Effects of Dietary Protein Levels on Body Composition of Zinc-Deficient Rats

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(Received September 7, 2009)

Summary This study examined why decreased protein intake retards zinc deficiency in zinc-deficient rats. Rats were freely provided zinc-deficient diets with either 10 or 20% protein. Experimental groups consisted of five rats that were fed experimental diets for 0, 3, 4, 12 and 25 d in Experiment 1. The body protein content in rats fed the 10% protein diet was similar to those fed the 20% protein diet for the duration of the experiment. The body zinc content in both dietary groups slowly decreased in a similar manner. Eventually, the body zinc/protein ratio in the 10% protein diet group decreased more slowly than that in the 20% protein diet group. Ingestion of the 10% protein diet also reduced the zinc/protein ratio in bone more slowly compared with that of the 20% protein diet, under zinc-deficient conditions, at 12 d in Experiment 2. However, there was no difference in the zinc/protein ratio of carcass total soft tissue between the two zinc-deficient groups. Decreased protein intake eventually slowed the reduction in both the body and bone zinc/protein ratios in zinc-deficient rats, resulting in retardation of zinc deficiency.

Key Words zinc deficiency, dietary protein level, body zinc, body protein, zinc/protein ratio

Under zinc-deficient conditions, serum alkaline phosphatase (ALPase, orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) activity and the zinc concentration in the serum and femur of rats fed a 10% protein diet have been shown to decrease slower than in those fed a 20% protein diet (1). In addition, the average survival time of zinc-deficient rats fed a 10% protein diet was longer than that observed in rats fed a 20% protein diet (1). Similar results were also found in zinc-deficient rats fed a protein-free diet compared with those fed a 20% protein diet (2). Furthermore, the enzyme activity, zinc concentration and survival time of zinc-deficient rats showed a significant negative correlation to protein intake (3). From these observations, it was determined that serum ALPase activity and the zinc concentration in the serum and femur of zinc-deficient rats are applicable as parameters of zinc status in the body (1) and that a decrease in protein intake retards zinc deficiency and lowers the zinc requirement in rats (3). It has been demonstrated experimentally that a high protein diet increases the zinc requirement in rats (4). Incidentally, the body weight of zinc-deficient rats fed a 20% protein diet and those fed a 10% protein diet were similar (1, 3).

Zinc-deficient rats fed alkali-treated soy protein (AP) showed a slower reduction in these zinc parameters and longer survival time than those fed intact soy protein. The nutritive value of soy protein was reduced by alkaline treatment. In AP, cysteine levels were below the detectable limit compared to untreated soy protein levels. This indicated that the zinc requirements of rats fed the AP diet were lower than those fed the intact soy protein diet, and that the nutritive value, as well as the amount of dietary protein, affected the zinc requirements of rats (5). Cystine supplementation of the AP diet resulted in a reduction in zinc parameters and survival time in zinc-deficient rats (6). Rapid decreases in zinc parameters were found to accompany larger body weight in zinc-deficient rats (5). These findings suggest that rats with a higher protein nutritional status are liable to exhibit severe zinc deficiency. The aim of this study was to examine why a decrease in protein intake retards zinc deficiency in rats.

In a previous study, rats were freely fed a zinc-deficient diet for a period of 4 d until growth ceased and were subsequently fed a zinc-deficient diet via intra-gastric tube feeding. Although growth was not restored, the body protein content continued to increase (7). Therefore, freely feeding rats a zinc-deficient diet may not produce an increase in body weight, even with an increase in body protein. In this study, therefore, the body protein content of zinc-deficient rats was measured.

MATERIALS AND METHODS
Diets. Demineralized soy protein (DP) was prepared as follows (1, 3). Isolated soy protein (Fujipro, Fuji Oil, Osaka, Japan) was suspended in deionized water (300 g/3 L). Protein in the soy protein suspension was precipitated at pH 4.4 by the addition of 6 mol/L HCl. Precipitated protein was washed in a >100-fold volume

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Abbreviations: ALPase, alkaline phosphatase; AP, alkali-treated soy protein; DP, demineralized soy protein.
of deionized water, dried by vacuum freezing and pulverized. This process resulted in a DP zinc content reduced to less than 1 mg/kg. Severe zinc-deficient diets (≤0.2 mg/kg Zn) were prepared using the prepared DP as the dietary protein source. Table 1 shows the composition of the zinc-deficient diets.

Animals. This study was conducted in accordance with the guidelines for the care and use of laboratory animals at Ube Frontier College. Three-week-old male Wistar rats (Japan SLC, Inc., Hamamatsu, Japan) were individually housed under temperature- (23˚C) and light-controlled conditions (lights on from 08:00–20:00). Rats had free access to the 20% casein diet and deionized water for 4 d (Experiment 1) and 6 d (Experiment 2) until the experiment was initiated.

Experiment 1. Deionized drinking water and diet (10 or 20% DP diet) were freely provided to rats. Body weight and food intake were measured daily at 09:00. The diet was provided in a glass bottle and deionized water was available from a plastic bottle with a silicon-Teflon stopper and a glass nozzle. In the morning of day 0, 3, 4, 12 and 25 of the feeding period, five rats per dietary group were anesthetized by intra-peritoneal injection of sodium pentobarbital (Nembutal, 60 mg/kg body weight, Abbot Laboratories, Chicago, IL, USA) and were bled by heart puncture. The femur was immediately excised and the contents of the stomach, cecum and large intestine were removed.

Experiment 2. This experiment was done under the same conditions as Experiment 1 and with the respective control groups. On day 12 of the experimental period, the rats were sacrificed.

Analytical methods. Carcasses were frozen and ground using a meat grinder. Ground carcasses were homogenized in a physiological saline solution containing 0.5% Triton X-100. The femur was cleaned after being boiled in 0.1 mol/L NaOH solution. Femurs and ground carcasses were dried at 110˚C and were ashed in a muffle furnace at 550˚C. The ash was resolved in a solution mixture of 0.7 mol/L HNO3 and 0.2 mol/L HCl. Zinc and calcium levels were analyzed by atomic absorption spectrophotometry. The calcium sample solution was prepared in 1% (0.33 mol/L) HCl containing 1.77% LaCl3 (1% lanthanum). Triglycerides (Triglyceride G-Test Wako), phospholipids (Phospholipid B-Test Wako) and cholesterol (Cholesterol CII-Test Wako) in the homogenate were analyzed using the indicated kits (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Nitrogen was analyzed by the Kjeldahl method and moisture was measured gravimetrically. ALPase activity (9) in the serum was measured and expressed in King-Armstrong (K-A) units: it takes 1 K-A unit of enzyme activity to produce 1 mg of phenol when 100 mL of serum reacts with a substrate for 15 min at 37˚C (1 K-A unit=7.08 IU/L).

Statistics. Data are expressed as means±SE. Data were analyzed by a repeated measures two-way analysis of variance (ANOVA). The Holm test (modified Bonferroni test) was used to compare means. Differences were considered significant at p<0.05. Calculations were performed using a personal computer with the relevant software (Excel Toukei, Version 5.0; Esumi Co., Tokyo, Japan).

RESULTS AND DISCUSSION

Effect of dietary protein level on changes in body composition in zinc-deficient rats (Experiment 1)

Figure 1 shows body weight, food intake and protein intake in zinc-deficient rats. Rats fed a 10% DP diet showed body weight similar to those fed a 20% DP diet, which was in agreement with previous reports (1, 3).
The time course of changes in zinc parameters in zinc-deficient rats is shown in Fig. 2. The zinc concentration in the femur of zinc-deficient rats fed a 10% DP diet decreased more slowly than those fed a 20% DP diet. Similar tendencies were shown for serum ALPase activity. These results indicate that the zinc deficiency in rats fed a 10% DP diet was not as severe as in those fed a 20% DP diet.

Changes in the carcass weight of zinc-deficient rats are shown in Fig. 3. Carcass wet weight of rats fed the 10% DP diet was similar to that of rats fed the 20% DP diet. On a dry weight basis, the carcass weights of both dietary groups were similar throughout the feeding period.

Figure 4 shows the changes in protein, lipids (total of triglycerides, phospholipids and cholesterol) and ash contents in the carcass of zinc-deficient rats. Body protein in rats fed the 10% DP diet was similar to those fed the 20% DP diet. Body lipid levels in rats fed the 20% DP diet decreased rapidly, and were lower than in those fed the 10% DP diet at 12 d. Ash content in both dietary groups exhibited gradual increases. The femur weight in both dietary groups increased similarly (data not shown). This is consistent with the observations of Brown et al. (10). They reported that femur weight increased in zinc-deficient rats (10). Whole bone weight certainly increased throughout the feeding period, despite dietary protein levels.

Figure 5 shows the time course of changes in the zinc level and zinc/protein ratio in the carcass of zinc-deficient rats. Zinc content in both dietary groups gradually declined similarly. In wet samples, zinc concentration was higher in the 10% DP diet group than in the 20% DP diet group at 3 and 25 d. However, the zinc concentration in dry samples was indistinguishable between the two dietary groups for the duration of the experiment. While there was no difference in carcass weight, on a wet or dry basis, between the two dietary groups (Fig. 3), the difference in carcass water content was responsible for the difference in zinc concentration between the wet and dry samples. No difference in carcass protein content between the two groups was
observed; however, calculation of the zinc/protein ratio was undertaken. The zinc/protein ratio in the 10% DP diet group decreased more slowly than that in the 20% DP diet group. The zinc/protein ratio was reflected in that rats fed the 10% DP diet did not exhibit severe zinc deficiency as compared to rats fed the 20% DP diet. Ingestion of a lower protein diet slowed the reduction in the body protein and is responsible for the slower development of zinc deficiency in rats fed 10% DP diet. The time-course change in zinc/protein ratios resembled that of the femur zinc concentration (Fig. 2). The zinc/protein ratio is certainly applicable as a parameter of zinc status in the body as well as the femur zinc concentration.

The zinc/protein ratio in the body is thought to be mainly affected by bone zinc, because bone contains a greater amount of zinc (1, 10). Thus, the zinc/protein ratio in soft tissue (other than bone) may not be higher in rats fed the 10% DP diet as compared to those fed the 20% DP diet under zinc-deficient conditions. The next experiment was carried out in order to measure the zinc/protein ratio in soft tissues.

**Effect of the dietary protein level on the zinc/protein ratio in soft tissue of zinc-deficient rats (Experiment 2)**

In Experiment 1, the largest difference in the zinc/protein ratio between two dietary groups was observed at 12 d. Therefore, the subsequent experiment was carried out for a 12-d period under the same conditions as in Experiment 1.

Growth and food intake of rats are shown in Table 2, and zinc parameters are shown in Table 3. There was no difference in serum zinc concentration between the two zinc-deficient groups. A similar tendency was observed in serum ALPase activity. Additionally, the femur zinc concentration of the 10% DP diet group was higher than that of the 20% DP diet group, and is in good agreement with the results in Experiment 1 at 12 d. In rats fed a zinc-deficient diet, serum zinc concentrations fell to their lowest level within a few days, while the decrease in serum ALPase activity was delayed in comparison to serum zinc concentrations (1, 5). In this experiment, these two parameters seem to have already fallen to their lowest level before 12 d.
Table 4. Zinc/protein ratio in soft tissue from zinc-deficient rats (Experiment 2).

### Carcass

|                | Dry weight (g) | Protein (g) | Zn (µg) | Zn/protein (µg/g) | Ca (g) |
|----------------|----------------|-------------|---------|-------------------|--------|
| −Zn 10% DP     | 21.1±0.86b     | 12.8±0.21b  | 1.203±21c | 57±1.6b           | 94±1.0b | 0.53±0.021b |
| −Zn 20% DP     | 18.8±0.33c     | 14.0±0.16b  | 1.236±21c | 66±0.92a          | 88±0.55d | 0.53±0.013b |
| +Zn 10% DP     | 23.8±0.66b     | 13.7±0.15b  | 1.572±17b | 66±1.7a           | 115±0.72a | 0.53±0.0091b |
| +Zn 20% DP     | 36.9±1.18a     | 22.4±0.59a  | 2.394±62a | 65±0.76a          | 107±1.2b | 0.70±0.015a |

ANOVA
A (Zn)          | <0.001         | <0.001      | <0.001   | <0.01            | <0.001<0.001 |
B (protein)     | <0.001         | <0.001      | <0.001   | <0.02            | <0.001<0.001 |
A×B             | <0.001         | <0.001      | <0.001   | <0.002           | ns<0.001   |

### Bone

|                | Dry weight (g) | Protein (g) | Zn (µg) | Zn/protein (µg/g) | Femur Ca (g/g dry) |
|----------------|----------------|-------------|---------|-------------------|-------------------|
| −Zn 10% DP     | 2.5±0.096b     | 0.77±0.027c | 242±8.7 | 96±1.7b           | 313±6.3c | 0.209±0.0051b |
| −Zn 20% DP     | 2.6±0.071b     | 0.88±0.015b | 195±8.9 | 74±1.8b           | 222±8.6d | 0.203±0.0030b |
| +Zn 10% DP     | 2.5±0.060b     | 0.77±0.020c | 460±6.1 | 186±5.7b          | 608±18a  | 0.215±0.0025ab|
| +Zn 20% DP     | 3.2±0.063a     | 1.12±0.022a | 606±18.3| 192±2.1a          | 559±11b  | 0.222±0.0016a |

ANOVA
A (Zn)          | <0.01          | <0.001      | <0.001  | <0.01             | <0.001<0.002 |
B (protein)     | <0.001         | <0.001      | <0.001  | <0.05             | <0.001ns    |
A×B             | <0.002         | <0.001      | <0.001  | <0.002           | nsns       |

### Total soft tissue (other than bone)

|                | Dry weight (g) | Protein (g) | Zn (µg) | Zn/protein (µg/g) | Femur Ca (g/g dry) |
|----------------|----------------|-------------|---------|-------------------|-------------------|
| −Zn 10% DP     | 18.6±0.81bc    | 12.1±0.21b  | 962±18c | 52±1.7b           | 80±0.51b        |
| −Zn 20% DP     | 16.2±0.28bc    | 13.2±0.15b  | 1.041±17bc | 64±1.2a           | 79±0.70b       |
| +Zn 10% DP     | 21.3±0.68b     | 12.9±0.16b  | 1.112±12b | 52±1.6b           | 86±0.68a       |
| +Zn 20% DP     | 33.7±1.18a     | 21.3±0.59a  | 1.788±59a | 53±0.52b          | 84±0.94a       |

ANOVA
A (Zn)          | <0.001         | <0.001      | <0.001  | <0.001            | <0.001         |
B (protein)     | <0.001         | <0.001      | <0.001  | <0.001            | ns             |
A×B             | <0.001         | <0.001      | <0.001  | <0.001           | ns             |

Means±SE of 6 rats.
1 Values not sharing a common superscript letter within a column are significantly different (p<0.05).
2 Femur was analyzed and bone weight was calculated.

Therefore, differences were not shown in the individual parameters between the two zinc-deficient groups. On the other hand, the femur zinc concentration continued to fall for several weeks following the onset of the experiment (1, 5). Femur zinc concentration had not yet fallen to its lowest level at 12 d. Accordingly, a significant difference in the femur zinc concentration was recognized at 12 d between the two zinc-deficient groups.
These findings show that each zinc parameter has an adequate period from the beginning of the experiment to observe zinc deficiency in the rats.

Carcass analyses in Experiment 2 are shown in Table 4. The carcass weights in the two zinc-deficient groups were similar on a dry weight basis. In zinc-deficient rats, the body protein content in rats fed the 10% DP diet did not differ from that in rats fed the 20% DP diet. Lipid and ash contents in the carcass of zinc-deficient rats were similar to results seen in Experiment 1 (data not shown). No difference in the carcass zinc content was observed between the two zinc-deficient groups. In the two zinc-deficient groups, carcass zinc concentration (on a dry basis) was lower in the 10% DP diet group than in the 20% DP diet group. The zinc/protein ratio in the carcass was higher in the 10% DP diet group than in the 20% DP diet group.

Ninety-nine percent of calcium in the body exists in bone (11). Therefore, bone calcium was considered to account for 99% of the calcium in the body, and carcass bone weight was estimated from calcium contents in the carcass and femur.

\[
\text{Carcass bone (g)} = \frac{\text{Carcass Ca (g)}}{\text{Femur Ca (g)}} \times \text{Femur (g)}
\]

While there was no difference in bone weight, lower bone protein content and higher bone zinc content in the 10% DP diet group compared to the 20% DP diet group were observed between the two zinc-deficient groups. Hence, the femur zinc/protein ratio in rats fed the 10% DP diet was higher than in those fed the 20% DP diet.

Soft tissue dry weight, and zinc and protein contents in the carcass were calculated from the difference between carcass and bone values.

\[
\text{Soft tissue weight (g)} = \text{Carcass weight (g)} - \text{Carcass bone weight (g)}
\]

In zinc-deficient rats, the soft tissue (dry weight) of the 10% DP diet group was similar to that in the 20% DP diet group. Similar tendencies were also observed in the contents of protein and zinc in the soft tissue. Consequently, there was no difference in the soft tissue zinc/protein ratio between the two zinc-deficient groups. These results show that the higher zinc/protein ratio in the carcass of rats fed the 10% DP diet compared to those fed 20% DP diet was caused by higher bone zinc/protein ratio of rats fed the 10% DP diet. In other words, bone zinc/protein ratio markedly affected that ratio in the body.

When rats are fed a zinc-deficient diet, rat growth ceases within a few days, and femur zinc concentration decreases; however, femur weight continues to increase (1, 5, 10). The femur of zinc-deficient rats continues to grow. From this finding, at a minimum, the amount of zinc required for bone growth is retained in the bone of zinc-deficient rats. Thus, it seems reasonable that the zinc in the bone of normal rats is stored at a higher level than is required for bone growth. Additionally, when greater amounts of zinc are administered to rats, the zinc in bone is remarkably increased compared to in the liver and kidney (12). The zinc content in the muscle (12), heart (12) and skin (13) are scarcely increased. These findings suggest that zinc storage ability in bone is much greater than that of the soft tissues. On the other hand, when a zinc-deficient diet is fed to rats, zinc in bone is remarkably decreased to a greater extent than in the pancreas (13), liver (13, 14) and kidney (13–15). Furthermore, the zinc content in the muscle (13), heart (13, 14) and skin (13) is scarcely changed. Skin and muscle occupies a large portion of the body; therefore, the zinc concentration and zinc/protein ratio in the soft tissues are scarcely changed compared with those in the bone.

Incidentally, much data regarding zinc concentration in tissues has been reported (12–18); however, references to zinc/protein ratios in tissues could not be found. A comparison between two zinc-deficient groups in Experiment 2 showed that the carcass zinc/protein ratio of the 20% DP diet group was lower than that of the 10% DP diet group, whereas the carcass zinc concentration of the 20% DP diet group was higher than that of the 10% DP diet group. In addition, the soft tissue zinc/protein ratio of the 20% DP diet group did not differ from that of the 10% DP diet group; however, the soft tissue zinc concentration of the 20% DP diet group was higher than that of the 10% DP diet group. These data show the differing results between zinc concentration and the zinc/protein ratio. The reasons for these differences require future examination.

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