The Bioavailability of Calcium in Spinach and Calcium-Oxalate to Calcium-Deficient Rats

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Summary We estimated the utilization of calcium in spinach and calcium-oxalate to calcium-deficient rats, and the effect of oxalic acid on absorption of dietary calcium by using calcium-deficient rats. The body weight gain of the calcium-deficient rats for 8 days receiving a calcium-deficient diet supplemented with raw-powdered spinach (R-sp), boiled-powdered spinach (B-sp), or calcium-oxalate (Ca-ox), and a control diet supplemented with oxalic acid (OX-C) were 4.8, 2.8, 4.9, and 5.1 g, respectively. The calcium content in the liver and kidney of the rats receiving R-sp, B-sp, Ca-ox, and OX-C diets significantly increased as compared with the calcium-deficient rats. Significant differences in the liver calcium levels were not observed among the rats receiving various additional diets, though the content in the kidneys of the rats receiving R-sp, B-sp, Ca-ox, and OX-C diets were 28.0, 21.5, 0.11, and 0.59 mg, respectively. An especially large amount of calcium was accumulated in the kidneys of the rats receiving R-sp and B-sp diets. The calcium concentration in the serum of the rats receiving Ca-ox and OX-C diets was higher than the calcium concentration in the serum of the R-sp, B-sp, and calcium-deficient rats. The calcium content in the left tibiae of the rats receiving Ca-ox and OX-C diets was higher than that of the rats receiving R-sp and B-sp diets. The breaking force of the right tibiae of the rats was highest in the OX-C group, and higher in the R-sp and Ca-ox groups than the breaking force of the right tibiae of the rats fed on B-sp diet. The alkaline phosphatase activity in the small intestines of the rats rose in the order of the R-sp, B-sp, and Ca-ox groups, although significant differences of the activity were not observed between the Ca-ox and the OX-C groups. The calcium retention of the rats receiving the calcium-deficient, R-sp, B-sp, Ca-ox, and OX-C diets was -18.5, 35.2, 25.6, 41.6, and 45.8%, respectively. About 35% of the calcium in the spinach was absorbed by the calcium-deficient rats, and oxalic acid depressed the calcium absorption in the rats.

Key Words calcium availability, calcium-deficient rat, spinach, calcium-

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oxalate, oxalic acid, alkaline phosphatase

Calcium Recommended Dietary Allowances (RDA) in Japan has been set at a level of 600 mg per day for adults, except for pregnant and lactating women who need more calcium than the normal. However, the statistical data showed that the average calcium intake of Japanese has not reached that level. Calcium is the only nutrient that does not reach RDA value in Japan (1). The National Nutrition Survey (Japan) 1980–1984 showed that calcium intake from vegetables accounted for an average 19% of all sources of calcium. Vegetables are one of the major sources of calcium and are only second to milk and dairy products (2). Though vegetables are known to be rich in oxalic acid, we estimated the content of calcium and oxalic acid in typical vegetables (3). The concentration of calcium and oxalic acid in spinach is the highest of the analyzed vegetables. The large amount of calcium and oxalic acid in spinach is a water-insoluble complex. This complex was thought to be a binding type of both calcium-oxalate and calcium-fiber (3, 4). Spinach is one of the most commonly consumed vegetables throughout the year. However, there are different reports that show spinach in the diet depressed calcium absorption (5–7), and did not adversely affect calcium balance (8–10). Also, bioavailability of iron in spinach was lower than that in cereal (11–13). But Gordon and Chao reported that dietary oxalic acid and phytic acid increased the relative biological value of iron ($\text{FeSO}_4 = 100\%$) (14). What amount of calcium in spinach is absorbed? We investigated the utilization of calcium in spinach and calcium-oxalate added to the diet, and the effect of oxalic acid on calcium utilization to calcium-deficient rats. These utilizations were determined according to the growth rate, calcium balance, calcium content of left tibiae and organs, breaking force of right tibiae, and alkaline phosphatase activity in the small intestines.

MATERIALS AND METHODS

Spinach (smooth leaved) was obtained from a vegetable store in Okayama City and was boiled in distilled-deionized water as described previously (3). Raw and boiled spinach were powdered with a mill after drying in a heat wind drier at 60 °C for 2 days. The amount of calcium and oxalic acid in the raw-powdered and boiled-powdered spinach was determined as described previously (3). The concentration of calcium and oxalic acid were 18.3 mg/g and 147.4 mg/g in raw-powdered spinach and 5.8 mg/g and 137.9 mg/g in boiled-powdered spinach, respectively. Crude protein was calculated as Kjeldahl N $\times$ 6.25 after measuring nitrogen according to the micro-Kjeldahl method (15) and neutral detergent fiber (NDF) was prepared according to the method of Van Soest and Wine (16). These values were 314.9 mg/g and 187.4 mg/g in raw-powdered spinach, respectively; and 298.5 mg/g and 234.0 mg/g in boiled-powdered spinach, respectively.

Weanling male Sprague-Dawley rats were purchased commercially (Japan Charles River Co., Kanagawa) and were used in all experiments. The rats, weighing about 36 g, were housed individually in stainless-steel wire cages in a temperature-controlled room (22–24°C) with a 12-h interval of light (6:00–18:00) and dark. The rats were fed on a purified diet, prepared as described below, containing calcium (586 mg Ca as CaCO_3 and 5 mg Ca as CaHPO_4 per 100 g diet), P (394 mg/100 g diet) and Mg (47 mg/100 g diet) for 7 days, and then were divided into the control and calcium-deficient groups. Distilled-deionized water was given *ad libitum*. The rats of the control and calcium-deficient groups were fed *ad libitum* a purified diet containing (in %): demineralized casein, 20; DL-methionine, 0.3; sucrose, 63.5; demineralized cellulose powder, 5; corn oil, 5; salt mixture (Harpers salt (17) with or without calcium, removed CaCO_3 in the salt mixture), 5; AIN vitamin mixture (100 IU of vitamin D_3 and other fat-soluble vitamins dissolved in corn oil in 100 g diet) (18), 1; choline bitrate, 0.2. Demineralized casein and cellulose powder were prepared by stirring vitamin-free casein in 0.1 M EDTA (Na_2) and cellulose powder in 3 M HCl and then washing them in distilled-deionized water. This treatment was repeated several times until no detection of calcium was made by an atomic absorption spectrophotometer (Shimadzu AA-610S). Demineralized casein and cellulose powder were dried in a heat wind drier at 60°C for 2 days.

When there were significant differences in the body weight of the rats among the control and calcium-deficient groups, the calcium-deficient rats were divided randomly into five groups. They were fed *ad libitum* for 8 days on: 1) calcium-deficient diet (mg/100 g diet: Ca, 7.2; P, 394; Mg, 47); 2) 100 g of calcium-deficient diet supplemented with 15 g of raw-powdered spinach (mg/100 g diet: Ca, 267; P, 507; Mg, 136); 3) 100 g of calcium-deficient diet supplemented with 15 g of boiled-powdered spinach (mg/100 g diet: Ca, 85; P, 479; Mg, 94); 4) 100 g of calcium-deficient diet supplemented with 24.6 mm of calcium-oxalate (mg/100 g diet: Ca, 952; P, 391; Mg, 47); 5) 100 g of control diet supplemented with 24.6 mm of oxalic acid (mg/100 g diet: Ca, 595; P, 391; Mg, 46), which is the same amount of oxalic acid contained in raw-powdered spinach. The content of sucrose, protein, and cellulose powder in these diets was adjusted to the 48.5, 20, and 5% levels, when raw-powdered or boiled-powdered spinach naturally containing crude protein and NDF was added.

At the end of the experimental period, the animals were decapitated and their organs and tibiae were removed quickly and weighed. These organs were stored in a deepfreeze until analyzed. The right tibiae of the rats were used for the measurement of breaking force by a rheodynacorder (Iio Electric Co., Tokyo).

The proximal 10 cm of intestine was rinsed *in situ* with ice-cold physiological saline. Crude enzyme solution for measuring alkaline phosphatase activity in the small intestinal mucosa was prepared according to the method of Hirano *et al.* (19). The alkaline phosphatase activity was determined according to the method of Norman *et al.* (20). The protein in the crude enzyme solution was determined according to the method of Lowry *et al.* (21) using bovin serum albumin as a

standard.

The left tibiae were dried at 105°C for 24 h and defatted in a mixture of chloroform-methanol (2:1, v/v) (22) for 24 h and in ethyl-ether for 24 h. They were then dried at 105°C until they obtained a constant weight. Feces and urine of the rats receiving various diets were collected at 10:00 on the 4th to 7th days, and were dried until a constant weight was obtained. Diets, serum, feces, urine, organs, and left tibiae were digested with 10 ml of concentrated HNO₃ using a Teflon crucible under the condition of 120°C for 6 h. Then the same volume of concentrated HNO₃-concentrated HClO₄ (1:1, v/v) was added to the wet ashed solution. It was gently heated on a hot plate until dried. The ash was dissolved in a small volume of 3 M HCl, and distilled-deionized water was added so that the final concentration of HCl became 0.1 M. This solution was used for the determination of minerals by an atomic absorption spectrophotometer and a direct current plasma emission spectroscopy (BECKMAN, Spectra Span V) as described previously (23, 24).

RESULTS

Weanling rats, weighing 36.2 ± 0.2 g, were fed on a purified diet containing calcium for 7 days. Then they were divided into control and calcium-deficient groups and were fed on a control or calcium-deficient diet for an additional 8 days, respectively. The body weight of the calcium-deficient rats increased from 69.6 ± 0.5 g to 77.9 ± 0.9 g after 4 days. But after that, it decreased with the advance of calcium-deficiency. Significant differences of body weight were observed between

Table 1. Food intake and body weight of the rats receiving various diets. Calcium-deficient rats were divided into five groups: calcium-deficiency, calcium-deficient diet supplemented with raw-powdered spinach (R-sp), boiled-powdered spinach (B-sp), or calcium-oxalate (Ca-ox); the rats in the fifth group were fed on a control diet supplemented with oxalic acid (OX-C). Values represent means $\pm$ SE. Significant differences were calculated by *t*-test.

Group	Beginning state	On 8th day		Weight gain
	Body weight (g)	Food intake (g)	Body weight (g)	(g)
Control	96.4 ± 1.3^a	75.6 ± 2.3^a	116.0 ± 1.5^a	19.6
Ca-deficiency	70.1 ± 1.9^a	59.8 ± 1.8^a	62.7 ± 4.1^{abcd}	-7.4
R-sp	69.0 ± 1.5^a	54.5 ± 4.8^a	74.9 ± 1.4^{ab}	4.8
B-sp	69.0 ± 2.3^a	55.5 ± 5.8^a	72.9 ± 2.3^{ad}	2.8
Ca-ox	69.8 ± 2.6^a	55.9 ± 2.6^a	75.0 ± 1.8^{ac}	4.9
OX-C	68.9 ± 1.2^a	61.5 ± 1.7^a	75.2 ± 1.4^{ab}	5.1

Significantly different at a level of ^a $p < 0.001$ between control and the other groups, and at a ^b $p < 0.001$, ^c $p < 0.01$, and ^d $p < 0.05$ between calcium-deficiency and the other additional groups.

the control and the calcium-deficient rats on the 8th day. Then the calcium-deficient rats were divided into calcium-deficient, R-sp, B-sp, Ca-ox, and OX-C groups.

The food consumption and changes in the body weight of the rats during the 8 days are represented in Table 1. The food consumption of the rats receiving control and calcium-deficient diets from the stage of separated group to the beginning stage were 75.4 ± 1.1 g and 70.3 ± 0.8 g, respectively, and there were no significant differences between both groups. But the body weight of the rats in the beginning stage higher in the control than that of the rats of the other groups. On the other hand, the food consumption and the body weight of the control rats were higher than those of the other groups. And there were no significant differences in food consumption among calcium-deficient and each of the additional groups, but the body weight of the rats in the additional groups was significantly higher than the body weight of calcium-deficient rats. No significant differences were observed in the body weight of the rats among each of the additional groups. The food consumption of the OX-C rats was higher ($p < 0.001$) in comparison with the R-sp rats. The body weight gain of the rats for 8 days receiving control, calcium-deficient, R-sp, B-sp, Ca-ox, and OX-C diets was 19.6, -7.4 , 4.8, 2.8, 4.9 and 5.1 g, respectively. During the 8 days, three out of eight calcium-deficient rats and one out of eight B-sp rats died from the beginning stage to and on the 8th day.

Table 2 shows liver and kidney weight and the calcium content in the liver,

Table 2. Weight of organs and calcium content in organs and serum of the rats receiving various diets.

Three out of eight calcium-deficient rats died in the course of 8 days from the beginning stage to and on the 8th day. Values represent means $\pm$ SE.

	No. of rats	Liver		Kidneys		Serum
		Weight (g)	Ca content (μg)	Weight (g)	Ca content (μg)	Ca content (mg/dl)
Beginning stage						
Control	8	3.8±0.1 ^a	222±9 ^a	1.16±0.03 ^a	71±3	9.1±0.2
Ca-deficiency	8	2.9±0.1 ^a	161±6 ^a	0.95±0.01 ^a	71±4	7.9±0.6
On 8th day						
Control	8	5.3±0.3 ^a	279±3 ^a	1.80±0.11 ^{ab}	99±3 ^a	9.8±0.5 ^c
Ca-deficiency	5	2.5±0.3 ^{aef}	150±3 ^{ae}	1.03±0.12 ^{bd}	36±2 ^{ade}	7.4±0.8 ^{cf}
R-sp	7	2.5±0.1 ^{ae}	171±3 ^{ae}	2.98±0.08 ^{ad}	28,036±1,509 ^{ad}	8.5±0.5
B-sp	6	3.3±0.1 ^{af}	176±3 ^{ae}	2.75±0.24 ^{bd}	21,534±1,942 ^{ad}	8.0±0.5 ^c
Ca-ox	7	3.3±0.1 ^{af}	175±4 ^{ae}	1.08±0.02 ^a	108±44 ^c	9.7±0.5 ^f
OX-C	7	2.8±0.1 ^a	172±4 ^{ae}	1.14±0.05 ^a	599±83 ^{ad}	9.8±0.5 ^d

Significantly different at a $^a p < 0.001$, $^b p < 0.01$, and $^c p < 0.05$ between control and the other groups, and at a $^d p < 0.001$, $^e p < 0.01$, and $^f p < 0.05$ between calcium-deficiency and the other additional groups.

kidneys, and serum of the rats receiving various diets. In the beginning stage, the liver and kidney weight of the control rats were higher than the calcium-deficient rats. The calcium content in the liver significantly decreased in the calcium-deficient rats in comparison with the calcium content in the livers of the control rats. But there were no significant differences in the calcium content of the kidney and serum of the rats of the control and calcium-deficient groups. On the 8th day, the weight and calcium content of the liver in the control rats were significantly higher than the weight and calcium content of the liver of the rats of the other groups. Liver weight was higher in the R-sp, B-sp, and Ca-ox groups than the liver weight of the calcium-deficient group, and was also higher in the R-sp ($p < 0.01$) and Ca-ox ($p < 0.05$) groups than the OX-C group. But significant differences in the liver weight were not observed between the calcium-deficient and OX-C groups, and between the R-sp and OX-C groups. The calcium content of the livers of the R-sp, B-sp, Ca-ox, and OX-C rats was higher in comparison with that of the calcium-deficient rats. On the other hand, the kidney weight of the control rats was higher than the kidney weight of the calcium-deficient, Ca-ox, and OX-C rats. No significant differences were observed in the kidney weight between the R-sp and B-sp groups, but the kidney weight of the R-sp and B-sp groups was from about 1.7 to about 2.8 times as high as that of the other groups. The calcium content of the kidneys was higher in the control rats in comparison with the calcium-deficient rats, and was lower in the control rats than in the R-sp, B-sp, and OX-C rats. The calcium content of the kidney in the R-sp ($p < 0.001$) and B-sp ($p < 0.001$) rats was much higher than that of the other rats. And there were no significant differences in the calcium content of the kidney between the control and Ca-ox groups, but in the Ca-ox rats, it was lower ($p < 0.001$) than the calcium content of the kidney of the OX-C rats. Significant differences in serum calcium content were not observed between the control and the calcium-deficient rats in the beginning stage. On the 8th day, the calcium concentration in the control rats was higher than that in the serum of the calcium-deficient rats. We observed no significant differences in the calcium concentration in serum among the R-sp, B-sp, and calcium-deficient groups, but the calcium concentration of the Ca-ox and OX-C groups was higher than the calcium concentration in the serum of the calcium-deficient group. Significant differences in the concentration were not found between the R-sp and the other groups except the OX-C group, but the calcium concentration of the B-sp group was lower ($p < 0.05$) than the calcium concentration in the serum of the control and OX-C groups.

Table 3 shows the calcium content and breaking force in the left tibiae of the rats receiving various diets. The calcium content and breaking force of the control rats in the beginning stage and on the 8th day were significantly higher than the calcium content and breaking force of the tibiae of the other groups. On the 8th day, the calcium content and breaking force of the tibiae of the control group were higher than those of the other groups, and the calcium content and breaking force of the additional groups were higher than those of the calcium-deficient rats. There were no significant differences in the calcium content of the tibiae between the R-sp

Table 3. Calcium content in left tibia and breaking force of right tibia of the rats receiving various diets.

Values represent means $\pm$ SE.

Group	Beginning stage		On 8th day	
	Ca content (mg)	Breaking force ($\times 10^6$ dyn)	Ca content (mg)	Breaking force ($\times 10^6$ dyn)
Control	29.9 ± 0.2^a	6.6 ± 0.4^a	43.4 ± 1.4^a	14.8 ± 0.6^a
Ca-deficiency	13.2 ± 0.3^a	3.3 ± 0.2^a	11.2 ± 0.1^{abc}	3.0 ± 0.1^{ab}
R-sp			13.6 ± 0.3^{ab}	4.7 ± 0.1^{ab}
B-sp			13.1 ± 0.4^{ac}	3.8 ± 0.1^{ab}
Ca-ox			14.8 ± 0.3^{ab}	4.7 ± 0.1^{ab}
OX-C			16.4 ± 0.2^{ab}	5.3 ± 0.1^{ab}

Significantly different at a level of ^a $p < 0.001$ between control and the other groups, and at a ^b $p < 0.001$ and ^c $p < 0.01$ between calcium-deficiency and the other additional groups.

Table 4. Alkaline phosphatase activity in small intestinal mucosa of the rats receiving various diets.

One unit of alkaline phosphatase activity is expressed as $1 \mu\text{mol}$ of liberated *p*-nitrophenol from *p*-nitrophenol phosphate per min. Values represent means $\pm$ SE.

Group	Beginning stage (unit/mg protein)	On 8th day (unit/mg protein)
Control	38.2 ± 1.9^a	36.5 ± 1.6^{ab}
Ca-deficiency	57.3 ± 2.9^a	31.4 ± 1.7^c
R-sp		66.4 ± 1.8^{ac}
B-sp		52.8 ± 2.2^{ac}
Ca-ox		43.9 ± 1.0^{bc}
OX-C		43.5 ± 0.7^{bc}

Significantly different at a level of ^a $p < 0.001$ and ^b $p < 0.01$ between control and the other groups, and at a level of ^c $p < 0.001$ between calcium-deficiency and the other additional groups.

and B-sp groups, but that of the R-sp ($p < 0.05$, $p < 0.001$) and B-sp ($p < 0.01$, $p < 0.001$) groups was lower in comparison with the Ca-ox and OX-C groups. The calcium content of the OX-C rats was higher ($p < 0.01$) than the calcium content of the Ca-ox rats. Also the breaking force of the R-sp group was higher ($p < 0.001$) in comparison with the B-sp group, and was not different from the breaking force of the Ca-ox group, and was lower ($p < 0.05$) than that of the OX-C group. The breaking force of the B-sp rats was lower ($p < 0.001$) in comparison with the Ca-ox

Table 5. Calcium absorption and balance of the rats receiving various diets. Feces and urine of the rats receiving various diets were collected at 10:00 on the 4th or 7th day. Values represent means $\pm$ SE.

Group	Intake (mg/day)	Feces (mg/day)	Urine (mg/day)	Absorption (%)	Retention (%)
Control	49.83 $\pm$ 1.10 ^a	19.78 $\pm$ 1.16 ^a	0.49 $\pm$ 0.06 ^{ab}	60.3 $\pm$ 2.1 ^a	59.3 $\pm$ 2.0 ^a
Ca-deficiency	0.54 $\pm$ 0.02 ^{ac}	0.49 $\pm$ 0.03 ^{ac}	0.15 $\pm$ 0.01 ^{bcd}	9.3 $\pm$ 2.6 ^{ac}	-18.5 $\pm$ 3.9 ^{ac}
R-sp	16.92 $\pm$ 0.82 ^{ac}	10.91 $\pm$ 0.50 ^{ac}	0.05 $\pm$ 0.004 ^{ad}	35.5 $\pm$ 1.6 ^{ac}	35.2 $\pm$ 1.6 ^{ac}
B-sp	5.89 $\pm$ 0.27 ^{ac}	4.32 $\pm$ 0.27 ^{ac}	0.06 $\pm$ 0.008 ^{ac}	26.7 $\pm$ 1.2 ^{ac}	25.6 $\pm$ 1.3 ^{ac}
Ca-ox	69.86 $\pm$ 1.64 ^{ac}	40.71 $\pm$ 2.06 ^{ac}	0.07 $\pm$ 0.02 ^{ad}	41.7 $\pm$ 1.8 ^{ac}	41.6 $\pm$ 1.8 ^{ac}
OX-C	42.06 $\pm$ 1.61 ^{ac}	22.74 $\pm$ 0.77 ^c	0.07 $\pm$ 0.007 ^{ac}	45.9 $\pm$ 2.1 ^{ac}	45.8 $\pm$ 2.1 ^{ac}

Significantly different at a level of ^a $p < 0.001$ and ^b $p < 0.01$ between control and the other groups, and at a ^c $p < 0.001$ and ^d $p < 0.01$ between calcium-deficiency and the other additional groups.

and OX-C groups. The breaking force of the OX-C rats was higher ($p < 0.01$) as compared with the Ca-ox group.

Table 4 shows the variation of alkaline phosphatase activity in the small intestinal mucosa of the rats receiving various diets. The alkaline phosphatase activity of the calcium-deficient rats in the beginning stage elevated significantly in comparison with the control rats. But its activity was not different between the calcium-deficient and the control group on the 8th day. It was higher in the additional groups than the alkaline phosphatase activity of the control and calcium-deficient groups. In various additional groups, its activity was highest in the R-sp rats, and was higher in the R-sp ($p < 0.001$) and B-sp ($p < 0.01$) rats as compared with the Ca-ox and OX-C rats. There were no significant differences in its activity between the Ca-ox and OX-C groups.

Table 5 shows the daily calcium intake, daily fecal and urinary calcium excretion, calcium absorption, and calcium retention of the rats receiving various diets. The rate of fecal calcium excretion to calcium intake of the rats fed with the R-sp, B-sp, Ca-ox, and OX-C diets was 64.5, 73.3, 58.2 and 54.1%, respectively. The calcium absorption and retention of the control rats were higher in comparison with the other groups. The calcium absorption and retention of the rats were higher in the R-sp ($p < 0.01$) than that of the B-sp rats, and were higher in the Ca-ox ($p < 0.05$, $p < 0.001$) as compared with the R-sp and B-sp groups. There were no significant differences in the absorption and retention of the rats between the Ca-ox and OX-C groups. Absorption and retention were high in the order of the control, OX-C, Ca-ox, B-sp, and calcium-deficient rats. The calcium retention of the calcium-deficient rats indicated negative values.

DISCUSSION

Vegetables are one of the major sources of calcium for the Japanese. Spinach has the highest consumption rate of vegetables throughout the year, and is known to be rich in oxalic acid and calcium. We estimated an availability of calcium from spinach and added calcium-oxalate in the diet on calcium utilization given to calcium-deficient rats.

The body weight of the rats receiving R-sp, B-sp, and Ca-ox diets on the 8th day did not increase in comparison with the body weight of the calcium-deficient rats in the beginning stage, but it was higher in the R-sp, B-sp, and Ca-ox groups than the body weight of the calcium-deficient group on the 8th day (Table 1). The body weight of the calcium-deficient rats decreased still more on the 8th day. These results suggest that calcium in spinach and calcium-oxalate in the Ca-ox diet are available to the rats. The body weight of the rats of each additional group did not attain the level of the control group on the 8th day. It is thought that the body weight gain of young rats reflects the change in nutritional balance, digestion and absorption in the digestive tract, and a change in the metabolism of several materials in the whole body. If, in this study, the rats of each additional group were killed after their body weight attained the level of the control group, the analyzed values, such as calcium content in organs and tibiae, enzyme activity in the intestinal mucosa, and the breaking force of the tibiae, would not show the difference among each group. In the present study, after about a week, the body weight of the rats which were fed with the several additional diets began to rise. It was necessary to assess the difference of the bioavailability of calcium in the R-sp, B-sp, Ca-ox, and OX-C diets to the rats by of all the data, the analyzed values.

As summarized in Table 2, the calcium content in the liver of the additional groups was higher than that of the calcium-deficient rats on the 8th day. But there were no significant differences between the calcium contents of the liver of the additional groups and the calcium-deficient group on the 8th day. The calcium contents and kidney weights of the rats receiving the R-sp and B-sp diets were much higher than the respective data of the other groups. The calcium content of the OX-C rats was higher than that of the Ca-ox, control and calcium-deficient rats. It was reported that water-soluble and insoluble oxalic acid in spinach was absorbed in 6.0(25) and 2.4%(26) in man, respectively. Cook(27) and Hautman *et al.*(28) reported that the majority of the stones in the kidney and urinary tract was formed by calcium-oxalate. And Grossman reported that the increased incidence of urinary calcium-oxalate stones in central Europe after the First World War resulted from an increased consumption of oxalate-rich vegetables(29). We did not measure the concentration of oxalic acid in the kidney, and did not examine the existence of calcium-oxalate stones in the kidneys of the rats receiving R-sp, B-sp, and OX-C diets in the present study. Accumulated calcium in the kidneys of the R-sp, B-sp, and OX-C rats might be made up of calcium-oxalate stones. There were no significant differences in calcium concentration in the serum of the Ca-ox, OX-C, R-

sp, and control groups, but the calcium concentration in the serum of the B-sp rats was lower than that of the control rats. An extremely large amount of calcium accumulated in the kidneys of the R-sp and B-sp rats, nevertheless, the calcium concentration in the serum of the B-sp rats was lower than the calcium concentration in the serum of the control rats. Januzovic and Štraus reported that serum ionic calcium rose markedly in patients with urinary calcium-oxalate stones, but that the total calcium and oxalate levels in the serum were normal (30). It was thought that calcium accumulated in the kidneys because of a large intake of dietary oxalate in spite of increased calcium requirements of the body. Kidney calcification was observed in rats which ingested the high phosphorus diet (31) and the magnesium-deficient diet (32–34). In the present study, magnesium intake of the rats receiving the R-sp and B-sp diets was from 2 to 3 times that of the other groups and was of an adequate amount. But phosphorus intake of the R-sp and B-sp groups was higher (1.2 to 1.3 times) than that of the other groups. The high intake of phosphorus of the rats receiving the R-sp and B-sp diets might be one of the many causes of calcium accumulation in the kidneys. It has been shown that the main risk factors of calcium stone formation in the urinary tract are calcium, oxalate, pH, acid mucopolysaccharides and uric acid (35). The rats of the R-sp and B-sp groups ingested 1,205 mg and 1,148 mg of oxalic acid, respectively, through intake of the diets for 8 days. Accumulation of calcium in the kidneys of the rats receiving the R-sp and B-sp diets might also be caused by a continuous intake of a large amount of oxalic acid. In the present study we thought that a continuous intake of a large amount of oxalic acid was the main factor causing calcium accumulation in the kidneys. It was thought that the intestinal absorption of oxalic acid from the Ca-ox and OX-C diets was lower than that of the R-sp and B-sp diets because oxalic acid formed insoluble calcium-oxalate bound with calcium in the diets and was excreted in feces. Accumulation of great quantities of calcium in the kidneys of the rats with hypocalcemia which have continuously consumed a large amount of the R-sp and B-sp is a very serious problem. This problem must be resolved by clarifying the relationships among the risk factors of kidney calcification such as oxalate, phosphorus, calcium, and silicon in spinach, and calcium accumulation in the kidneys.

The calcium content and breaking force of the tibiae of the rats receiving various additional diets significantly increased in comparison with the calcium content and breaking force of the calcium-deficient rats on the 8th day (Table 3). These showed the same tendency as the results obtained in the change of body weight (Table 1) and the calcium content of the liver and serum (Table 2). Crenshaw reported that there was a high positive correlation between bone calcium content and bone strength (36). In the present study, the bone calcium content and bone strength indicated that calcium taken from various additional diets was absorbed by the calcium-deficient rats.

The alkaline phosphatase activity in the small intestinal mucosa is known to rise by dietary calcium restriction, and it plays a role in enhancing intestinal calcium

transport (37, 38). Its activity in the rats significantly increased in the calcium-deficient rats as compared with that of the control rats in the beginning stage (Table 4). This increased activity in the calcium-deficient rats led to the same results reported by Krawitt *et al.* (38). But, in the present study, the activity decreased in the calcium-deficient rats on the 8th day. We thought that the activity decreased just before death, toward the end of calcium-deficiency, as three of the eight calcium-deficient rats died from the beginning stage to and on the 8th day. The alkaline phosphatase activity of the rats receiving various additional diets was higher than that of the control rats. These results suggested that the rats receiving various additional diets were in the stage of marginal calcium-deficiency, and were vigorously absorbing calcium in their diets.

The calcium absorption (in %) from the diets of the rats fed on the control, R-sp, B-sp, Ca-ox, and OX-C diets was 60.3, 35.5, 26.7, 41.7, and 45.9, respectively (Table 5). In general, absorption of dietary calcium is known to increase in response to elevated physiological calcium requirements of the body (39). The absorption of dietary calcium in the rats receiving various additional diets was lower than that of the control rats; and the dietary absorption of the R-sp and B-sp rats was lower as compared with that of the Ca-ox rats. These results indicated that the calcium contained in spinach was extremely difficult for the rats to absorb. Also, it was thought that oxalic acid added to the control diet prevented the absorption of calcium in the OX-C diet. From the results of our previous experiment (3), we hypothesized that the utilization of calcium in rats would increase in boiled-powdered spinach as compared with raw-powdered spinach because water-soluble oxalic acid, which had been thought to depress calcium absorption, was leached out in boiling water. But the calcium absorption and retention of the B-sp rats were lower in comparison with the R-sp rats in the present study. Water-soluble oxalic acid in spinach, included in a small quantity (3), might have no interference with calcium absorption. From the present study's results about rats' calcium absorption from spinach, it was thought that the absorption of calcium in spinach by rats was equal (11, 13) to or lower (12, 14, 40) than the iron absorption rate from spinach as reported by other investigators. In the present study, calcium absorption from raw-powdered spinach and boiled-powdered spinach by calcium-deficient rats was about 36 and 27%, respectively. We had to conclude that the quality of spinach as a calcium source was very low in comparison with milk and dairy products (31, 41).

REFERENCES

- 1) Ministry of Health and Welfare (1986): Kokumineiyō no Genjō (The result of National Nutrition Survey 1984, Japanese).
- 2) Ministry of Health and Welfare (1982-1986): Kokumineiyō no Genjō (The result of National Nutrition Survey 1980-1984, Japanese).
- 3) Kikunaga, S., and Takahashi, M. (1985): Determination of oxalic acid in vegetables by isotachopheresis. *Nippon Eiyō Shokuryō Gakkaishi (J. Jpn. Soc. Nutr. Food Sci.)*, **38**, 123-128.

- 4) Kikunaga, S., and Takahashi, M. (1986): The availability of calcium in foods (IV). Distribution of calcium in spinach. Notre Dame Seishin University KIYO, Studies in: *Food Nutr.*, **10**, 11–15.
- 5) Fincke, M. L., and Garrison, E. A. (1938): Utilization of calcium of spinach and Kale. *Food Res.*, **3**, 575–581.
- 6) Pingle, U., and Ramasastri, B. V. (1978): Absorption of calcium from a leafy vegetable rich in oxalates. *Br. J. Nutr.*, **39**, 119–125.
- 7) Kelsay, J. L., and Prather, E. S. (1983): Mineral balances of human subjects consuming spinach in a low-fiber diet and in a diet containing fruits and vegetables. *Am. J. Clin. Nutr.*, **38**, 12–19.
- 8) McLaughlin, L. (1927): Utilization of the calcium of spinach. *J. Biol. Chem.*, **74**, 455–562.
- 9) Bonner, P., Hummel, F. C., Bates, M. F., Horton, J., Hunscher, H. A., and Macy, I. G. (1938): The influence of a daily serving of spinach or its equivalent in oxalic acid upon the mineral utilization of children. *J. Pediatr.*, **12**, 188–196.
- 10) Frances, A. J., Thelma, J. M., and Gladys, D. F. (1952): Calcium retained by young women before and after adding spinach to the diet. *J. Am. Diet Assoc.*, **28**, 933–938.
- 11) Layrisse, M., Cook, J. D., Martinez, C., Roche, M., Kuhn, I., Walker, R. B., and Finch, C. A. (1969): Food iron absorption: A comparison of vegetable and animal foods. *Blood*, **33**, 430–443.
- 12) Chao, L. S., and Gordon, D. T. (1983): Influences of fish on the bioavailability of plant iron in the anemic rat. *J. Nutr.*, **113**, 1643–1652.
- 13) Zhang, D., Hendricks, D. G., Mahoney, A. W., and Cornforth, D. P. (1985): Bioavailability of iron in green peas, spinach, bran cereal, and cornmeal fed to anemic rats. *J. Food Sci.*, **50**, 426–428.
- 14) Gordon, D. T., and Chao, L. S. (1984): Relationship of components in wheat bran and spinach to iron bioavailability in the anemic rat. *J. Nutr.*, **114**, 526–535.
- 15) Horwitz, W. (1975): Association of official analytical chemists, in *Official Methods of Analysis*, 12th Ed. sec. 2.049, AOAC, Washington, DC.
- 16) Van Soest, P. J., and Wine, R. H. (1967): Use of detergents in the analysis of fibrous feeds. IV. Determination of plant cell-wall constituents. *J. Assoc. Offic. Anal. Chem.*, **50**, 50–55.
- 17) Rogers, Q. R., and Harper, A. E. (1965): Amino acid diets and maximal growth in the rat. *J. Nutr.*, **87**, 267–273.
- 18) Bieri, J. G., Stoewsand, G. S., Briggs, G. M., Phillips, R. W., Woodard, J. C., and Knapka, J. J. (1977): Report of the American Institute of Nutrition Ad Hoc Committee on standards for nutritional studies. *J. Nutr.*, **107**, 1340–1348.
- 19) Hirano, K., Iizumi, Y., Mori, Y., Toyoshi, K., Sugiura, M., and Iino, S. (1985): Role of alkaline phosphatase in phosphate uptake into brush border membrane vesicles from human intestinal mucosa. *J. Biochem.*, **97**, 1461–1466.
- 20) Norman, A. W., Mircheff, A. K., Adams, T. H., and Spielvogel, A. (1970): Studies on the mechanism of action of calciferol. III. Vitamin D-mediated increase of intestinal brush border alkaline phosphatase activity. *Biochim. Biophys. Acta*, **215**, 348–359.
- 21) Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951): Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.
- 22) Folch, J., Lees, M., and Slavestavly, G. H. (1957): A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.*, **226**, 497–509.
- 23) Kikunaga, S., Arimori, M., and Takahashi, M. (1984): Effects of dietary phytate on

- mineral bioavailability in rats. *Nippon Eiyō Shokuryō Gakkaishi (J. Jpn. Soc. Nutr. Food Sci.)*, **37**, 77–84.
- 24) Kikunaga, S., Arimori, M., and Takahashi, M. (1985): An examination of content of minerals in the biological material difference between direct current plasma emission spectroscopy and atomic absorption spectrophotometry. Notre Dame Seishin University KIYO, Studies in: *Food Nutr.*, **9**, 61–65.
 - 25) Earnest, D. L., Johnson, G., Williams, H. E., and Admirand, W. H. (1974): Hyperoxaluria in patients with ileal resection: An abnormality in dietary oxalate absorption. *Gastroenterology*, **66**, 1114–1122.
 - 26) Prenen, J. A. C., Boer, P., and Mess, E. J. D. (1984): Absorption kinetics of oxalate from oxalate-rich food in man. *Am. J. Clin. Nutr.*, **40**, 1007–1010.
 - 27) Cook, S. A. R. (1971): The distribution of calcium in the human kidney: method and results. *Br. J. Urol.*, **43**, 130–139.
 - 28) Hautman, R., Lehmann, A., and Komor, S. (1980): Intrarenal distribution of oxalic acid, calcium, sodium, and potassium in man. *Eur. J. Clin. Invest.*, **10**, 173–176.
 - 29) Grossman, W. (1938): The current stone wave in central Europe. *Br. J. Urol.*, **10**, 46–54.
 - 30) Januzovic, N., and Štraus, B. (1981): Blood-serum and urinary concentrations of calcium and oxalate ions in patients with calcium-oxalate stones. *Acta Pharm. Jugosl.*, **31**, 105–110.
 - 31) Greger, J. L., Krzykowski, C. E., Khazen, R. R., and Krashoc, C. L. (1987): Mineral utilization by rats fed various commercially available calcium supplements or milk. *J. Nutr.*, **117**, 717–724.
 - 32) Hurley, L. S., Cosens, G., and Theriault, L. L. (1976): Magnesium, calcium and zinc levels of maternal and fetal tissues in magnesium deficient rats. *J. Nutr.*, **106**, 1261–1264.
 - 33) Woodard, J. C., and Jee, W. S. S. (1984): Effects of dietary calcium, phosphorus and magnesium on intranephronic calculosis. *J. Nutr.*, **114**, 2331–2388.
 - 34) Forbes, R. M., and Parker, H. M. (1980): Effect of magnesium deficiency on rat bone and kidney sensitivity to parathyroid hormone. *J. Nutr.*, **110**, 1610–1617.
 - 35) Robertson, W. G., Peacock, M., Heyburn, P. J., Marshall, D. H., and Clark, P. B. (1978): Risk factors in calcium stone disease of the urinary tract. *Br. J. Urol.*, **50**, 449–454.
 - 36) Crenshaw, T. D. (1986): Reliability of dietary Ca and P levels and bone mineral content as predictors of bone mechanical properties at various time periods in growing swine. *J. Nutr.*, **116**, 2155–2170.
 - 37) Morrissey, R. L., and Wasserman, R. H. (1971): Calcium absorption and calcium-binding protein in chicks on differing calcium and phosphorus intakes. *Am. J. Physiol.*, **220**, 1509–1515.
 - 38) Krawitt, E. L., Stubbert, P. A., and Ennis, P. H. (1973): Calcium absorption and brush border phosphatase following dietary calcium restriction. *Am. J. Physiol.*, **224**, 548–551.
 - 39) Allen, L. H. (1982): Calcium bioavailability and absorption: a review. *Am. J. Clin. Nutr.*, **35**, 783–808.
 - 40) Van Campen, D. R., and Welch, R. M. (1980): Availability to rats of iron from spinach: Effects of oxalic acid. *J. Nutr.*, **110**, 1618–1621.
 - 41) Wong, N. D., and Lacroix, D. E. (1980): Biological availability of calcium in dairy products. *Nutr. Rep. Int.*, **21**, 673–680.