Role of Gastrointestinal Microflora in Nitrogen and Mineral Balances in Young Mice Fed on Autoclaved and Irradiated Diets

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Summary Male germ-free (GF) and conventional (CV) mice were fed on steam-sterilized (autoclaved for 30 min at 121°C) and gamma irradiation-sterilized (5 Mrad with 60Co) diets for a one-week adjustment period from 4 weeks of age. During the subsequent week, the amounts of feed eaten were determined, and the feces and urine were collected daily. The effects of the mode of diet sterilization and intestinal microflora on the feed consumption, body weight gain, feed efficiency (body weight gain/feed consumption), feed N efficiency (body weight gain/feed N consumption), and the excretion, absorption and retention of N, Ca, Mg and P and the bone deposits of these minerals were investigated.

1) The method of sterilization of the diet did not appreciably influence these parameters, except for the apparent digestibility of N and ratio of N retention. Compared to autoclaved diet-fed mice, irradiated diet-fed mice showed a higher apparent digestibility of N and lower ratio of N retention.

2) Feed consumption in both GF and CV mice showed no major differences. Body weight gain, feed efficiency and feed N efficiency were higher in GF mice than in CV mice.

3) GF conditions increased the apparent digestibility of N, Ca, Mg and P. Lower total excretion of N, Ca and P in the feces and urine, a higher retention of Ca and P, and higher ratios of retention of N and these minerals were observed in GF mice.

4) The results in GF mice indicated a higher weight of moisture- and fat-free bone and a higher Ca, Mg and P concentration in the bones (femurs and tibias with fibulas) with respect to the body weight minus the weight of the digesta.

Key Words germ-free mice, conventional mice, autoclaved diet, irradiated diet, nitrogen, calcium, magnesium, phosphorus
The effect of treatment of germ-free (GF) animal diets by gamma irradiation to eliminate their bacterial load has received considerable attention in recent years, particularly in relation to heat sterilization.

It has been observed that gamma irradiation has a minimal influence on protein quality in commercial rat diet and that autoclaving reduces protein quality (1–3). However, no comparison has been made concerning the digestibility and retention of N in mice after treatment of diet by autoclaving or gamma irradiation. Moreover, no work has been done to compare the effects of the two kinds of sterilization on the mineral balances in any experimental animals except rats (4).

Pleasants et al. (5) observed urinary calculi and soft tissue calcification in GF mice on a particular diet while the conventional (CV) controls remained healthy. This observation indicates the possibility of basic differences in the metabolism of minerals, including Ca, Mg and P, which could lead to pathological conditions in GF mice.

The previous study showed that GF adult rabbits excreted a higher percentage of ingested Ca and P in the urine than CV rabbits (6). The absence of viable microflora in the digestive tract was associated with an increase in the retention of Ca, Mg and P in young Japanese quails (7). Reddy et al. (8, 9) extended this finding to adult rats, showing that GF rats had higher retentions and bone concentrations of Ca, Mg and P than CV animals. At the same time, Garnier and Sacquet (10) found that GF rats have higher retentions of Ca and P. On the other hand, no comparisons of the retention of Ca, Mg and P in GF and CV mice have been reported.

Therefore, the studies reported here were designed for young mice to elucidate the effect of the mode of sterilization and the intestinal microflora of (a) the absorption and retention of N, Ca, Mg and P, and (b) the bone distribution of Ca, Mg and P.

EXPERIMENTAL

Animals and housing. GF and CV mice from a CF#1 closed colony (Institute of Medical Sciences, University of Tokyo) were used in this investigation. The GF and CV mice were maintained in plastic rearing cages with wire screen bottoms (two or three mice per cage) in Trexler flexible plastic isolators and kept in the animal room at a temperature of 23±3°C. The isolator for GF mice was equipped with a germicidal trap through which samples of feces and urine were removed. The body weight of the GF and CV mice was measured at 4 weeks of age. Mice from the same litters were separated into four groups with almost the same body weight levels, and pairs of animals were fed on each diet. Feed intake and body weight were measured for one week from 5 weeks of age, and the feces and urine were collected daily. Both diet and distilled water were given ad libitum.

Mice with a terminal body weight of 23–29 g were killed by etherization 2 days
Table 1. Formula of diet L-485.

| Ingredient                                | g/100 g |
|-------------------------------------------|---------|
| Ground maize                              | 58.9625 |
| 50% soybean meal                          | 30.0    |
| 17% alfalfa meal                          | 3.5     |
| Corn oil                                  | 3.0     |
| Salt, iodized                             | 1.0     |
| Dicalcium phosphate CaHPO₄·2H₂O           | 1.0     |
| Calcium carbonate CaCO₃                   | 0.5     |
| DL-Methionine (feed grade)                | 0.5     |
| L-Lysine (feed grade)                     | 0.5     |
| Antioxidant (BHT)                         | 0.0125  |
| Trace mineral mixture                     | 0.025a  |
| Vitamin premix                            | 1.0b    |

a In mg/100 g of diet: Mn 6.58, Fe 2.74, Cu 0.22, Zn 1.31, I 0.13 and Co 0.066. b In mg/100 g of diet: vitamin A 2,632 IU, vitamin D₃ 101, vitamin E 21.9, vitamin K₃ 8.77, riboflavin 3.07, pantothenic acid 28.51, niacin 6.58, choline chloride 197, vitamin B₁₂ 0.44, thiamine 6.58, pyridoxine hydrochloride 2.19, folic acid 1.10 and para aminobenzoic acid 5.04.

Table 2. Mean content of moisture, N, Ca, Mg and P in the diet (%).

|                | Moisture | N   | Ca   | Mg   | P    |
|----------------|----------|-----|------|------|------|
|                | 11.39    | 4.09| 0.766| 0.174| 0.568|

after they became 6 weeks of age. Both the right and left femurs and tibias with the fibulas were removed. The GF status of mice was verified at the beginning and the end of the experiment by the routine procedures (11, 12).

Diet. Mice were given sterilized diet L-485 (13) containing 1% Cr₂O₃ (Table 1). The diet was sterilized by autoclaving for 30 min at 121°C or by 5 Mrad of irradiation with ⁶⁰Co. Crude protein, Ca, Mg and P contents of the diet are shown in Table 2. The diet contained 1% Cr₂O₃ for use as a marker, and complete collection of feces was achieved but samples were not analyzed for chromium.

Analysis. The feces was dried with circulating air at 60°C after spraying with a 10% HCl solution. The urine was collected with four drops of conc. H₂SO₄ in the bottom of the cages. The diet and dried feces were ground finely and these samples and the urine with a few drops of CHCl₃ added were kept in a refrigerator until the analysis.

Total N was estimated by the semi-micro Kjeldahl method, ash was measured by incandescence in an electric furnace (550°C), and Ca and Mg were determined with an atomic absorption spectrophotometer (Shimadzu Model MAF 1) in ashed samples diluted with distilled water. This solution also contained 0.1% of LaCl₃ to
suppress interference in the flame (14). Total P was analyzed by the Allen method (15) after wet ashing of the samples with HClO₄.

The data were analyzed statistically by the analysis of variance. The difference between the means was considered significant if \( p < 0.05 \).

RESULTS

In the present experiment, with the exception of N balance, there were no significant differences in the results with respect to the process of sterilization of the diet. Therefore, except for N balance study, no comparison was made between the autoclaved diet-fed group and the irradiated diet-fed group.

Table 3 summarizes the feed consumption, body weight gain, feed efficiency and feed N efficiency for GF and CV mice eating diets sterilized by two kinds of processes for one week from 5 weeks of age.

With respect to feed consumption, the GF and CV groups were found to eat the same amounts of diets. Body weight gain of GF mice in the experimental period was higher than that of CV mice. Thus, the average feed efficiency and feed N efficiency, measured as grams of weight gain per gram of feed consumed and per gram of feed N consumed respectively, were higher for GF mice.

The intake, fecal and urinary excretion, apparent digestibility, retention and rate of retention of N, Ca, Mg and P during the experiment are shown in Tables 4 and 5.

Almost the same N intake was observed between the groups fed on the autoclaved and irradiated diets, but less fecal N excretion was shown in the group

| Group                  | Feed consumption (g) | Body weight gain (g) | Feed efficiency a | Feed N efficiency b |
|------------------------|----------------------|----------------------|-------------------|---------------------|
| Germ-free mice         |                      |                      |                   |                     |
| Autoclaved diet (4)*   | 29.1 ± 2.06**        | 4.05 ± 0.94          | 0.139 ± 0.027     | 3.37 ± 0.63         |
| Irradiated diet (4)    | 29.2 ± 2.67          | 4.53 ± 1.02          | 0.156 ± 0.034     | 3.78 ± 0.79         |
| Conventional mice      |                      |                      |                   |                     |
| Autoclaved diet (4)    | 31.5 ± 3.36          | 2.81 ± 0.81          | 0.089 ± 0.024     | 2.19 ± 0.60         |
| Irradiated diet (4)    | 30.0 ± 4.09          | 3.06 ± 0.73          | 0.102 ± 0.036     | 2.56 ± 0.75         |
| Statistical summary    |                      |                      |                   |                     |
| Sterilization method   | –                    | –                    | –                 | –                   |
| Microbial status       | –                    | ++                   | ++                | ++                  |
| Interaction            | –                    | –                    | –                 | –                   |

* Body weight gain (g)/feed consumption (g). ** Body weight gain (g)/feed N consumption (g). * Number of cages. ** Mean ± SD. ++, Significant difference at \( p < 0.01 \).
Table 4. N, Ca, Mg and P balances (per mouse per day).

|          | Intake (mg) | Excretion (mg) | Apparent digestibility (%) | Retentiona (mg) | Ratio of retentionb (%) |
|----------|-------------|----------------|----------------------------|-----------------|-------------------------|
|          |             | Feces | Urine | Total |                     |                         |                         |
| N        |             |       |       |       |                     |                         |                         |
| Germ-free mice |             |       |       |       |                     |                         |                         |
| Autoclaved diet | 171 ± 13*          | 32 ± 4 | 84 ± 14 | 116 ± 14 | 81.1 ± 2.4 | 55 ± 9 | 32.2 ± 5.3 |
| Irradiated diet    | 171 ± 15            | 25 ± 2 | 103 ± 14 | 128 ± 16 | 85.6 ± 1.2 | 44 ± 11 | 25.6 ± 6.0 |
| Conventional mice  |             |       |       |       |                     |                         |                         |
| Autoclaved diet    | 186 ± 20           | 48 ± 6 | 92 ± 15 | 140 ± 17 | 74.1 ± 2.5 | 46 ± 7 | 24.8 ± 4.4 |
| Irradiated diet     | 174 ± 24           | 33 ± 5 | 106 ± 10 | 139 ± 10 | 81.2 ± 1.8 | 35 ± 19 | 20.1 ± 8.4 |
| Ca        |             |       |       |       |                     |                         |                         |
| Germ-free mice     |             |       |       |       |                     |                         |                         |
| Autoclaved diet    | 31.6 ± 2.3         | 20.1 ± 1.7 | 2.4 ± 0.4 | 22.5 ± 1.4 | 36.4 ± 6.0 | 9.2 ± 2.2 | 28.8 ± 5.3 |
| Irradiated diet     | 31.7 ± 3.4         | 20.9 ± 3.3 | 2.3 ± 0.5 | 23.2 ± 2.9 | 34.4 ± 3.7 | 8.5 ± 0.8 | 27.0 ± 2.1 |
| Conventional mice  |             |       |       |       |                     |                         |                         |
| Autoclaved diet    | 35.8 ± 3.6         | 26.8 ± 3.3 | 1.4 ± 0.4 | 28.2 ± 3.1 | 25.2 ± 3.5 | 7.5 ± 1.5 | 21.1 ± 3.4 |
| Irradiated diet     | 33.5 ± 4.6         | 26.0 ± 3.4 | 1.2 ± 0.1 | 27.2 ± 3.5 | 22.3 ± 3.4 | 6.3 ± 1.6 | 18.7 ± 3.3 |
| Mg        |             |       |       |       |                     |                         |                         |
| Germ-free mice     |             |       |       |       |                     |                         |                         |
| Autoclaved diet    | 7.2 ± 0.6         | 3.8 ± 0.4 | 2.1 ± 0.2 | 5.9 ± 0.4 | 47.7 ± 2.9 | 1.4 ± 0.3 | 18.4 ± 2.4 |
| Irradiated diet     | 7.3 ± 0.9         | 3.9 ± 0.6 | 2.2 ± 0.1 | 6.1 ± 0.7 | 45.9 ± 1.5 | 1.2 ± 0.2 | 15.8 ± 1.7 |
| Conventional mice  |             |       |       |       |                     |                         |                         |
| Autoclaved diet    | 8.4 ± 0.9         | 5.7 ± 0.9 | 1.3 ± 0.2 | 7.0 ± 1.0 | 32.7 ± 6.6 | 1.4 ± 0.5 | 16.6 ± 6.4 |
| Irradiated diet     | 7.9 ± 1.1         | 5.6 ± 1.1 | 1.2 ± 0.4 | 6.8 ± 1.4 | 29.5 ± 8.3 | 1.9 ± 0.7 | 13.7 ± 10.2 |
| P         |             |       |       |       |                     |                         |                         |
| Germ-free mice     |             |       |       |       |                     |                         |                         |
| Autoclaved diet    | 23.6 ± 1.8        | 12.9 ± 1.6 | 4.4 ± 0.7 | 17.3 ± 1.3 | 45.5 ± 6.4 | 6.3 ± 1.2 | 26.5 ± 4.0 |
| Irradiated diet     | 23.6 ± 2.1        | 13.0 ± 1.8 | 4.6 ± 0.2 | 17.6 ± 2.1 | 44.8 ± 5.6 | 6.0 ± 1.4 | 25.5 ± 5.3 |
| Conventional mice  |             |       |       |       |                     |                         |                         |
| Autoclaved diet    | 25.2 ± 2.7        | 17.1 ± 1.7 | 3.8 ± 0.8 | 20.9 ± 2.5 | 31.8 ± 3.1 | 4.3 ± 0.9 | 17.0 ± 3.5 |
| Irradiated diet     | 24.0 ± 3.3        | 17.2 ± 2.0 | 3.0 ± 1.2 | 20.2 ± 3.0 | 28.3 ± 4.8 | 3.8 ± 1.6 | 15.9 ± 6.8 |

*Intake (mg) – total excretion (mg). *Relation (mg)/intake (mg). *Mean ± SD.
Table 5. Statistical summary of N, Ca, Mg and P balances.

|   | Intake | Excretion | Apparent digestibility | Retention | Ratio of retention |
|---|--------|-----------|------------------------|-----------|-------------------|
|   |        | Feces     | Urine | Total |                     |                   |
| N | Sterilization method | – | ++ | + | – | ++ | – | ++ |
|   | Microbial status    | – | ++ | – | + | ++ | – | ++ |
|   | Interaction         | – | – | – | – | – | – | – |
| Ca| Sterilization method | – | – | – | – | – | – | – |
|   | Microbial status    | – | ++ | ++ | ++ | ++ | + | ++ |
|   | Interaction         | – | – | – | – | – | – | – |
| Mg| Sterilization method | – | – | – | – | – | – | – |
|   | Microbial status    | – | ++ | ++ | – | ++ | – | – |
|   | Interaction         | – | – | – | – | – | – | – |
| P | Sterilization method | – | – | – | – | – | – | – |
|   | Microbial status    | – | ++ | + | + | ++ | + | ++ |
|   | Interaction         | – | – | – | – | – | – | – |

+, Significant difference at $p<0.05$. ++, Significant difference at $p<0.01$. 
Table 6. Weight and Ca, Mg and P contents of bone* (44 days of age).

| Moisture- and fat-free bone weight (mg) | Bone ash (mg) | (%)* | Ca (mg) | (%)* | Mg (mg) | (%)* | P (mg) | (%)* |
|---------------------------------------|---------------|-------|---------|-------|---------|-------|--------|-------|
| Germ-free mice                        |               |       |         |       |         |       |        |       |
| Autoclaved diet                       | 143           | 84.0  | 65.3    | 29.5  | 20.3    | 0.75  | 0.52   | 15.8  | 11.1  |
| ± 5*                                  | ± 6.2         | ± 7.6 | ± 1.4   | ± 2.1 | ± 0.01  | ± 0.04| ± 0.8  | ± 0.9 |
| Irradiated diet                       | 146           | 83.9  | 57.4    | 29.6  | 20.3    | 0.75  | 0.51   | 16.1  | 11.1  |
| ± 15                                  | ± 9.1         | ± 0.5 | ± 1.9   | ± 1.7 | ± 0.07  | ± 0.02| ± 0.7  | ± 0.8 |
| Conventional mice                     |               |       |         |       |         |       |        |       |
| Autoclaved diet                       | 135           | 78.8  | 60.5    | 26.9  | 20.6    | 0.61  | 0.47   | 13.5  | 10.4  |
| ± 22                                  | ± 14.1        | ± 1.0 | ± 5.1   | ± 1.3 | ± 0.11  | ± 0.02| ± 2.0  | ± 0.4 |
| Irradiated diet                       | 132           | 78.7  | 59.5    | 28.0  | 21.2    | 0.62  | 0.47   | 13.8  | 10.5  |
| ± 24                                  | ± 15.8        | ± 3.1 | ± 5.5   | ± 0.7 | ± 0.11  | ± 0.01| ± 2.3  | ± 0.6 |

Statistical summary
- Sterilization method
- Microbial status
- Interaction

* Femur and tibia with fibula. *% in moisture- and fat-free bone. * Mean ± SD. +, Significant difference at p < 0.05. ++, Significant difference at p < 0.01.
fed on the irradiated diet. Therefore, the apparent digestibility of N was higher in mice fed on the irradiated diet. Compared with mice fed on the autoclaved diet, mice fed on the irradiated diet excreted more urinary N and had a lower ratio of N retention.

The intakes of N, Ca, Mg and P were approximately comparable in the GF and CV groups, and the fecal excretion of N, Ca, Mg and P was less in GF mice than in CV mice. Therefore, the apparent digestibilities of N and the three minerals were higher in the GF group. The urinary excretion of these minerals was greater, and total excretion of N, Ca and P from the feces and urine was less in GF mice. The retention of Ca and P, and the ratio of retention of N and these minerals were greater in GF mice than in CV mice.

Tables 6 shows the bone (the femur and tibia with fibula) weight and the contents of ash, Ca, Mg and P in the bones of GF and CV mice at 44 days of age. There was no substantial difference between these weights and contents in GF and CV mice except for Mg % and P mg.

The distention of the cecum is one of the major abnormalities seen in GF rodents including mice (16). Therefore, the bone weight and ash, Ca, Mg and P contents in the bone with respect to the body weight minus the weight of the digesta were calculated from Table 6, and are shown in Fig. 1. The weights of moisture- and fat-free bone and levels of ash, Ca, Mg and P contents in the bone were affected by the microbial status of the animals, and were higher in GF mice than in CV animals, although the bone ash content did not reveal any statistically significant
difference between the GF and CV groups.

DISCUSSION

Autoclaving of diets has been reported by several workers to reduce the N digestibility in rodents. Irradiation of diets, on the other hand, was shown to have a negligible influence (1, 2) or an advantageous effect (3) on the N digestibility. Compared with irradiation, autoclaving decreased the apparent digestibility of N in this study. The decrease in the N digestibility after autoclaving might have been caused by modification of the proteins (2).

In spite of the lower apparent digestibility of N, a higher ratio of N retention was observed after autoclaving than after irradiation. Further studies concerning the utilization of N compounds should be undertaken when mice are fed on diets sterilized by different procedures.

Andrieux et al. (4) observed that body weight gain and the apparent digestibility of P were greater in rats fed on an autoclaved diet than in those fed on an irradiated diet. This disagreement with our result in which there was no difference due to the diet sterilization process may be attributed either to the use of a different animal species or a different diet from ours.

The findings for feed consumption in Table 3, in which no difference was obtained between GF and CV mice, agree with reports for guinea-pigs by Newton and De Witt (17), for rats by Luckey (18), and for rabbits by Yoshida et al. (6). On the other hand, the result was different from that of Yamanaka et al. (19) who obtained a lower body weight gain in the GF group of mice. It is possible that this difference may be due to the use of different strains of mice or different diets.

While our results, which showed that the feed efficiency, feed N efficiency, apparent digestibility of N and ratio of N retention in GF mice were higher than in CV mice, were in agreement with those of Yamanaka et al. (20) on N digestibility of mice and those of Forbes and Park (21) on the feed efficiency of chicks, they were contrary to the results obtained from experiments on N digestibility in rats (22–25) and on feed efficiency and feed N efficiency in Japanese quails (7). This may indicate that there are species differences in the effects of the intestinal microflora on feed efficiency, feed N efficiency and N digestibility.

Cardiac output in young adult GF rats was found to be approximately 30% lower than in CV controls (26, 27), and adult GF rats (27) and mice (28) had O₂ consumption values lower by 24% and 12%, respectively than those found in the comparable CV animals. Since GF rodents have a lower metabolic rate than their CV counterparts, it is quite possible that GF mice have a higher feed efficiency, feed N efficiency and N retention than CV mice.

In addition to the apparent digestibility of N, the fact that GF conditions in mice increased the apparent digestibility and net retention of Ca and P, which has already been observed in Japanese quails (7) and rats (8–10), is shown in Table 4. The increase in retention of these minerals was reflected in increased deposition of
the minerals in mice bones (Fig. 1) as was shown for rats (8).

Since the intestines of GF animals are thinner than those of their CV counterparts (29), it can be expected that the thinner GF gut may transport N, Ca and P more rapidly so that less remains in the gut (30).

Most workers agree that trypsin survives longer in the gut of GF animals (31). It seems possible, therefore, that GF mice improved N digestibility.

Another factor has been shown to increase the absorption and retention of Ca and P. It is considered that enhanced levels of intestinal brush border Ca\(^{2+}\)-stimulated ATPase [EC 3.6.1.3] and alkaline phosphatase [EC 3.1.3.1] together with mucosal calcium-binding protein (32) may be responsible for the increased Ca and P absorption observed in GF mice.

A third possibility is the effect of steroid compounds on the Ca absorptive system. GF rats secrete and retain bile acids mostly as conjugates (33), whereas in CV animals bile acids are present mainly in the unconjugated form (34). Conjugated bile salts, such as taurocholate or taurodeoxycholate, have been reported to enhance the absorption of Ca (35, 36), in addition to their possible influence on vitamin D uptake. Moreover, it is conceivable that enteric microflora might alter the vitamin D structure as observed with other steroid compounds.

In conclusion, this investigation indicates that the absence of viable intestinal microflora in mice plays an important role in the absorption and retention of N, Ca and P, and the bone concentrations of these minerals. It is expected that future studies of this nature will be directed towards establishing optimal concentrations of N and these minerals in the diets fed to GF animals. It will also be of value to understand more clearly the mechanism of the increased absorption of N and these minerals in GF animals.

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