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Effect of phytate and other dietary factors on intestinal phytase and bone calcification in the rat

BY ANN HEULWEN ROBERTS AND JOHN YUDKIN

Department of Nutrition, Queen Elizabeth College, University of London

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The work described here began as an extension of our previous studies on enzyme induction. We were interested to see whether an increase in dietary phytate resulted in an increase in intestinal phytase. This question is of particular interest in relation to the effects of diets rich in phytate on calcium absorption in man. Walker, Fox & Irving (1948), amongst others, have claimed that an increase in dietary phytate produces only a temporary loss of Ca from the body; after a few weeks, Ca begins to be retained again. One way in which this adaptation could occur would be by an increased destruction of phytate, caused by an increased amount of phytase evoked by the increased dietary phytate.

There is a great deal of published work on phytate and on plant phytases, but little on intestinal phytase. This enzyme was first reliably demonstrated in rats by Patwardhan (1937). Some of its properties were defined by Spitzer & Phillips (1945). A study of the effects of some dietary changes showed only that phytase activity existed in the presence or absence of vitamin D, or of inorganic or bound phosphorus (Spitzer, Maruyama, Michaud & Phillips, 1948). A more detailed study of the effects of vitamin D in a variety of diets was made by Steenbock, Krieger, Wiest & Pileggi (1953) and by Pileggi, De Luca & Steenbock (1955). In all instances, vitamin D increased the amount of intestinal phytase, but there was no evident relationship between enzyme activity and the severity of rickets when the rats were given rachitogenic diets. Pileggi, De Luca, Cramer & Steenbock (1956) showed that the addition of citrate to the diet reduced the amount of intestinal phytase.

Thus, although we were chiefly concerned with the effects of dietary phytate on the amount of intestinal phytase, we extended our study to cover the effects of other dietary factors such as vitamin D and Ca. Since these all affect bone calcification, we included measurements of bone ash in most of our experiments.

EXPERIMENTAL AND RESULTS

Animals

Hooded rats of the Lister strain were used. They were weaned at 23 days and given ground stock diet and milk for 24 h. From 24 days onwards they were fed on the experimental diets. For some of the preliminary experiments, and for the experiments on the induction and healing of rickets, the animals were kept on sawdust, six to eight

in a cage. For the experiments in which they were given purified diets, they were housed on grids in individual cages.

Food and water were provided freely for all animals. When B vitamins were to be administered 1 μg vitamin B_{12} was given weekly and 1 ml of a solution containing the other B vitamins was given daily. It supplied 5 mg choline chloride, 1·1 mg inositol, 500 μg nicotinic acid, 500 μg calcium-D-pantothenate, 25 μg thiamine, 150 μg riboflavin, 40 μg pyridoxine, 1 μg biotin, 3 μg *p*-aminobenzoic acid and 2 μg folic acid. Vitamin A (120 i.u.) and vitamin K (menaphthone, 800 μg), dissolved in 80 mg arachis oil, were given weekly and also, on a different day, α-tocopherol (1 mg in 15 mg arachis oil). The different experimental diets are described with the different experiments.

Measurements of bone ash

The method of Hume (1922) was used. The femur, tibia and fibula of the right leg were cleansed of all adhering tissue and dried at 100° overnight. They were tied in a silk bag and extracted in a Soxhlet apparatus for 7 h with a mixture of equal parts of industrial ethyl alcohol and light petroleum (b.p. 40°–60°). The bones were dried and weighed, and then ashed at 650° for 2 h.

Measurement of intestinal phytase

Each animal was killed by a blow at the back of the neck. The whole of the small intestine was removed and the proximal 12 in. cut off from the remainder. Patwardhan (1937) and Spitzer & Phillips (1945) had used the whole of the intestine. However, the concentration of several enzymes is higher in the proximal part than in the distal part (Heyman, 1930; Laskowski, 1937), which is also true of phytase (Steenbock *et al.* 1953). We have found phytase activity to be three times greater in the proximal 12 in. than in the remainder. The proximal portion was placed in ice-cold water in a Petri dish, surrounded by crushed ice. Both it and the remainder were cleaned of adhering matter and the contents removed by gently blowing water through the lumen from a wash-bottle. They were then blotted on filter-paper and weighed. The distal portion was discarded, and the proximal portion cut up finely with scissors in the Petri dish on ice. It was then homogenized by the technique of Potter & Elvehjem (1936) in 20 ml ice-cold water for exactly 2 min at 2000 rev/min. Coarse particles were removed by filtering through butter muslin. A portion of the homogenate was taken for the determination of dry weight by heating in an oven at 100° for 4 h.

We chose homogenization rather than the autolysis used by Patwardhan (1937) and Spitzer & Phillips (1945); it was not only quicker but also yielded more active extracts. Thus, in one of our experiments, autolysis gave a phytase activity of 63 units (defined below), and homogenization an activity of 182 units.

Enzyme activity was determined in two samples of homogenate, one diluted in water 1 in 4, the other 1 in 5. Determinations were made in duplicate in boiling-tubes. Each tube contained 5 ml veronal buffer, pH 8·2, 1 ml sodium-phytate solution (20 mg/ml), 1 ml $MgSO_4$ solution (0·012M), 2 ml water and 1 ml diluted extract. 'Blank' determinations without sodium phytate were also made. The tubes were incubated at

37° for 16 h, and then 5 ml of a 10% (w/v) solution of trichloroacetic acid were added. After 30 min the protein was filtered off and inorganic P determined in 1 ml filtrate by the molybdate method of Allen (1940). The results were expressed in arbitrary phytase units, 1 unit being defined as mg inorganic P liberated by 1 g dry tissue in 16 h at 37°.

One batch of sodium phytate (Ciba Laboratories Limited) was used throughout, and it contained 0·28% inorganic P. A suitable correction was also made in all calculations for the amount of free inorganic P in the intestinal extracts. It proved to be very small, amounting to 10% or less of the liberated P. Since this value did not vary in the early experiments, a constant correction for this source of P was applied in the later experiments.

It was not necessary to apply a correction for inorganic P liberated during incubation from other compounds of P, since there was no measurable increase in the control tube containing no added sodium phytate. No chemical hydrolysis of the phytate resulted from the mixture standing in contact with trichloroacetic acid at the end of incubation, or was caused by the reagents subsequently used in the estimation of inorganic P; these procedures therefore did not contribute to the value of the blank. Liberation of inorganic P was found to be linear up to a level of enzyme activity three or four times that studied.

Although the pH optimum for intestinal phytase is said to be 7·8 (Patwardhan, 1937; Spitzer & Phillips, 1945; Mellanby, 1950), it has never been determined very precisely. Our own experiments showed that the optimum is pH 8·2; at pH 7·8, the activity is nearly 10% lower. We also found that phytase activity decreased when there was less than 10 mg of substrate in the medium; the use of 20 mg in routine estimations ensured that at least 10 mg remained at the end of incubation. As Patwardhan (1937) had shown, the addition of Mg ions increased the activity; we too found that the optimal concentration was about 0·001 M, and this concentration of added Mg increased the activity by nearly 50%.

Statistical treatment of results

The standard deviations given in Tables 1–8 are descriptive of the spread of values within each group. The significances of differences between groups have been tested by analysis of variance to take account of the use of litter-mates.

Preliminary experiments

The first experiments were designed to give general information on the effect of age and vitamin D on intestinal phytase in young rats. Bone ash was not measured.

Rats weaned at 23 days on to the stock cube diet had, after 1 week, about 250 phytase units in the proximal part of the small intestine. After 4 weeks, the value was the same, and after 9 weeks it had increased to about 300 units. In most of our experiments on the effect of diet on phytase, the diets were given for 28 days from weaning, so that 250 phytase units may be accepted as a basal value for rats of this age fed on the stock diet.

Rats given from weaning the rachitogenic diet of McCollum, Simmonds, Shipley & Park (1921) had about 170 phytase units after 4 weeks. The administration to litter-mates of vitamin D, 20 i.u. weekly, increased the value to about 360 units. This finding is in accord with the observations of Steenbock *et al.* (1953) and Pileggi *et al.* (1955). Their observation that vitamin D can increase phytase activity of animals fed on a non-rachitogenic diet was also confirmed. Rats given the stock diet for 3 weeks from weaning were divided into three groups; one received no supplementary vitamin D, the second 20 i.u. on alternate days for the 3rd week, and the third 20 i.u. on alternate days for the 2nd and 3rd weeks. The first group had an enzyme activity of 270 units, and each experimental group an activity of 380 units. Vitamin D did not increase the weight of the animals, but it did increase the weight of the small intestine by the small though significant amount of about 7%.

Phytase and bone ash during induction and healing of rickets

For these experiments we used diet 2965 of Steenbock & Black (1925), in order more closely to follow the conditions used by Steenbock *et al.* (1953) and Pileggi *et al.* (1955). This diet consists of finely ground yellow maize 76, wheat gluten 20, $CaCO_3$ 3 and NaCl 1 part.

Table 1. *Expt* 1. *Effect of induction of rickets on bone ash and intestinal phytase in four litters, each of three male or three female rats, fed on the Steenbock–Black diet* 2965 *from* 24 *days of age*

(Mean values with standard deviations)

Group	Days on diet	No. of rats	Wt of rats (g)	Wt of small intestine (g)	Bone ash (%)	Phytase (units)*
a	12	4	56 ± 5·3	2·77 ± 0·25	40·9 ± 0·91	340 ± 57
b	19	4	63 ± 5·6	2·78 ± 0·14	39·4 ± 0·88	198 ± 73
c	26	4	66 ± 4·4	2·71 ± 0·16	32·3 ± 3·16	146 ± 29

* See p. 459.

Expt 1. *Induction of rickets.* Twelve animals from four litters were fed on the rachitogenic diet from 24 days, and one rat from each of the four litters was killed after 12, 19 and 26 days. The animals grew little during the 26 days. From the 12th day, there was no change in the weight of the small intestine (Table 1), and both bone-ash content and phytase activity decreased. The chief decrease in the bone ash occurred in the latter part of the experiment, and in the enzyme activity in the early part.

Expt 2. *Healing of rickets.* Twenty-two animals were fed on the rachitogenic diet from the 24th day. After 5 weeks, they were distributed in such a way that the animals killed at various intervals were always pairs of litter-mates, one of which had been given vitamin D. The vitamin (20 i.u.) was given at weekly intervals, from the beginning of the 6th week, for 3 weeks.

Vitamin D made no difference to the growth of the animals, but it produced an increase in the weight of the small intestine; at the end of the 8 weeks of experiment the weights were 3·27 ± 0·09 g in the rats given vitamin D, and 2·57 ± 0·17 g in those

not given vitamin D. The changes in bone-ash content and phytase activity are shown in Fig. 1. In the absence of vitamin D, both values remained low, but in its presence there was an increase in both. The main increase in bone ash was in the latter part of the experiment, and in the enzyme in the early part.

These experiments, and others of a similar nature, showed that the fall or rise in the enzyme activity occurs chiefly in the 1st week after the change in diet, whereas the fall or the rise in bone-ash content occurs mainly in the 2nd or 3rd weeks after the change.

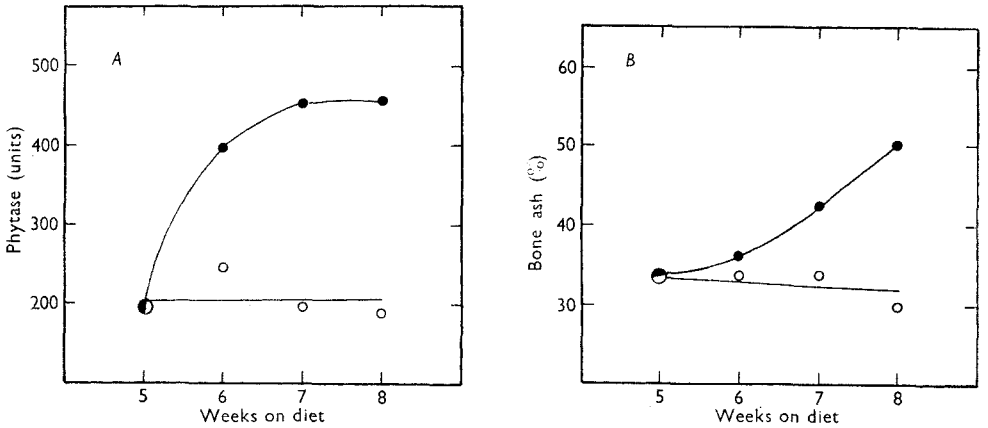

Fig. 1. Effect of healing of rickets on (*A*) intestinal phytase and (*B*) bone ash in the rat. Animals were fed on the Steenbock diet 2965 from 24 days. After a further 5 weeks, each rat in the treated group was given 20 i.u. vitamin D weekly. ●—●, with vitamin D; ○—○ without vitamin D.

Phytase and bone ash in rats on cereal diets

In order to determine whether intestinal phytase is an adaptive enzyme, that is whether additional dietary phytate would cause an increase in enzyme activity, rats were fed on diets based on cereals, to which were added bran or 'pure' sodium phytate. From the experiments already described, however, it was clear that it was necessary to determine the effect, not only of phytate, but also of vitamin D. As before, this meant that relevant information could be obtained by a concurrent study of calcification. These considerations led us to include an investigation of the effect of other factors, such as availability of Ca and of phosphate, which are known to affect calcification.

Expt 3

Four rats from each of eight litters were housed in individual cages from the age of 24 days. They were given a basal cereal diet, with or without added bran, and with or without added vitamin D. The basal diet contained flour (70% extraction) 70, dried yeasts 15, arachis oil 6, salt mixture 4, and low-vitamin casein 5 parts. In the diet with bran, 10 parts of the flour were replaced by 10 parts of bran. Analysis of the ingredients, and of the final diets, showed that the basal diet contained 17 mg phytate P/100 g, and the bran diet contained 145 mg/100 g. Vitamins A and D (120 and 20 i.u. respectively) and 18 μg menaphthone (vitamin K) were given daily to one group of eight animals on the basal diet, and one group on the bran diet. The other groups

received vitamins A and K only. The salt mixture used in all the diets contained 700 mg Ca and 550 mg inorganic P in 100 g diet. Thus, the Ca:P ratio in the basal diet was 1·3:1, and was reduced to about 1:1 by the presence of bran. Whereas, therefore, the bran diet contained about eight times as much phytate as the basal one, both diets had a Ca:P ratio well within the range considered optimal for calcification. Rats in all groups grew well and equally. They were killed at the end of 4 weeks.

Neither bran nor vitamin D had any effect on the weight of the small intestine (Table 2). Vitamin D increased bone-ash content and enzyme activity slightly in the absence of bran and more in its presence. The addition of bran to the diet produced no significant changes in bone-ash content. There was no indication that it made the cereal diet rachitogenic. It did not affect the enzyme activity in the absence of vitamin D, but in its presence it produced an increase of about 25 %.

Table 2. *Expt* 3. *Effect of a supplement of bran or vitamin D or both on bone ash and intestinal phytase in eight litters, each of four female rats, fed on a diet based on* 70 % *extraction flour, for* 4 *weeks from* 24 *days of age*

(Mean values with standard deviations)

Group	Diet	Vitamin D	No. of rats	Wt of rats (g)	Wt of small intestine (g)	Bone ash (%)	Phytase (units)*
a	Flour	−	8	99 ± 14·1	3·77 ± 0·33	50·8 ± 1·58	226 ± 61
b	Flour	+	8	97 ± 10·7	3·46 ± 0·32	52·6 ± 1·07	291 ± 66
c	Flour + bran	−	8	100 ± 9·0	3·74 ± 0·42	49·0 ± 1·61	207 ± 46
d	Flour + bran	+	8	99 ± 9·6	3·65 ± 0·53	53·7 ± 2·04	366 ± 83

* See p. 459.

It seemed, therefore, that dietary phytate could increase the content of intestinal phytase, but only in the presence of vitamin D. It will be recalled that high levels of phytase activity were also found with the Steenbock rachitogenic diet and vitamin D. This diet is said to contain 0·170 % phytate P (Pileggi *et al.* 1955), which is very close to the 0·145 % found in our bran diet.

Expt 4

This experiment was designed to study the effect of omitting the calcium carbonate from the Steenbock rachitogenic diet, or of adding to it purified sodium phytate, in both the presence and absence of vitamin D. Six litters each of six to eight rats were used. Each of three diets was given either with or without vitamin D. These diets were: (1) the Steenbock rachitogenic diet 2965 which contains 76 % yellow maize and 3 % $CaCO_3$ (high-Ca cereal diet); (2) as (1) but with 79 % maize and no $CaCO_3$ (low-Ca cereal diet); (3) as (1) but with 75 % maize, 3 % $CaCo_3$ and 1 % sodium phytate (phytate diet). Vitamin supplements were given as in Expt 3.

Growth. Table 3 shows that there was no significant difference in weight gain between the groups receiving vitamin D. Without vitamin D growth was very poor on the low-Ca diet, but much better on the other two diets. The highest weight gain in the absence of vitamin D was on the phytate diet, i.e. the addition of phytate to a

rachitogenic diet produced a small but significant improvement in growth. The weights of the small intestine were related to the body-weights of the rats.

Bone ash. In the absence of vitamin D, the bone-ash content was lowest on the rachitogenic and low-Ca diets; the addition of phytate improved calcification appreciably. In the presence of vitamin D, bone ash was lowest on the low-Ca diet and was appreciably higher on the rachitogenic diet; again, the addition of phytate produced a considerable improvement in calcification. The addition of vitamin D increased bone ash with the rachitogenic and phytate diets, but was without significant effect with the low-Ca diet (Table 3).

Table 3. *Expt* 4. *Effect of cereal diets high or low in calcium, or high in calcium with added sodium phytate, on bone ash and intestinal phytase of eight litters, each of six to eight male rats, fed on the diets for* 4 *weeks from* 24 *days of age*

(Mean values with standard deviations)

Group	Diet	Vitamin D	No. of rats	Wt of rats (g)	Wt of small intestine (g)	Bone ash (%)	Phytase (units)*
a	Steenbock high-Ca	−	7	79 ± 4·6	2·35 ± 0·38	34·4 ± 2·45	183 ± 49
b	Steenbock high-Ca	+	8	84 ± 5·6	2·58 ± 0·16	44·8 ± 1·84	470 ± 129
c	Low-Ca	−	6	62 ± 6·0	2·38 ± 0·22	32·5 ± 1·55	166 ± 40
d	Low-Ca	+	7	90 ± 9·7	3·29 ± 0·24	34·0 ± 1·76	348 ± 84
e	High-Ca + phytate	−	8	84 ± 4·4	2·68 ± 0·36	43·5 ± 2·14	197 ± 59
f	High-Ca + phytate	+	6	90 ± 5·3	2·95 ± 0·38	50·5 ± 1·52	321 ± 93

* See p. 459.

Phytase activity. In the absence of vitamin D the amount of enzyme activity was low and similar on all three diets. In the presence of vitamin D it was moderate and similar on the low-Ca and phytate diets, and appreciably higher on the rachitogenic diet. The addition of vitamin D increased the activity considerably on all diets, especially on the rachitogenic diet (Table 3).

These results show that bone-ash content was not affected by the addition of vitamin D to a low-Ca diet. On the rachitogenic diet, bone ash was increased by sodium phytate both in the presence and absence of vitamin D, so that in these conditions sodium phytate acted as an antirachitogenic agent. It presumably does so by immobilizing some of the excessive Ca in the rachitogenic diet, in which the Ca:P ratio is 4:1. These results agree with the accepted view that bone-ash content depends both on the total availability of Ca and on a suitable Ca:P ratio.

On the other hand, the intestinal phytase appeared to be affected by the availability of dietary Ca and not by the Ca:P ratio. In the presence of vitamin D, the enzyme activity fell with the decrease in dietary Ca or with the addition of sodium phytate, which presumably immobilized some of the Ca in the high-Ca diet. In the absence of vitamin D, neither of these effects was observed, presumably because the availability of Ca was limited by the absence of the vitamin.

The experiments provide evidence that increase in bone ash need not necessarily be associated with increased enzyme activity. When sodium phytate was added to the

rachitogenic diet with vitamin D, there was an increase in bone-ash content but a fall in phytase activity.

Hence the results obtained with diets containing cereals are not likely to be easy to interpret. The phytate of such diets is associated with variable and unknown amounts of the cations hydrogen, K, Mg and Ca. It will therefore be able to immobilize variable amounts of dietary Ca from other sources. Hence subsequent experiments were carried out with purified diets, to which known amounts of sodium phytate and other substances could be added.

Effect of phytate in purified diets on phytase and bone ash

Preliminary experiments showed that sodium phytate was toxic. With 10% of it in the diet, seven out of eight rats died within a week. At levels of 5%, the rats had diarrhoea, lost weight and showed various other signs, but only one died after 2 weeks. The probability that this toxicity is a manifestation of Mg deficiency is described elsewhere (Roberts & Yudkin, 1960).

Expt 5. *Effect of* 5% *sodium phytate.* Four to six rats from each of four litters were arranged into four groups. They were given a purified diet with or without sodium phytate, and with or without vitamin D. The basal diet consisted of sucrose 60, low-vitamin casein 20, arachis oil 15 and salt mixture 5%. As described above, the salt mixture provided 700 mg Ca and 550 mg inorganic P in 100 g diet. The phytate diet contained 5% sodium phytate, which replaced the same amount of sucrose. All rats received supplements of the B vitamins, and of vitamins A and K (see p. 458). In addition, animals in two of the groups received 20 i.u. vitamin D weekly. Measurements of bone ash and intestinal phytase were made after 4 weeks.

Phytate decreased the weight gain of the animals by a third or more, and vitamin D increased the weight gain (Table 4).

Table 4. *Expt* 5. *Effect of sodium phytate,* 5% *in a purified diet, on bone ash and intestinal phytase of four litters of four to six male and female rats fed on the diet for* 4 *weeks from* 24 *days of age*

(Mean values with standard deviations)

Group	Diet	Vitamin D	No. of rats	Wt of rats (g)	Wt of small intestine (g)	Bone ash (%)	Phytase (units)*
a	Basal	−	6	115 ± 19·6	3·18 ± 0·64	49·6 ± 2·76	217 ± 56
b	Basal	+	6	126 ± 12·6	3·44 ± 0·46	53·2 ± 2·39	341 ± 89
c	With phytate	−	5	68 ± 5·8	2·13 ± 0·18	42·7 ± 1·43	80 ± 39
d	With phytate	+	4	81 ± 10·3	2·84 ± 0·52	45·6 ± 1·82	136 ± 52

* See p. 459.

The bone-ash content was increased by vitamin D, and decreased by phytate. The activity of intestinal phytase was considerably increased by vitamin D and considerably decreased by phytate.

These results provide another, though less striking, instance of the lack of relationship between calcification and phytase activity. The addition of 5% phytate had only

a moderate rachitogenic effect, yet it produced a profound fall in the enzyme activity. Indeed, the values for enzyme activity were the lowest observed throughout this work, although several much lower values of bone ash were encountered.

A similar experiment was made with 1 % sodium phytate instead of 5 %; the effects were similar but less pronounced (Table 5).

It is possible that the decrease in intestinal phytase activity produced by dietary phytate is caused by a reduction in available Ca, as was suggested in relation to Expt 4. The effect of adding citrate both to the rachitogenic and to the purified diets was therefore examined.

Table 5. *Expt 5a. Effect of sodium phytate, 1 % in a purified diet, on bone ash and intestinal phytase of four litters of seven or eight male rats fed on the diet for 4 weeks from 24 days of age*

(Mean values with standard deviations)

Group	Diet	Vitamin D	No. of rats	Wt of rats (g)	Wt of small intestine (g)	Bone ash (%)	Phytase (units)*
a	Basal	−	7	139 ± 19·6	3·47 ± 0·48	52·4 ± 1·26	282 ± 57
b	Basal	+	7	156 ± 10·3	3·67 ± 0·27	54·2 ± 0·95	432 ± 151
c	With phytate	−	8	138 ± 16·9	3·26 ± 0·47	49·8 ± 1·63	185 ± 60
d	With phytate	+	7	141 ± 18·4	3·41 ± 0·18	53·4 ± 0·61	325 ± 114

* See p. 459.

Table 6. *Expt 6. Effect of 10 % of a citrate mixture* in the Steenbock diet 2965 on bone ash and intestinal phytase of four litters of four male rats fed on the diet for 4 weeks from 24 days of age*

(Mean values with standard deviations)

Group	Diet	Vitamin D	No. of rats	Wt of rats (g)	Wt of small intestine (g)	Bone ash (%)	Phytase (units)†
a	Steenbock	−	4	79 ± 6·2	2·33 ± 0·09	35·0 ± 3·46	274 ± 64
b	Steenbock	+	4	80 ± 11·0	2·42 ± 0·07	43·9 ± 3·25	491 ± 162
c	Steenbock + citrate	−	4	71 ± 8·5	2·04 ± 0·05	44·6 ± 6·07	267 ± 33
d	Steenbock + citrate	+	4	64 ± 9·9	1·82 ± 0·24	47·4 ± 1·51	364 ± 104

* See below. † See p. 459.

Effect of citrate on phytase and bone ash

The citrate was added as a mixture of 60 % sodium citrate and 40 % citric acid, which had been shown to be more effective than either the salt or the acid alone (Shohl, 1937; Hathaway & Meyer, 1939).

Expt 6. Effect of 10 % citrate in the Steenbock rachitogenic diet. Four groups of four litter-mate rats were used. Two groups were given the Steenbock rachitogenic diet, and two were given a similar diet in which 10 % of the maize was replaced by the citrate mixture. One group on each diet received vitamin D in addition. The results after 4 weeks are shown in Table 6.

There were unusually large variations between the animals in this experiment. Our conclusions from it are therefore somewhat tentative.

Citrate reduced the weight gain, but only when no vitamin D was administered. Citrate increased the bone-ash content, but the reduction in enzyme activity was not significant.

Expt 7. Effect of 10% citrate in purified diets. Four groups of six litter-mate male rats were used. Two were given the purified diet with the vitamin supplements described for Expt 5. Two were given similar diets in which the citrate mixture replaced 10% of the sugar. One group on each diet was given vitamin D as in Expt 5.

Citrate tended to reduce weight gain and vitamin D to increase it, but neither effect was significant (Table 7).

Table 7. *Expt 7. Effect of 10% of a citrate mixture* in a purified diet on bone ash and intestinal phytase of four litters of six male rats fed on the diet for 4 weeks from 24 days of age*

(Mean values with standard deviations)

Group	Diet	Vitamin D	No. of rats	Wt of rats (g)	Wt of small intestine (g)	Bone ash (%)	Phytase (units)†
a	Basal	−	6	147 ± 16·9	3·38 ± 0·31	51·1 ± 1·59	299 ± 96
b	Basal	+	6	151 ± 12·6	4·00 ± 0·33	53·9 ± 1·13	494 ± 86
c	Basal + citrate	−	6	133 ± 18·6	3·15 ± 0·57	49·9 ± 1·75	249 ± 63
d	Basal + citrate	+	6	140 ± 13·7	3·59 ± 0·31	52·2 ± 0·92	395 ± 82

* See p. 465. † See p. 459.

Table 8. *Expt 7a. Effect of 20% of a citrate mixture* in a purified diet on bone ash and intestinal phytase of four litters of four male rats fed on the diet for 4 weeks from 24 days of age*

(Mean values with standard deviations)

Group	Diet	Vitamin D	No. of rats	Wt of rats (g)	Wt of small intestine (g)	Bone ash (%)	Phytase (units)†
a	Basal	−	4	137 ± 12·5	3·24 ± 0·51	50·9 ± 1·94	276 ± 68
b	Basal	+	4	149 ± 9·9	3·47 ± 0·84	53·4 ± 2·50	369 ± 55
c	Basal + citrate	−	4	100 ± 4·6	2·79 ± 0·24	45·4 ± 2·34	165 ± 29
d	Basal + citrate	+	4	125 ± 6·6	3·40 ± 0·24	48·5 ± 1·33	212 ± 55

* See p. 465. † See p. 459.

Vitamin D increased bone-ash content and considerably increased the enzyme activity. Citrate reduced both bone-ash content and enzyme activity.

A similar experiment with 20% citrate in a purified diet gave similar results but with greater differences (Table 8).

These experiments with citrate showed that its effects on bone-ash content and enzyme activity are similar to those produced by phytate. For example, at lower levels, both citrate and phytate reduce enzyme activity significantly only in the presence of vitamin D but at higher levels they do so in the absence of vitamin D also. Again,

at the higher levels, both citrate and phytate effect considerable reduction in enzyme activity but relatively small reduction in bone-ash content. Further, both have an antirachitogenic action in a cereal diet and a rachitogenic action in a purified diet. More citrate is needed than phytate to produce these effects; for example, 10% citrate produced about the same effect on bone-ash content and enzyme activity as 1% phytate.

DISCUSSION

Effect of vitamin D on intestinal phytase and bone ash

The addition of vitamin D to the diets always increased intestinal phytase activity and bone-ash content, even with the stock diet which was not apparently deficient in vitamin D. The magnitude of the effect varied with the diet. It was greatest with the cereal rachitogenic diet, and least with the same diet to which phytate or citrate had been added.

On diets composed mainly of wheat, vitamin D increased the contents of enzyme and bone ash only a little, but when bran was added the increases were greater. On purified diets, the vitamin produced small increases. The addition of phytate or citrate did not affect the increase in bone ash but reduced the increase of the enzyme.

These results fit reasonably well the hypothesis that the level of intestinal phytase is determined by the amount of Ca that can be absorbed (see p. 463).

Relationship between phytase and calcification

With all but one of the diets, changes in calcification were paralleled by changes in enzyme activity. The exception was the cereal rachitogenic diet. The addition of phytate or citrate to this diet produced a considerable increase in bone-ash content, but no increase in enzyme activity. In order to understand this apparent anomaly, and the quantitative relationships between phytase and calcification, the values for enzyme activity and bone ash found in all the experiments were plotted (Fig. 2) by joining together the two points obtained on a given diet in a single experiment, in the presence or absence of vitamin D.

It will be seen that the results for the rachitogenic diet alone or with various additions, or for the corresponding diet low in Ca, give points which lie lower in the graph than those for the remaining diets. Moreover, the results for these remaining diets give points which all appear to lie on one curve, for convenience referred to as the 'standard curve' (A). These diets were either the purified diets or the quite different wheat diets; one common feature, however, was that they all contained salt mixtures with adequate inorganic phosphate.

These findings suggest that, in the presence of adequate inorganic phosphate, there is a correlation between phytase activity and bone-ash content, independent of the nature of the diet or of the presence or absence of vitamin D. The asymptotic nature of the curve suggests that bone-ash content increases to a maximum of about 54%, but that enzyme activity may in appropriate conditions be increased beyond the highest levels found in our experiments.

A variety of factors determine the position of the points along the 'standard curve' relating phytase activity and bone-ash content. Vitamin D is one of these factors, increasing both enzyme and bone ash. Irrespective of the presence or absence of vitamin D, sodium phytate in the diet decreased the enzyme activity, which shows clearly that phytase does not adapt specifically to an increased concentration of its substrate. The reduction in enzyme activity by phytate, and also by citrate, is due, we believe, to a reduction in available Ca.

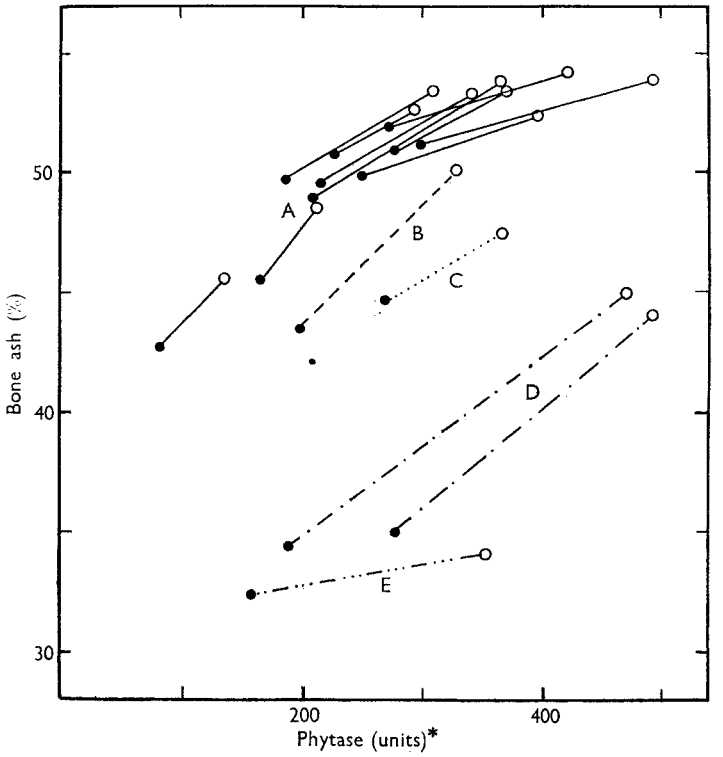

Fig. 2. Relationship between intestinal phytase and bone ash. The figure shows results of all experiments reported in this paper in which bone ash and phytase were measured in animals with and without vitamin D. Lines join results obtained in a given experiment, with a given diet without vitamin D (●) and with vitamin D (○). A, Diets with added phosphate; B, rachitogenic diet+phytate; C, rachitogenic diet+citrate; D, rachitogenic diet; E, low-Ca diet.

* See p. 459.

It remains to be explained why bran in the presence of vitamin D produced an increase of about 25% in phytase activity. This was the only instance where phytate caused an increase in enzyme activity. The bran gave a concentration of phytate P of about 170 mg/100 g diet. The addition of 1% sodium phytate to a purified diet gave a concentration of about 200 mg phytate P, yet led to a decrease of phytase activity of about 25%. The changes in bone-ash content produced by the bran were not significant, but there was a tendency to an increase in the presence of vitamin D and a decrease in its absence. The explanation of these differences might be that some of the phytate in bran contains calcium. During hydrolysis of the phytate in the intestine,

some of the calcium would be released. Since, however, the amount of calcium in bran is small (about 0·1 %), it may be that there is some other reason for the unusual effect of bran in increasing phytase activity.

In the experiments with cereal diets, where the points lie below the 'standard curve', there was a low level of dietary inorganic phosphate. There were two experiments with the cereal rachitogenic diet alone (D, Fig. 2) with the results in good agreement. In the absence of vitamin D, the ash content was very low, and the addition of the vitamin increased both it and the enzyme more than it did with any other diet.

The addition of phytate to the rachitogenic diet produced a considerable increase in bone ash (B, Fig. 2). In the absence of vitamin D, the enzyme activity was not much changed by the addition of phytate; in the presence of vitamin D, it was much lower with added phytate than with the rachitogenic diet alone. The addition of phytate to the rachitogenic diet brought the values both in the presence and absence of vitamin D much closer to the 'standard curve' relating bone-ash content to enzyme activity.

The addition of citrate to the rachitogenic diet produced changes similar to those produced by sodium phytate (C, Fig. 2). There was a considerable increase in bone ash, little change in the enzyme content in the absence of vitamin D and a decrease in the presence of vitamin D.

If it is accepted that the 'standard curve' represents the relationship between phytase activity and bone-ash content when there is adequate phosphate, it must be concluded that both phytate and citrate increase the content of available phosphate in the rachitogenic diet. This they can do by reducing the excessive Ca in this diet. The addition of vitamin D, however, produced a smaller increase in bone ash with citrate than with phytate—7 % against 15 %. As a result, the value for the bone ash approached more closely the 'standard curve' with the phytate diet with vitamin D than with the citrate diet with vitamin D. This finding is explicable on the basis of a higher level of available phosphate which comes from the hydrolysis of part of the phytate. Thus, the antirachitogenic action of phytate is based on the two different ways in which it increases the availability of inorganic phosphate. One is that it reduces the excessive calcium of a rachitogenic diet, and this property it shares with citrate. The other is that it increases the amount of inorganic phosphorus by that which is formed by its hydrolysis.

The lowest values for bone ash occurred with the cereal diet low in Ca (E, Fig. 2). The addition of vitamin D produced a considerable increase in phytase activity but no significant change in bone-ash content, the only instance where there was an increase in the enzyme without an improvement in calcification. The most probable explanation is that this diet was deficient both in Ca and in inorganic phosphate. Mellanby (1949) also found that vitamin D produced no improvement in calcification on a diet high in phytate and low in Ca.

It thus appears that, in the presence of an adequate supply of inorganic phosphate, there is a relationship between the activity of intestinal phytase and the degree of calcification. This relationship holds for a variety of diets, both in the presence and absence of added vitamin D. The exact contents of the enzyme and bone ash appear

to be determined by the amount of available Ca. When the supply of inorganic P is inadequate, there is a large increase in bone ash for given values of phytase. In such rachitogenic diets the relationship between the enzyme and calcification is restored toward normal by citrate or soluble phytate. The citrate removes excessive Ca, so increasing availability of inorganic P. Soluble phytate does it too, but at the same time also provides additional inorganic P from its own hydrolysis. Because of the increased availability of inorganic P, there is a rise in bone ash. Because of the reduction in Ca, there is a fall in phytase content.

Thus, phytate in ordinary diets can have complex effects upon calcification. These will depend first on the anions with which the phytate is associated, and secondly on the absolute and relative amounts of inorganic P in the diet. If there is a limiting amount of Ca, dietary phytate with a relatively high amount of the soluble phytate will remove Ca and so may reduce calcification. On the other hand, dietary phytate containing a significant proportion of Ca may supply Ca and so may improve calcification. If there is a limiting amount of inorganic P, perhaps with an excessive amount of Ca, dietary phytate may again improve calcification. It would do it by supplying inorganic P from its own hydrolysis, and also perhaps by removing excessive Ca.

SUMMARY

1. The activity of phytase in the proximal part of the small intestine has been measured in rats kept on a variety of diets. In most experiments, the proportion of ash in the bones of the leg was also measured.

2. The diets were based on wheat, on maize or on purified ingredients, alone or with the addition of vitamin D, calcium carbonate, bran, sodium phytate, or a sodium citrate–citric acid mixture.

3. During the induction of rickets there was a fall, and during its healing a rise, both in phytase and in bone-ash content.

4. The administration of vitamin D, even with non-rachitogenic diets, always increased the amount of phytase.

5. The administration of bran or of sodium phytate usually decreased rather than increased the amount of phytase. The enzyme is thus not 'adaptive', i.e. it is not inducible by its substrate.

6. When the diets contained adequate inorganic phosphorus, there was a relationship between phytase and bone-ash content, determined by the amount of available calcium. Thus, it was affected by vitamin D, phytate and citrate.

7. When the diets contained inadequate inorganic P, bone-ash content was lower for a given amount of enzyme. With the high-Ca rachitogenic diets, the availability of the inorganic P was increased by phytate and by citrate. Both combined with excessive Ca; in addition, phytate gave an increased amount of inorganic P from its own hydrolysis.

8. Phytate in ordinary diets, consisting of compounds with varying proportions of the cations hydrogen, potassium, magnesium and calcium, can thus have varying effects on calcification. It can increase or decrease available Ca, or increase available

inorganic P. The effects on calcification will therefore depend on the other components of the diet.

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