Note

The Effect of Gut Flora on the Utilization of Ca, P and Zn in Rats Fed a Diet Containing Phytate

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Our previous experiment⁽¹⁾ showed that appreciable amounts of extra phytate P supplemented to a diet was utilized by young germfree (GF) and especially by young conventional mice. Hence, it is assumed that partial substitution of inorganic P by phytate P in purified diets will have no inhibitory effect on the growth of young rodents, even if the total P content of the diet is around the P requirement level. Although there are simple comparisons of Zn status^(2,3) or phytate hydrolysis⁽⁴⁾ between GF and conventional animals, no work has been reported on the effect of gut flora on the interaction between the bioavailability of Zn and phytate, which is known as a cation binding agent.⁽⁵⁾ Therefore, the experiment described in this paper was performed to estimate the effect of gut flora on the utilization of dietary Zn and phytate P by feeding phytate-containing and -free diets, which had the same levels of P, to GF and conventionalized (CVZ) rats. Since it is generally recognized that Ca is a predominant metal in the diet and exhibits a synergistic effect with Zn to increase the precipitation of metallic phytate,^(6,7) Ca analysis was undertaken simultaneously in the present experiment.

Male inbred Fischer strain (F344/Yit) GF rats, and CVZ rats obtained by orally intubating the fresh cecal contents of conventional rats diluted with physiological saline solution at 3 weeks of age, were used. The GF and CVZ rats at 4 weeks of age with an average body weight of 64.8 g were divided into groups of four animals each, and fed a phytate-free diet, L-488FY (0% phytate diet, a modification of L-488F^(8,9)) or a 1% phytate-containing diet, L-488FYPh (Table I). To obtain equal levels of dietary P, the 1% phytate diet was formulated by complete substitution by sodium phytate of the Na₂HPO₄ in the 0% phytate diet. Theoretically, the Na level of these two kinds of diet was roughly the same. The rats were housed

individually in stainless steel metabolism cages for 3 weeks at 22 ± 1°C under 12 hr light-dark periods. The diets were sterilized by 5 Mrad of γ -irradiation with ⁶⁰Co, and the contents of N (g), Ca (mg), P (mg) and Zn (mg) in 100 g of dry matter of the sterilized diets were analyzed as 3.29, 878, 620 and 3.62 for the 0% phytate diet, and 3.34, 787, 599 and 3.77 for the 1% phytate diet (mean values of two lots), respectively. Distilled deionized water after autoclaving and sterilized diets were available *ad libitum*. Since bacterial contamination occurred during the initial experimental period, the experiment for the GF groups fed the 1% phytate diet from a different lot was repeated at a later period.

Body weight and feed intake were recorded daily during the 2-week period after one week of preliminary feeding, and the feces and urine were collected daily. The feces were

TABLE I. COMPOSITION OF EXPERIMENTAL DIETS

	L-488FY*	L-488FYPh
	(g/100 g)	
Casein	24.0	24.0
Corn starch	60.4	60.4
Cellulose ^a	5.0	5.0
DL-Methionine	0.3	0.3
Corn oil ^b	5.0	5.0
myo-Inositol	0.1	0.1
Vitamin mixture ^c	0.5	0.5
Mineral mixture ^d	3.7	3.7
Na ₂ HPO ₄	1.0	—
Sodium phytate ^e	—	1.0

^a Toyo filter paper for feed type No. D (40 ~ 60 mesh).

^b Corn oil : modification of Ladek-55 = 3 : 2. Modification of Ladek-55** containing 1,600 IU of vitamin A (acetate + palmitate), 100 IU of vitamin D₃, 47.5 mg of *dl*- α -tocopheryl acetate, 10 mg of vitamin K₃, and corn oil to make 2 g.

^c Vitamin B mixture** contained 6 mg of thiamine, 3 mg of riboflavin, 5 mg of nicotinamide, 30 mg of calcium pantothenate, 200 mg of choline chloride, 2 mg of pyridoxine HCl, 0.4 mg of pyridoxamine dihydrochloride, 0.1 mg of biotin, 1 mg of folic acid, 5 mg of *p*-amino-benzoic acid, 25 mg of a trituration of 0.1% vitamin B₁₂ in mannitol, and corn starch carrier to make 500 mg.

^d Mineral mixture contained 515 mg of iodized NaCl, 400 mg of MgSO₄, 60 mg of FeC₆H₅O₇ · nH₂O, 20 mg of MnCO₃, 2.5 mg of CuO, 2.5 mg of ZnO, 0.05 mg of CoCl₂ · 6H₂O, 1.00 mg of NaF, 0.005 mg of MoO₃, 0.01 mg of KBr, 0.01 mg of Na₂SeO₃, 1,700 mg of CaCO₃, and 1,000 mg of K₂HPO₄ (modification of Reddy, B. S. *et al.****).

^e Sigma Chemical Co., U.S.A.

* Modification of L-488F^(8,9)

** B. S. Reddy, *et al.*, *J. Nutr.*, **97**, 327 (1969).

*** B. S. Reddy, *et al.*, *J. Nutr.*, **99**, 353 (1969).

dried in an electric oven at 70°C after spraying with a 5% HCl solution, and then weighed. The urine was collected in a cylinder containing a 5% HCl solution. The rats were sacrificed, using ether, two days after they had reached 7 weeks of age. The right femur and the right tibia with the fibula were removed, dried and defatted by ether. The dietary N was estimated by the semi-micro Kjeldahl method, total ash of the bone was measured by incandescence in an electric furnace (550°C), and Ca and Zn were determined with an atomic absorption spectrophotometer¹⁰⁾ (Shimadzu Model AA-640) after wet-ashing of

the sample with HNO₃-HClO₄ (4:1, v/v). Total P was analyzed by Allen's method¹¹⁾ after digestion of the samples with HClO₄. The data were analyzed statistically by an analysis of variance, and the differences between the means were considered significant at $p < 0.05$. When interaction among the four experimental groups was found, the data between the GF and CVZ groups and between the 0% and 1% phytate diet groups were analyzed for statistical significance by the *t*-test ($p < 0.05$).

The average initial (5 weeks of age) and final (7 weeks of age) body weights of the rats were 84.9 g and 135.5 g,

TABLE II. DAILY INTAKE, EXCRETION, RETENTION AND BONE^a CONTENT OF Ca, P AND Zn IN GERMFREE AND CONVENTIONALIZED RATS FED A 0% OR 1% SODIUM PHYTATE DIET (mean value and standard deviation for four rats)

Group	Intake	Excretion as % of intake			Bone ^a		
		Feces ^b	Urine	Total ^c	Total ash	Mineral content	Mineral % ^d
Ca Germfree rats	(mg)				(mg)	(mg)	(%)
0% phytate diet	77.4±3.2	38.2±3.9	2.1±0.2	40.3±3.8	233±19	77.6±9.2	20.2±2.1
1% phytate diet	63.6±2.6	48.2±1.3	0.7±0.2	48.8±1.2	236±15	79.6±3.8	21.1±0.7
Conventionalized rats							
0% phytate diet	82.5±7.9	51.6±2.5	2.0±0.4	53.6±2.2	217±22	75.1±7.1	21.0±0.4
1% phytate diet	81.6±5.7	49.9±3.4	2.7±0.3	52.6±3.1	222±8	75.5±2.1	21.4±1.2
P Germfree rats	(mg)					(mg)	(%)
0% phytate diet	54.6±2.3	23.8±2.3	29.6±6.4	53.4±7.2		38.6±3.7	10.1±0.6
1% phytate diet	54.7±2.3	33.0±0.9	10.4±1.0	43.4±1.8		37.8±2.5	10.0±0.2
Conventionalized rats							
0% phytate diet	58.3±5.6	34.5±3.1	30.8±1.7	65.3±3.7		36.9±4.5	10.3±0.4
1% phytate diet	55.5±3.9	44.7±5.4	17.4±0.9	62.1±5.2		35.8±2.2	10.1±0.5
Zn Germfree rats	(μg)					(μg)	(mg%)
0% phytate diet	319±13	56.1±6.7				133±17	34.6±4.0
1% phytate diet	340±15	64.7±2.1				111±6	29.4±0.8
Conventionalized rats							
0% phytate diet	340±32	62.8±4.6				112±12	31.4±0.8
1% phytate diet	322±22	65.1±5.5				90±10	25.4±1.2
Statistical summary							
Ca Microbial status	**	**	**	**	—	—	—
Phytate content	*	*	*	*	—	—	—
Interaction	*	**	**	**	—	—	—
P Microbial status	—	**	*	**	—	—	—
Phytate content	—	**	**	*	—	—	—
Interaction	—	—	—	—	—	—	—
Zn Microbial status	—	—				**	*
Phytate content	—	*				**	**
Interaction	—	—				—	—

^a Femur and tibia with fibula at 2 days after 7 weeks of age.

^b 100—fecal excretion as % of intake=apparent digestibility (%).

^c 100—total (=fecal and urinary) excretion as % of intake=retention rate (%).

^d % or mg % in moisture- and fat-free bone.

* Significant difference at $p < 0.05$.

** Significant difference at $p < 0.01$.

respectively, and the average feed intake per day was 10.2 g during the 2-week experimental period. Daily body weight gains (g) for GF rats fed the 0% or 1% phytate diet and for CVZ counterparts fed the 0% or 1% phytate diet were averaged as 3.6 or 3.9 and 3.5 or 3.4, respectively. Although there were no significant differences among body weights or total feed intakes in the four experimental groups, GF rats and the 1% phytate groups showed a lower Ca intake than CVZ rats and the 0% phytate groups. Therefore, the fecal and urinary data analysed are expressed as the percentage of feed intake. The fecal, urinary and total (=fecal + urinary) excretion percentage of Ca and P and the fecal excretion percentage of Zn during the experimental periods, together with the contents of ash and the three minerals in the bones of the rats two days after 7 weeks of age, are given in Table II. Since the urinary excretion of Zn was less than 1% of the intake, coinciding with the result of Reddy *et al.*,²⁾ and the urine stock for analysis was minimal, an analysis of urinary Zn was not conducted.

A comparison of balance studies showed a higher percentage of fecal and total excretions, *i.e.* lower apparent digestibilities and retention rates, of Ca and P in the CVZ groups than in the GF groups. Our previous study¹⁰⁾ using mice also gave the same results and we discussed the reasons for this. Although there was no difference in Zn balance, a similar tendency to those of Ca and P was obtained from the fecal excretion of Zn.

Concerning the mineral balance, dietary phytate had a definite effect: the percentages of fecal excretions of Ca, P and Zn and that of the total excretion of Ca were higher in the 1% phytate groups than in the 0% phytate groups, with the exception of Ca in the CVZ rats. For Ca and P, the same tendencies have already been obtained in mice.¹⁾

However, interactions among the fecal, urinary or total excretion of Ca for the four experimental groups were found. The results of statistical analyses show that rats fed the 1% phytate diet excreted more fecal and total Ca than those fed the 0% phytate diet under GF conditions, whereas no difference was found in the fecal and total Ca excretion between both the CVZ-group rats fed the 0% and 1% phytate diets. (No discussion is made here on the result of urinary Ca because of the negligible amount of excretion.) These observations show the possibility that the Ca-phytate formed in the digestive tract, which under practical dietary conditions may be a predominantly insoluble metal-phytate complex at neutral pH,^{5,12)} is actively hydrolysed by the gut flora of rats and Ca is absorbed from the intestine. Accordingly, it is assumed that a similar tendency for the fecal excretion of P to that of Ca is obtained. However, fecal P excretion was higher in the 1% phytate group irrespective of microbial status, and thus it appears that the hydrolysis of phosphate P from phytate that released Ca occurred only partially. Moreover, it is necessary to investigate the possibilities that there was higher absorptive activity on Ca than P in the lower parts of the intestine and/or that the endogenous

excretory patterns of Ca and P into the intestine were different.

In spite of the higher percentage of fecal P excretion, a lower percentage of total P excretion was obtained in the 1% phytate groups, unlike the case of Ca. This is not in agreement with the previous study,¹⁾ in which mice fed a diet extra-supplemented with 1% phytate showed a higher percentage of total excretion of P (= a lower retention rate of P), possibly since the diet with 1% phytate had a much higher P content than the with phytate-free diet. Although it is suggested in the present study that the lower P absorption in the 1% phytate groups resulted in a lower P excretion into the urine due to the homeostasis of P retention and thus brought about lower total P excretion, experiments should be undertaken to clarify this discrepancy between mice and rats.

Studies based on a balance test²⁾ and on a single oral dose of labeled Zn¹³⁾ showed that in rats fed a phytate-free diet, the apparent Zn absorption was 16.1%²⁾ and 13.5%¹³⁾, respectively. These values are lower than that (100% - 62.8% = 37.2%) obtained from CVZ rats consuming the 0% phytate diet in the present experiment. This disagreement of apparent Zn digestibility is attributable, at least in part, to the younger rats used in our study.

The data on bones in Table II indicate that there were no differences in bone ash, Ca and P contents, and Ca and P % in the bone ash between the GF and CVZ rats, and between the 0% and 1% phytate groups. These values are roughly same as those of Reddy *et al.*¹⁴⁾ who fed a similar kind of diet to that used in this study to rats until 115 days of age. When the Zn levels of the bones are compared, on the other hand, a lower content and percentage of Zn were obtained in the CVZ rats and 1% phytate groups, respectively. Our results for the comparison between the GF and CVZ animals agree with the observation of Reddy *et al.*,²⁾ in which the Zn content in the femur was significantly lower in conventional rats than in GF rats and this tendency was also shown by Smith *et al.*,³⁾ whereas there was no difference between the two groups in the percentage of the fecal and total excretion of Zn. The fact that the 1% phytate groups, which excreted a higher percentage of fecal Zn, had lower levels of bone Zn than the 0% phytate groups could be attributed to the inhibitory effect of dietary phytate on Zn bioavailability, irrespective of the gut flora.

Even though there were no differences between the body weight gains in the 0% and 1% phytate groups, the bone Zn levels tended to be lower in the 1% phytate groups. Morris and Ellis¹⁵⁾ also observed that a dietary phytate/Zn molar ratio of 10 did not depress the growth of rats but decreased the bone Zn accumulation. These results indicate that bone Zn deposition is a more sensitive indicator of Zn bioavailability than body weight gain, in agreement with Močilović *et al.*¹⁶⁾ and Forbes *et al.*¹⁷⁾ However, the experiment by Forbes *et al.*¹⁷⁾ showed that a phytate/Zn molar ratio of 20, the same as with our experimental diet as shown later, reduced the body weight

gain of rats at a moderate dietary Ca level of 0.8%. One reason for this disagreement with our results of body weight gain may be a lower dietary Zn level (12 mg/kg) in the experiments of Forbes *et al.* than in our study (37.7 mg/kg).

Bone Zn concentrations of the CVZ rats obtained from the present experiment were approximately the same as those of Flanagan *et al.* (24.2 mg/100 g dry femur),¹⁸⁾ who fed a phytate-free diet containing 0.00485% of Zn to growing rats. However, Reddy *et al.*²⁾ showed a lower level of Zn (17.4 mg/100 g dry femur) in adult rats fed a phytate-free diet containing a higher level of Zn. This discrepancy in results obtained from these experiments might be due to the different ages of the rats and/or the different analytical methods for Zn, *i.e.* dry-ashing in Reddy *et al.* and wet-ashing in the other two studies.

The phytate-containing diet (L-488FYPh) used in our experiment was supplemented with approximately 1% sodium phytate, and the Ca and Zn contents of the diet were analyzed as 0.787% and 0.00377%, respectively. Therefore, the molar ratio of Ca-phytate-Zn in the diet was calculated roughly as 20:1.2:0.058 (=340:20:1). The *in vivo* experiments indicated that a high Ca intake with a molar ratio of Ca/phytate of 15⁴⁾~19¹⁹⁾ decreased the utilization of phytate in rats, and the Zn bioavailability was usually predicted by the phytate/Zn molar ratio of the diets and the amounts of Ca; the ratios of phytate/Zn above 10 were generally associated with impaired Zn bioavailability,^{13,15,20~23)} and the ratios were lowered by higher dietary Ca levels.^{17,22,24)} Since the 1% phytate diet used in the present experiment had high molar ratios of Ca/phytate (17) and phytate/Zn (20), higher fecal excretion of Ca, P and Zn and lower Zn accumulation in the bones were obtained in the rats fed the 1% phytate diet than in those fed the 0% phytate diet.

In conclusion, the present experiment on young rats showed: (1) that gut flora increased the fecal and total excretion as a percentage of the intake of Ca and P and decreased the bone Zn level, (2) that substitution of 1% sodium phytate in the diet increased the fecal excretion percentage of P and Zn, but reduced total excretion percentage of P and the bone Zn level, irrespective of microbial status, and (3) that dietary phytate increased the fecal and total excretion percentage of Ca in GF rats but not in the CVZ counterparts.

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