevented the deposition of large amounts of calcium in the kidney of female rats. There was no effect of any of the diets on kidney calcium concentration in male rats.

**Experiment B2.** Because only female rats are subject to kidney calcification when fed purified diets with Ca:P molar ratios of <1, we were interested in whether this phenomenon was hormonally related. In this experiment, we determined the effect of
ovariectomy with and without estrogen replacement on kidney calcification. Table 5 shows the effects of these treatments on final body weight. Ovariectomized rats fed AIN-76A were 50% heavier than intact rats fed a similar diet. When 17β-estradiol from a subcutaneous implant was supplied constantly to ovariectomized rats during the last 7 wk of the experiment, their body weights were not different from those of intact rats.

Figure 4 shows the effect of these treatments on kidney calcification. Kidney calcium concentration in intact rats fed the AIN-76A diet was high, but in ovariectomized rats fed the same diet, it was normal. However, when the ovariectomized rats were given estrogen replacement, kidney calcium concentration was comparable to that found in intact rats. Serum 17β-estradiol concentrations (nmol/L, mean ± SEM)

![Graph showing calcium levels in male and female rats.](image)

**Figure 3** (Experiment B1) The concentration of calcium in kidneys of rats fed Laboratory Rodent Diet 5001 (LRD-5001, PMI Feeds, Richmond, IN), AIN-76A with 0.3% L-cystine substituted for 0.3% DL-methionine (AIN-76A Cys), and AIN developmental formulation 2 (AIN-DF2). Data are means ± SEM of ten rats per group. Because of the large variation among groups, equal variance could not be achieved; therefore, the Kruskal-Wallis rank test with pair wise comparisons using the t statistic were used to determine significant differences between means. Separate tests were done for each sex. Female [Kruskal-Wallis] P < 0.0001, different letters between bars signify that the means differ at P < 0.0001. There were no significant diet effects for male rats.

**Figure 4** (Experiment B2) The effect of ovariectomy (OVX) with and without estrogen replacement therapy on kidney calcification in female rats. Data are means ± SEM of 10 rats per group. Because of the large variation among groups, equal variance could not be achieved; therefore, the Kruskal-Wallis rank test with pair wise comparisons using the t statistic were used to determine significant differences between means. Kruskal-Wallis, P < 0.0003, different letters between bars signify that the means differ at P < 0.0001.

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**Table 5**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Intact</th>
<th>OVX</th>
<th>OVX + estrogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>197 ± 7</td>
<td>292 ± 8</td>
<td>186 ± 3</td>
</tr>
<tr>
<td>g/100 g body wt</td>
<td>1.58 ± 0.15</td>
<td>2.15 ± 0.17</td>
<td>1.05 ± 0.10</td>
</tr>
<tr>
<td>Liver weight, g/kg</td>
<td>2.52 ± 0.02</td>
<td>2.26 ± 0.01</td>
<td>3.47 ± 0.08</td>
</tr>
</tbody>
</table>

1Values are means ± SEM of 10 rats per group. A one-way ANOVA showed a significant (P < 0.0001) effect of diet for all three parameters measured. Single degree of freedom comparison: Body wt., intact vs. OVX, P < 0.0001; OVX vs. OVX + estrogen, P < 0.0001; Fat pad, intact vs. OVX, P > 0.9; OVX vs. OVX + estrogen, P < 0.0003; Liver, intact vs. OVX, P < 0.03; OVX vs. OVX + estrogen, P < 0.0001.

2OVX = ovariectomized.

3Perirenal fat pads.

4Tissues were weighed immediately upon removal from the animals.
were as follows: intact rats, 0.24 ± 0.001; ovariec-
tomized rats, 0.19 ± 0.007; ovariecutomized rats given
estrogen, 1.78 ± 0.13. This suggests that estrogen
might be one of the causative factors in kidney cal-
cification in female rats.

The weights of perirenal fat pads of ovariecutomized
rats were not different from those of intact rats but
were significantly heavier \( P < 0.0003 \) than those of
rats receiving estrogen replacement therapy. Liver
weight relative to body weight of ovariecutomized rats
was significantly \( P < 0.03 \) less than that of intact
rats. Liver weight of ovariecutomized rats receiving
estrogen replacement was 50% greater \( P < 0.0001 \)
than that of ovariecutomized rats. This difference
might have been caused by the greater accumulation
of fat in the ovariecutomized rats compared with
ovariecutomized rats given estrogen.

These data show clearly that a purified diet formula-
tion very similar to that recommended for AIN-93G
provides a rate of growth and final body weight in rats
similar to that of rats fed a commercially available
cereal-based, nonpurified diet. The final body weight
of male but not female mice fed the purified diet was
significantly greater than those fed the cereal-based
diet. The new formulation was shown to prevent
kidney calcification in female rats and mice for at
least 16 wk of feeding.

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