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Lowering dietary phosphorus concentrations reduces kidney calcification, but does not adversely affect growth, mineral metabolism, and bone development in growing rabbits

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New Zealand White rabbits were used to investigate the influence of increasing dietary P concentrations on growth performance, mineral balance, kidney calcification and bone development. The minimum dietary P requirement of 0·22 % (National Research Council) is usually exceeded in commercial natural-ingredient chows, leading to undesirable kidney calcifications. In order to study the optimal dietary P level, rabbits were fed semi-purified diets with four different P levels (0·1, 0·2, 0·4, and 0·8 %; w/w) at a constant dietary Ca concentration (0·5 %) during an 8-week period. Body weight and growth were not influenced by the dietary P level. During two periods (days 20–23 and 48–51), faeces and urine were collected quantitatively for the analysis of Ca, Mg and P and balances were calculated. Increased dietary P intake caused increased urinary and faecal P excretion and P apparent absorption and retention. Faecal Ca excretion increased with higher dietary P levels, whereas urinary Ca excretion reacted inversely. The apparent absorption of Ca became reduced at higher dietary P concentrations, but Ca retention was unchanged. The response of Mg was in a similar direction to that of the Ca balance. Kidney mineral content increased with higher dietary P levels, indicating the presence of calcified deposits. Nephrocalcinosis became more severe in kidney cortex and medulla at increasing dietary P levels, as was confirmed by histological analysis. Femur bone length was not differentially influenced by dietary P. Bone density (g/cm^3) of the femur diaphysis became significantly lower at the 0·8 % dietary P level as compared with the 0·2 % P group only. The bone Mg content was significantly increased on the 0·8 % P diet, both in the diaphysis and epiphysis. Plasma P concentration increased and plasma Ca decreased with higher dietary P levels, whereas plasma Mg levels were unaffected. The present study shows that the current recommended minimum dietary P level of 0·2 % for rabbits, as advised by the National Research Council in 1977, leads to a normal growth and bone development, but also causes some degree of kidney calcifications at a dietary Ca level of 0·5 %. As the dietary P level of 0·1 % virtually prevented kidney calcification and at the same time did not give evidence for any deleterious effects on growth and bone development, this indicates that the current recommended dietary P level for rabbits should be regarded as a maximum advisable concentration, and that a lower P level may be more optimal.

Dietary phosphorus requirement: Mineral balance: Kidney calcification: Bone mineralisation: Rabbits

The minimum dietary P requirement for obtaining the maximum growth rate in rabbits was set at 0·22 % (w/w) by the National Research Council (1977). This recommendation was obtained from only one experimental study with New Zealand White rabbits, which were fed diets composed of natural ingredients mainly, that were provided *ad libitum* during growth (Mathieu & Smith, 1961). The dietary P levels in that study ranged from 0·07 to 0·32 % (w/w). The exact dietary Ca level was not given and varied with the change in P, as dicalcium phosphate was added for obtaining higher dietary P levels (Mathieu & Smith, 1961). The main parameters used for the evaluation of the dietary P requirement in rabbits were growth and bone development. In order to study the dietary P requirement in New Zealand White rabbits during growth under more well-defined conditions, the present experiment was performed by feeding semi-purified diets with precisely defined increasing dietary P levels at a constant Ca concentration. In addition to bone development, kidney calcification was analysed as an important parameter, for identifying the optimal dietary P requirement. Although the minimum requirement for

Abbreviation: PTH, parathyroid hormone.

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growth is only 0·22 % P (National Research Council, 1977), diets usually contain 0·5–0·8 % P to ensure a satisfactory intake in lactating does as well (Lang, 1981). M Ritskes-Hoitinga, O Skott, TR Uhrenholt, I Nissen, I Lemmens and AC Beynen (unpublished results) analysed two commercially available rabbit chow diets and found dietary P concentrations to be as high as 0·6 %. Dietary P concentrations were analysed, as up to 40 % of the rabbits had a relatively severe degree of nephrocalcinosis, making the kidneys unsuitable for physiological *in vitro* measurements (M Ritskes-Hoitinga, O Skott, TR Uhrenholt, I Nissen, I Lemmens and AC Beynen, unpublished results). From dietary studies in the rat it is well known that dietary P concentration is a major inducing factor for nephrocalcinosis (Ritskes-Hoitinga *et al.* 1989, 1992, 1993), which has been the reason for reducing the minimum recommended dietary P level for rats from 0·4 to 0·3 % (National Research Council, 1995).

In the rabbit little is known about the influence of high dietary P levels on metabolism. The availability of P may depend on the form it is presented in. P from organically bound components in the diets is thought to be poorly available in rabbits as is stated by Mathieu & Smith (1961). However, the National Research Council (1977) states that it can be expected that all dietary P in the form of phytate is available to the herbivorous rabbit due to intestinal bacterial phytase activity. Besides the absolute dietary P level, the absolute dietary Ca concentration, as well as the ratio between them, are important factors in order to evaluate the precise requirements reliably. Heinemann *et al.* (1957) reported retarded growth, impaired breeding efficiency, and abnormal bone development in rabbits at dietary Ca:P ratios exceeding 10 (at dietary P levels ranging from 0·12 to 0·26 %). When dietary P levels are relatively high, Ca content should be at least at the same concentration in order to establish normal growth in rabbits (Chapin & Smith, 1967a; Lang, 1981). High (1 %) levels of P can be unpalatable, causing feed rejection (Chapin & Smith, 1967b; National Research Council, 1977). High dietary P levels of 1·3–1·5 % are stated to be tolerated only when the Ca:P ratio is 1 or higher (Chapin & Smith 1967b; Lang, 1981). At dietary P levels of 1 %, a Ca:P ratio of 0·5 and lower will lead to insufficient growth and bone calcification (Chapin & Smith, 1967b).

In the present study the influence of increasing dietary P levels (0·1, 0·2, 0·4, 0·8 %) on growth performance, mineral metabolism, kidney calcification and bone development in male New Zealand White rabbits was examined. Semi-purified diets with a constant dietary Ca level (0·5 %) were fed daily in a standardised amount per animal, during an 8-week period. It was hypothesised that at a constant dietary Ca concentration of 0·5 %, a higher dietary P level (over 0·22 %) would cause (more) kidney calcification and that a Ca:P ratio of at least 1 would be sufficient for obtaining optimal bone calcification.

Materials and methods

Animals and diets

Male New Zealand White rabbits (Broekman Instituut, Someren, The Netherlands) were used, of 5 weeks of age on arrival at the laboratory at the Small Animal Centre, Wageningen, The Netherlands. Only male rabbits were used, as there was no indication of a sex difference in the P requirement (Mathieu & Smith, 1961), or in the frequency and degree of kidney calcifications registered (M Ritskes-Hoitinga, O Skott, TR Uhrenholt, I Nissen, I Lemmens and AC Beynen, unpublished results). The rabbits were housed individually in cages with wire-mesh bases of galvanised steel in a room with controlled temperature (18–19°C), lighting (lights on from 06.00 to 18.00 hours) and relative humidity (60 ± 10 %). For the first 3 weeks the rabbits received commercial pellets (LK-04; Hope Farms, Woerden, The Netherlands) and tap water *ad libitum*. Then (day 0) the rabbits were divided into four groups of eight rabbits each with similar mean body weight in each group. The rabbits had free access to demineralised water. During a 3 d period, the rabbits gradually switched to semi-purified experimental diets, which differed in the amount of P (Table 1). On day 1 the rabbits received a diet consisting of 50 % LK-04 and 50 % of one of the experimental diets; on day 2 this was

Table 1. Composition of the experimental diets

	Dietary P concentration			
	0·1 % P	0·2 % P	0·4 % P	0·8 % P
Ingredients (g)				
Maize starch:dextrose, 1:1	275·9/275·9	274·2/274·2	270·9/270·9	264·2/264·2
$NaH_2PO_4.2H_2O$	–	5·0	15·1	35·2
Na_2CO_3	11·9	10·3	6·8	–
Constant components*	436·4	436·4	436·4	436·4
Total	1000	1000	1000	1000
Chemical analysis (g/100 g)				
Ca	0·43	0·46	0·45	0·46
Mg	0·074	0·078	0·076	0·076
P	0·15	0·24	0·45	0·88
Ca/P	2·9	1·9	1·0	0·5

* The constant components consisted of (g): casein, 160; molasses, 100; maize oil, 10; coconut fat, 10; cellulose, 150; $CaCO_3$, 10·0; $MgCO_3$, 1·4; NaCl, 5·0; $KHCO_3$, 18·0; mineral premix, 10·0; vitamin premix, 12·0. The mineral and vitamin premixes have been described by Van der Meer *et al.* (1985).

25 and 75 % respectively. On day 3 the rabbits received only one of the experimental diets. The rabbits in each dietary group were fed restrictedly (this was 'near *ad libitum*', as the 77 g/rabbit that was provided daily was never eaten completely) with their respective diets (0·1, 0·2, 0·4 and 0·8 % P) in order to maintain similar performances between the four dietary groups. The rabbits could practice coprophagy.

Collection of samples and chemical analyses

From days 20–23 and 48–51 faeces and urine were collected quantitatively for each rabbit. Part of the urine was acidified to pH 1 with 6 M-HCl and was used for the analysis of Ca, Mg, and P. The faeces were freeze-dried, homogenised and weighed. The samples were ashed at 500°C for 17 h, dissolved in 6 M-HCl and brought to 50 ml with demineralised water. Feed samples were processed as described for the faecal samples. Ca and Mg were analysed in acidified urine, faeces and feed samples by using atomic absorption spectroscopy in the presence of 1 % lanthanum chloride with the use of a Varian AA-475 (Varian Techtron, Springvale, Australia). Total P was analysed with a test combination MA-kit 10 Roche Phosphate and a Cobas-Bio autoanalyser from Roche Diagnostica (Basel, Switzerland). On days 0, 28 and 56, blood was collected from the marginal ear vein into heparinised tubes. After centrifugation, plasma was collected and frozen at −20°C until analysis. Ca and Mg in plasma were analysed in the presence of 0·021 M-EDTA-Na_2 by atomic absorption spectroscopy. Total P was analysed as described for feed samples.

On day 57 the rabbits were killed by a blow to the head followed by exsanguination. The kidneys and femurs of the left hindleg were removed. After removing the capsules of the kidneys, the right kidney was weighed, minced and homogenised in 1 vol. of demineralised water. After overnight drying at 106°C, the samples were ashed, dissolved and analysed for Ca, Mg, and P as described for faeces. The left kidney was fixed in 10 % (v/v) neutral phosphate-buffered formalin for histological analysis, and stained with haematoxylin–eosin and Von Kossa as described by Hoek *et al.* (1988). The severity of nephrocalcinosis was graded on a scale from 0 (absence of nephrocalcinosis) to 3 (severe nephrocalcinosis). Femurs were sterilised in closed glass bottles for 15 min at 120°C and muscle remnants could then easily be removed from the bones. Femur volume was determined by weighing in air and under water. Femur length and circumference were determined. Then two different parts of the femur (5 mm of the medial diaphysis and of the epiphysis (femoral head)) were taken for mineral analysis. These parts were dried, ashed and processed as for the faeces and feed samples for the determination of mineral content.

Statistics

Differences between diet-group means were statistically evaluated by a one-way ANOVA followed by the Tukey B test. Results of the different dietary groups, with unlike superscript letters in the tables, are significantly different. The data of the nephrocalcinosis scores were evaluated by the Mann–Whitney U test, because of their ordinal character. The group results with unlike superscript letters in Table 5 are significantly different. The level of statistical significance was pre-set at $P<0·05$. All statistical analyses were performed using the SPSS-PC + software package (SPSS Inc., Chicago, IL, USA).

Results

Final body weights, growth and feed intake were not significantly different between the dietary groups (Table 2).

During the first collection period, the 0·1 % P diet caused a significant reduction in urinary volume as compared with the 0·8 % P diet (Table 3). The mean urinary production on the 0·1 % P diet was also lower than on the 0·2 and 0·4 % P diet; however this did not reach statistical significance. No significant differences in urine volume were detected during the last collection period (Table 4). During both collection periods, the 0·8 % P diet caused a significantly lower urinary pH (Tables 3 and 4). Faecal production was similar in all groups during the first collection period (Table 3). Faecal dry weight was significantly higher on the 0·4 % P diet as compared with the 0·1 and 0·2 % P diets during the second collection period (Table 4).

In the first collection period, the diet containing a P level of 0·4 % caused significantly more calciuria as compared

Table 2. Performance of the rabbits fed four experimental diets*
(Means and standard deviations for eight rabbits per dietary group)

	Dietary P concentration							
	0·1 % P		0·2 % P		0·4 % P		0·8% P	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Body weight (kg)								
Initial	1·77	0·11	1·77	0·10	1·77	0·10	1·77	0·10
Final	2·46	0·28	2·42	0·41	2·66	0·27	2·41	0·29
Growth								
Absolute (kg)	0·68	0·27	0·65	0·37	0·89	0·24	0·64	0·28
Relative (g/d)	12·2	4·8	11·6	6·6	15·8	4·2	11·4	5·0
Feed intake (g/d)	66·8	6·9	66·9	9·4	73·1	7·6	68·9	9·5

* For details of diets and procedures, see Table 1 and pp. 368–369.

Table 3. Mineral balance during days 20–23 of rabbits fed four experimental diets*
(Means and standard deviations for eight rabbits per dietary group)

	Dietary P concentration							
	0·1 % P		0·2 % P		0·4 % P		0·8 % P	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Urine production (ml/3 d)	518[a]	124	703[a,b]	153	711[a,b]	226	779[b]	179
Urine pH	9·49[b]	0·14	9·42[b]	0·16	9·36[b]	0·15	8·20[a]	0·35
Faeces dry weight (g/3 d)	30·3	7·6	28·5	9·0	33·0	12·6	32·4	9·4
Ca								
Intake (mg/d)	286	42	317	32	321	45	326	46
Urine (mg/d)	88·7[c]	30·0	96·8[c]	16·7	49·6[b]	23·5	1·48[a]	0·43
Faeces (mg/d)	15·6[a]	3·6	24·7[a]	11·0	129[b]	58	215[c]	68
Retention (mg/d)	182	42	196	33	142	77	110	89
Apparent absorption (mg/d)	271[b,c]	40	292[c]	40	192[b]	80	111[a]	89
Apparent absorption (% of intake)	94·5[c]	1·2	91·9[c]	4·1	58·2[b]	21·4	32·8[a]	24·4
Mg								
Intake (mg/d)	49·4	7·2	53·3	5·3	57·1	1·9	54·2	7·6
Urine (mg/d)	32·5[c]	3·3	33·8[c]	5·1	23·3[b]	5·4	15·3[a]	4·2
Faeces (mg/d)	3·17[a]	2·20	3·96[a]	1·60	16·8[b]	7·7	27·8[c]	9·6
Retention (mg/d)	13·7	4·8	15·5	4·3	16·9	6·0	11·0	11·2
Apparent absorption (mg/d)	46·2[b]	6·4	49·3[b]	5·9	40·2[b]	7·8	26·3[a]	14·2
Apparent absorption (% of intake)	93·8[c]	3·9	92·4[c]	3·2	70·5[b]	13·4	46·4[a]	23·5
P								
Intake (mg/d)	99·4[a]	14·6	163[b]	16	336[c]	11	630[d]	88
Urine (mg/d)	2·65[a]	2·77	22·4[b]	5·8	73·4[c]	12·6	238[d]	23
Faeces (mg/d)	17·6[a]	9·5	24·2[a]	9·8	126[b]	51	227[c]	55
Retention (mg/d)	79·1[a]	10·3	116[a,b]	5	136[a,b]	53	165[b]	110
Apparent absorption (mg/d)	81·8[a]	10·3	138[a,b]	22	209[b]	49	403[c]	110
Apparent absorption (% of intake)	82·9[b]	7·6	84·8[b]	6·5	62·4[a]	14·9	63·0[a]	11·4

[a,b,c,d]Mean values within a row with unlike superscript letters were significantly different ($P < 0·05$) (Tukey B test).
* For details of diets and procedures, see Table 1 and pp. 368–369.

Table 4. Mineral balance during days 48–51 of rabbits fed four experimental diets*
(Means and standard deviations for eight rabbits per dietary group)

	Dietary P concentration							
	0·1 % P		0·2 % P		0·4 % P		0·8 % P	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Urine production (ml/3 d)	681	122	730	234	894	232	793	250
Urine pH	9·53[b]	0·14	9·23[b]	0·34	9·35[b]	0·23	8·04[a]	0·30
Faeces dry weight (g/3 d)	23·8[a]	11·8	22·8[a]	15.1	39·1[b]	11·9	30·0[a,b]	6·2
Ca								
Intake (mg/d)	275	110	243	141	345	66	291	66
Urine (mg/d)	111[b]	55	136[b]	61	80·6[b]	58·8	3·25[a]	1·84
Faeces (mg/d)	14·2[a]	6·5	29·5[a]	21·8	189[b]	75	196[b]	48
Retention (mg/d)	150	88	77·0	84·7	74·8	73·1	92·0	58·2
Apparent absorption (mg/d)	259[b]	112	213[a,b]	129	155[a,b]	104	95·3[a]	59·2
Apparent absorption (% of intake)	87·3[b]	21·0	84·1[b]	13·0	42·5[a]	27·0	30·9[a]	17·6
Mg								
Intake (mg/d)	47·5	18·9	40·8	23·7	58·2	11·1	48·3	11·0
Urine (mg/d)	34·9[b]	8·7	34·6[b]	10·3	28·0[a,b]	10·5	17·1[a]	6·3
Faeces (mg/d)	3·34[a]	1·26	6·52[a]	4·93	26·6[b]	10·0	25·2[b]	4·1
Retention (mg/d)	9·22	11·10	0·79	16·10	3·72	8·05	5·94	6·53
Apparent absorption (mg/d)	44·2	18·2	31·8	18·8	31·3	12·1	23·1	11·2
Apparent absorption (% of intake)	91·1[b]	5·8	81·6[b]	13·9	53·3[a]	16·3	45·3[a]	15·2
P								
Intake (mg/d)	95·7[a]	38·0	125[a]	72	343[b]	65	562[c]	128
Urine (mg/d)	8·71[a]	8·38	65·7[b]	17·5	112[b]	44	250[c]	64
Faeces (mg/d)	16·3[a]	6·6	31·7[a]	20·4	160[b]	52	198[b]	52
Retention (mg/d)	70·7[a,b]	38·6	27·1[a]	73.0	69·7[a,b]	65·9	114[b]	65
Apparent absorption (mg/d)	79·4[a]	33·9	92·8[a]	60·0	182[b]	76	364[c]	107
Apparent absorption (% of intake)	80·3[b]	8·8	67·0[a,b]	26·9	51·9[a]	16·7	64·0[a,b]	8·1

[a,b,c,d]Mean values within a row with unlike superscript letters were significantly different ($P < 0·05$) (Tukey B test).
* For details of diets and procedures, see Table 1 and pp. 368–369.

with the 0·8 % P diet (Table 3). Urinary Ca excretion on the 0·1 and 0·2 % P diets was significantly higher than for the 0·4 and 0·8 % dietary P groups (Table 3). In the second collection period, the urinary Ca excretion of the 0·8 % dietary P group was significantly lower as compared with the other three dietary groups (Table 4). On the other hand, faecal Ca excretion increased significantly at increasing dietary P levels; in the first collection period, a significantly higher faecal Ca excretion was found in the 0·8 % P group as compared with the other three groups (Table 3). Faecal Ca excretion in the 0·4 % P group was also significantly higher than in the 0·1 and 0·2 % P groups (Table 3). During the second collection period, faecal Ca excretion of the two highest P groups was significantly higher than that in the two lowest P groups (Table 4). As a result, Ca retention was unaffected by the dietary P content (Tables 3 and 4). The absolute and relative apparent Ca absorption showed an inverse relationship with dietary P concentrations. The absolute apparent Ca absorption of the 0·8 % P group was significantly lower than that of the other three groups in the first collection period (Table 3). In the 0·4 % P group, absolute apparent Ca absorption was significantly lower than that measured in the 0·2 % P group (Table 3). The absolute apparent Ca absorption during the second collection period was significantly lower after feeding the 0·8 % P diet, as compared with the 0·1 % P group (Table 4). Upon expressing the apparent Ca absorption as a percentage of intake, a significantly lower value was found in the 0·8 % P group, as compared with the other three groups during the first collection period (Table 3). The relative apparent Ca absorption of the 0·4 % P group was significantly lower than that of the 0·1 and 0·2 % P groups (Table 3). During the second collection period, the relative apparent Ca absorption in the 0·1 and 0·2 % P group was significantly higher than that of the 0·4 and 0·8 % P groups (Table 4).

Urinary Mg excretion in urine followed the trends as seen for Ca. During the first collection period, a significantly lower urinary Mg excretion occurred in the 0·8 % P group, as compared with the other three groups (Table 3). Urinary Mg excretion of the 0·4 % P group was significantly lower than that of the 0·1 and 0·2 % P groups (Table 3). Urinary Mg excretion of the 0·8 % P group was significantly lower than that of the 0·1 and 0·2 % P groups during the second collection period (Table 4). During the first and second collection periods, faecal Mg excretion in the 0·1 and 0·2 % P groups was significantly lower than that of the 0·8 and 0·4 % P groups (Tables 3 and 4). The 0·8 % P diet resulted in a significantly higher faecal Mg excretion as compared with the 0·4 % P diet during the first period only (Table 3). Mg retention was similar in all groups in both collection periods (Tables 3 and 4). The absolute apparent Mg absorption in the 0·8 % P group was significantly lower in the 0·8 % P group as compared with the other three groups in the first collection period (Table 3), whereas there were no significant differences between the groups during the second period (Table 4). The direction of the effects of the various dietary P levels on the relative apparent Mg absorption was comparable with that described for Ca (see earlier, Tables 3 and 4).

In line with the design of the study, an increased dietary P level led to a significantly increased P intake (Tables 3 and 4); only the increase in P intake from the 0·1 to the 0·2 % P group during the second collection period did not reach statistical significance. The increasing dietary P concentrations caused significant increases in urinary P excretion, where all groups were significantly different from each other during the first collection period (Table 3). The same trend occurred during the second period; however, the urinary P excretion in the 0·2 and 0·4 % P group did not differ significantly from each other (Table 4). Faecal P excretion levels increased at higher dietary P concentrations (Tables 3 and 4). During the first collection period, faecal P excretion in the 0·8 % P group was significantly higher than that in the other three groups (Table 3). Faecal P excretion in the 0·4 % P group was significantly higher than that in the 0·1 and 0·2 % P groups (Table 3). During the second collection period, faecal P excretion in the 0·1 and 0·2 % P groups was significantly lower as compared with the 0·4 and 0·8 % P groups (Table 4). Even though (significantly) increased urinary and faecal P excretions occurred, this could not prevent an increased P retention at the higher dietary P levels. A significantly lower P retention occurred in the 0·1 % P group as compared with the 0·8 % P group during the first collection period (Table 3). During the second collection period, only the P retention in the 0·2 % P group was significantly lowered as compared with the 0·8 % P group (Table 4). The increased P retention on higher dietary P levels was not as marked as the increase in P intake. The absolute apparent P absorption increased at higher dietary P levels; a significantly higher value was found in the 0·8 % P group as compared with the other three groups in the two collection periods (Tables 3 and 4). A significantly lower value for the absolute apparent P absorption occurred in the 0·1 % P group than that found in the 0·4 % P group during the first collection period (Table 3). In the second collection period, the 0·4 % P group had a significantly higher absolute apparent P absorption than that of the 0·1 and 0·2 % P groups (Table 4). When expressed as a percentage of intake (relative apparent P absorption), the values of the 0·1 and 0·2 % P groups were significantly higher than of the other two groups in the first period (Table 3). During the second period, the relative apparent P absorption in the 0·1 % P group was significantly higher than that of the 0·4 % P group only (Table 4).

Dietary P concentrations had no significant influence on kidney weights (wet and dry weight and weight expressed as percentage of body weight) (Table 5). Higher dietary P concentrations led to higher mean kidney Ca, Mg and P levels. At the 0·8 % dietary P level, significantly increased values of kidney Ca and P were found in comparison with the other three dietary groups (Table 5). After the ingestion of the 0·8 % P diet, kidney Mg concentrations were significantly increased as compared with the two lowest dietary P groups (Table 5). Increased kidney mineral concentrations indicated that nephrocalcinosis had occurred. Histological scores of kidney calcification confirmed this. Both the haematoxylin–eosin- and Von Kossa-stained kidney sections gave higher scores for calcified deposits at higher

Table 5. Mineral composition of the right kidney and histological score of the left kidney of rabbits fed four experimental diets*
(Means and standard deviations for eight rabbits per dietary group)

	Dietary P concentration							
	0·1 % P		0·2 % P		0·4 % P		0·8 % P	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Wet weight (g)	7·54	1·39	7·34	1·41	8·71	1·63	8·12	1·17
Dry weight (g)	1·54	0·21	1·44	0·19	1·60	0·25	1·46	0·16
Weight (% body weight)	0·31	0·06	0·31	0·05	0·33	0·06	0·34	0·05
Ca (% dry weight)	0·05[a]	0·01	0·07[a]	0·04	0·34[a]	0·65	1·40[b]	1·51
Mg (% dry weight)	0·09[a]	0·01	0·09[a]	0·01	0·10[a,b]	0·01	0·11[b]	0·03
P (% dry weight)	1·43[a]	0·10	1·50[a]	0·10	1·61[a]	0·15	2·08[b]	0·60
Histological score†	Absolute frequency on a 0–3 scale							
	0 1 2 3		0 1 2 3		0 1 2 3		0 1 2 3	
Von Kossa-stained								
Cortex	8 – – –[A]		6 2 – –[A]		5 2 1 –[A]		– 3 4 1[B]	
Medulla	5 3 – –[A]		5 2 1 –[A]		– 2 6 –[B]		– 1 7 –[B]	
Haematoxylin–eosin-stained								
Cortex	7 1 – –[A]		6 2 – –[A]		6 1 1 –[A]		– 3 4 1[B]	
Medulla	4 3 1 –[A]		3 3 2 –[A,B]		– 3 5 –[B]		– 3 5 –[B]	

[a,b]Mean values within a row with unlike superscript letters were significantly different ($P<0·05$) (Tukey B test).
[A,B]Group values within a row with unlike superscript letters were significantly different ($P<0·05$) (Mann–Whitney U test).
* For details of diets and procedures, see Table 1 and pp. 368–369.
† Score 0, no calcium deposits; score 1, a few calcified deposits; score 2, multiple deposits; score 3, a band of calcification throughout the entire kidney section.

dietary P concentrations. In the medulla, a slight degree of calcification was detected in three animals and multiple calcified deposits in one animal at the 0·1 % dietary P level, where cortical calcifications were virtually absent. At rising dietary P levels, cortical calcifications became more prominent; the highest score was found at the 0·8 % P dietary level, which was significantly different from all the other groups. Medullary calcifications became also more severe with increasing dietary P levels. The Von Kossa staining showed a significantly increased calcification in the two highest dietary P groups as opposed to the two lowest P groups. The haematoxylin–eosin staining demonstrated a significantly higher score in the two highest dietary P groups as compared with the 0·1 % P group only (Table 5).

Of the femur, two different functional parts were taken; namely the diaphysis and epiphysis, because of their different function in bone development. The length and the diameter of the complete bone did not differ between the groups (Table 6). The bone density of the diaphysis

Table 6. Composition of total bone and the diaphysis of the femur of rabbits fed four experimental diets*
(Means and standard deviations for eight rabbits per dietary group)

	Dietary P concentration							
	0·1 % P		0·2 % P		0·4 % P		0·8 % P	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Complete bone								
Length (mm)	90·8	1·1	90·6	2·2	91·1	1·8	92·3	1·7
Diameter (mm)	8·15	0·46	8·10	0·54	8·11	0·43	7·74	0·20
Diaphysis								
Density (g/cm^3)	2·07[a,b]	0·03	2·13[b]	0·15	2·07[a,b]	0·06	1·95[a]	0·14
Ash percentage (% dry weight)	76·4	1·2	76·6	1·0	76·9	0·9	76·0	1·1
Ca								
% Dry weight	27·6	1·8	26·8	1·8	25·7	3·1	26·6	2·1
% Ash	36·0	2·1	34·9	2·5	33·5	4·1	35·1	2·5
mg/cm^3	496	33	492	47	466	56	440	64
Mg								
% Dry weight	0·40[a]	0·02	0·40[a]	0·02	0·42[a]	0·01	0·48[b]	0·04
% Ash	0·53[a]	0·02	0·52[a]	0·03	0·55[a]	0·02	0·64[b]	0·05
mg/cm^3	7·23	0·31	7·39	0·74	7·63	0·34	7·99	1·19
P								
% Dry weight	13·4	0·9	13·7	0·9	14·1	1·4	13·7	1·0
% Ash	17·5	1·2	17·9	1·1	18·4	1·9	18·1	1·3
mg/cm^3	241	13	253	26	255	26	227	29

[a,b]Mean values within a row with unlike superscript letters were significantly different ($P<0·05$) (Tukey B test).
* For details of diets and procedures, see Table 1 and pp. 368–369.

Table 7. Composition of the epiphysis of the femur of rabbits fed four experimental diets*
(Means and standard deviations for eight rabbits per dietary group)

	Dietary P concentration							
	0. 1 % P		0·2 % P		0·4 % P		0·8 % P	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Density (g/cm^3)	1·20	0·07	1·19	0·06	1·24	0·05	1·23	0·07
Ash percentage (% dry weight)	49·2	2·3	50·6	1·3	49·7	4·7	49·7	2·2
Ca								
% Dry weight	16·9	0·9	17·8	0·6	16·7	1·5	16·9	0·9
% Ash	33·9	1·8	35·2	0·7	33·7	2·1	34·1	1·2
mg/cm^3	162	26	170	16	164	29	167	36
Mg								
% Dry weight	0·22^a	0·02	0·24^a	0·02	0·23^a	0·04	0·29^b	0·03
% Ash	0·45^a	0·04	0·47^a	0·02	0·47^a	0·03	0·59^b	0·07
mg/cm^3	2·12^a	0·32	2·26^a	0·28	2·30^a	0·42	2·90^b	0·76
P								
% Dry weight	7·49	0·88	8·06	0·29	7·79	0·98	8·31	0·96
% Ash	15·0^a	1·4	15·9^{a,b}	0·14	15·7^{a,b}	0·8	16·8^b	1·7
mg/cm^3	72·0	13·8	76·9	8·4	76·1	13·0	82·7	20·8

^{a,b}Mean values within a row with unlike superscript letters were significantly different ($P<0·05$) (Tukey B test).
* For details of diets and procedures, see Table 1 and pp. 368–369.

on the 0·8 % P diet was significantly reduced as compared with the 0·2 % P diet, which gave the highest mean value (Table 6). The ash percentage, and the Ca and P content of the diaphysis did not show significant differences between the groups (Table 6). The diaphysis' Mg content expressed as percentage of dry weight and percentage of ash content was significantly increased on the 0·8 % P diet as compared with the other three groups, but when expressed as mg/cm^3, significant differences were no longer detected (Table 6). In contrast to the diaphysis, the density of the epiphysis did not show any differences (Table 7). Epiphysial ash percentage remained unchanged in the various groups (Table 7). Ca content of the epiphysis was not influenced by dietary P level (Table 7). Mg content expressed as percentage of dry weight, as percentage of ash content, and as mg/cm^3 was significantly elevated on the 0·8 % P diet as compared with the other three groups (Table 7). P concentration was only significantly increased at the highest dietary P level as compared with the lowest dietary concentration, when expressed as percentage of ash content (Table 7).

Plasma Ca levels were significantly decreased on the 0·4 and 0·8 % P diets as compared with the two lowest P groups after 28 d (Table 8). After 56 d, only the plasma Ca in the 0·8 % P group was significantly lower than concentrations in the other three groups. Plasma Mg concentration was not influenced by the P concentration in the diets. Plasma P concentration was significantly elevated in the 0·8 % P group after 28 and 56 d (Table 8).

Discussion

The minimum P requirement of 0·22 % for growing rabbits, as published by the National Research Council (1977), is often exceeded in the current commercial chow diets on the market, which coincides with the presence of calcifications in the kidneys (M Ritskes-Hoitinga, O Skott, TR Uhrenholt, I Nissen, I Lemmens and AC Beynen,

Table 8. Mineral composition of plasma of rabbits fed four experimental diets
(Means and standard deviations for eight rabbits per dietary group)

	Dietary P concentration							
	0·1 % P		0·2 % P		0·4 % P		0·8 % P	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Ca (mmol/l)								
Day 28	3·05^b	0·38	2·97^b	0·34	2·81^a	0·29	2·55^a	0·23
Day 56	3·14^b	0·31	3·16^b	0·09	3·21^b	0·41	2·71^a	0·20
Mg (mmol/l)								
Day 28	0·65	0·04	0·63	0·09	0·68	0·07	0·65	0·23
Day 56	0·73	0·14	0·64	0·09	0·63	0·18	0·64	0·18
P (mmol/l)								
Day 28	1·81^a	0·10	1·92^a	0·19	2·06^a	0·17	2·51^b	0·36
Day 56	1·86^a	0·16	1·80^a	0·28	1·84^a	0·12	2·29^b	0·57

^{a,b}Mean values within a row with unlike superscript letters were significantly different ($P<0·05$) (Tukey B test).
*For details of diets and procedures, see Table 1 and pp. 368–369.

unpublished results). In the rat and the rabbit, a relationship is found between the presence of kidney calcifications and the level of P intake (Jowsey & Balasubramaniam, 1972; Schoenmakers *et al.* 1989). The present experiment was conducted in order to obtain more insight into the consequences of various dietary P concentrations on growth, mineral metabolism and bone development in New Zealand White rabbits. The aim was to define a dietary P level that would prevent kidney calcifications, but at the same time maintain normal growth and bone development.

In the present study, food intake was standardised for all individuals in all dietary groups. The present study results revealed no differences in body-weight development at dietary P levels of 0·1, 0·2, 0·4 and 0·8 %, at a constant dietary Ca level of 0·5 %, when using a diet mainly composed of purified ingredients. In contrast to the present study, Heinemann *et al.* (1957) found a significantly retarded growth on a diet containing 0·1 % P as compared with 0·2 % P. The difference with the present study is that Heinemann *et al.* (1957) used a diet mainly based on natural ingredients (lucerne), with a high Ca level (1·5 %). Mathieu & Smith (1961) reported a significantly lower body-weight gain when the dietary P level was 0·07 and 0·14 % as opposed to 0·28 % P. In this last study, hay was the sole source of P in the 0·07 % P diet. The Ca level was not reported. When using natural ingredients such as hay as the dietary P source, the bioavailability may be negatively influenced. The present study shows that, in case P arises from casein and sodium phosphate, a dietary P level as low as 0·1 % does not appear to influence body-weight development adversely.

Urinary pH was significantly lowered at the 0·8 % dietary P concentration during both balance periods. The high urinary P excretion can in itself be the cause of a reduced pH. Usually, most P excretion occurs *via* the faeces (Lang, 1981), but in the present experiment an excessive excretion was also seen in the urine. With increasing dietary P concentrations, dietary carbonates become lower simultaneously, which will also contribute to a reduction of urinary pH. The increased urinary production at 0·8 % dietary P during days 20–23 can be considered a coincidental finding, as this difference had disappeared during days 48–51. However, one cannot rule out the possibility that a temporary decline in kidney function occurred around the time nephrocalcinosis became established, which became repaired again due to the large possibilities of compensatory action of the kidney tissue, just as is seen in rats (Ritskes-Hoitinga *et al.* 1989). The reason for the increase in faecal dry weight in the 0·4 % P diet during the second collection period is unclear, and is considered coincidental.

The present results demonstrate that increasing P levels at a constant Ca concentration in the diet led to a dramatic decrease in hypercalciuria and an increase in faecal Ca output. Also in human subjects (Zemel, 1988) and young, female rats (Schoenmakers *et al.* 1989) an increased dietary P level leads to a decreased urinary and increased faecal Ca output. So there appears to be a shift in the route of Ca excretion from urine to the faeces at increasing dietary P levels. A higher dietary P level leads to higher intestinal and faecal P levels, which through the formation of $CaPO_4$ complexes may give rise to this high faecal Ca excretion. As well as P interfering with intestinal Ca absorption, it also exerts a hypocalciuric effect through stimulating renal tubular Ca reabsorption (Zemel, 1988). In rabbits, urine is the major route for Ca excretion, whereas in most animal species the biliary excretion is more important (National Research Council, 1977). In the rabbit (National Research Council, 1977), urinary Ca varies directly with serum Ca, and serum Ca reflects dietary Ca levels directly. This may imply that a decreased urinary Ca excretion is the result of a reduced intestinal absorption due to the formation of insoluble complexes more than from an increased biliary excretion. In other words, the dietary P concentration influences the availability of Ca for the metabolism, which is also illustrated by the reduction in apparent Ca absorption and plasma Ca levels at dietary P levels of 0·4 and 0·8 %. The ultimate result was that Ca retention remained similar in all dietary groups.

The direction of the influence of dietary P concentration on Mg metabolism was comparable with the effects on Ca metabolism. Mg and phosphates can also form insoluble complexes in the intestine, just as described for Ca. In addition, the effects can also be mediated through the influence of parathyroid hormone (PTH), as PTH exerts a similar influence on the metabolism of Ca and Mg (Ritskes-Hoitinga & Beynen, 1992).

Higher dietary P intakes coincided with higher urinary and faecal excretion levels, and higher apparent absorption and retention levels of P. Higher levels of dietary P intakes caused a higher incidence and more severe degree of nephrocalcinosis, as demonstrated by the kidney Ca and P analysis, as well as the histological scores. This was also seen in other investigations (Jowsey & Balasubramaniam, 1972; Berg *et al.* 1979) and comparable with what occurs in rats (Ritskes-Hoitinga *et al.* 1989, 1992; Schoenmakers *et al.* 1989; Ritskes-Hoitinga & Beynen, 1992). Histologically, nephrocalcinosis in rats is characterised by the deposition of calcium phosphate crystals at the corticomedullary junction and is almost exclusively seen in females due to the influence of oestrogens (Ritskes-Hoitinga *et al.* 1989). In rabbits, calcified deposits are seen in the kidney medulla as well as the cortex, and are therefore spread out over a larger area in the kidney than in rats. Male rabbits develop significant degrees of kidney calcification as is shown in the present study, but females also are sensitive (M Ritskes-Hoitinga, O Skott, TR Uhrenholt, I Nissen, I Lemmens and AC Beynen, unpublished results). This suggests that oestrogen hormones do not play an important role in the aetiology of kidney calcification in rabbits, in contrast to rats. Medullary calcifications occurred already at a dietary P level as low as 0·1 %; it may be that the medullary calcifications are a normal physiological event, as rabbit urine always contains mineral deposits. Increasing degrees of calcifications occurred in the kidney cortex and medulla at increasing dietary P levels, which confirms our hypothesis that more nephrocalcinosis would occur at dietary P levels over 0·22 %. The minimum recommended dietary P level

of 0·2 % also led to kidney calcifications in the medulla and cortex of a number of animals.

The influence of dietary P levels on bone development was examined in two functionally different parts of the femur bone. The analysed mineral concentrations in the diaphysis were higher than those in the epiphysis, especially when expressed as mg/cm^3, which illustrates the physiological difference between these two tissues. The diaphyseal bone is cortical haversian bone, which is heavily mineralised, whereas the epiphyseal bone has a large component of growth-plate cartilage, which is poorly mineralised. When changing from the 0·2 to the 0·8 % dietary P level, a reduction in the bone density occurred in the diaphysis, but not in the epiphysis (femoral head). The dietary Ca:P ratio in this group was 0·63, and the bone-density result is therefore in line with the expectation that a Ca:P ratio of 1 or more would lead to a more optimal bone calcification. Jowsey & Balasubramaniam (1972) detected an increased cortical bone porosity in the tibia and femur in rabbits after P supplementation. By supplying extra dietary P, an increase in serum P and a decrease in Ca will be induced, which in turn will lead to the release of PTH (Jowsey & Balasubramaniam, 1972). PTH causes increased Ca reabsorption in the kidney, and activates vitamin D so that Ca absorption in the intestine is stimulated (Shoback & Strewler, 2000). Bone Ca release from a rapidly exchangeable pool of bone Ca is also stimulated under the influence of PTH, in order to restore blood Ca levels. PTH affects kidney P handling in such a way that urinary P excretion is increased (Shoback & Strewler, 2000). PTH acts on bone in two steps; initially Ca and P are rapidly mobilised from a compartment in direct contact with extracellular fluids, which results in bone resorption. Thereafter, Ca is mobilised by bone-matrix dissolution and changes in the bone remodelling process, which ultimately will lead to a stimulated bone formation again, since the processes of resorption and formation are coupled (Shoback & Strewler, 2000). It may be that the reduction in bone density in the diaphysis in the 0·8 % P group is a result from the initial rapid mobilisation of Ca and P by PTH. As Ca and P concentrations measured in the diaphysis were not influenced by dietary P concentrations, this cannot explain the reduced bone density, but a different bone structure may have been formed. A gradual reduction of the mean bone Ca:P ratio with increasing dietary P levels may indicate that bone composition changes gradually, which can have implications for bone structure as well (mean values of Ca:P for the diaphysis (Table 6) are 2·06, 1·95, 1·82, and 1·94; mean values for the epiphysis (Table 7) are 2·26, 2·21, 2·15, and 2·03 in the 0·1, 0·2, 0·4 and 0·8 % P groups respectively). Jowsey & Balasubramaniam's (1972) measurements of a change in bone porosity support the concept of an altered bone structure due to P supplementation. The increased Mg content in the ash and dry weight in the high P group may also be an indication of an altered bone structure in the diaphysis. The Mg content was significantly altered in the epiphysis as well, and also when expressed as mg/cm^3. However, no change in total bone-density measurement in that part of the bone was measured. Different metabolic mechanisms and different pressure forces in various bone parts may be the basis for this variation.

Higher dietary P concentrations caused reduced plasma Ca levels, which is in agreement with other studies (Jowsey & Balasubramaniam, 1972; National Research Council, 1977; Berg *et al.* 1979). Increased plasma P concentrations as a consequence of high dietary P levels have also been seen by other investigators (Jowsey & Balasubramaniam, 1972; Berg *et al.* 1979). It is probable that the formation of insoluble calcium phosphates in the intestinal tract leads to a reduced apparent absorption of Ca, contributing to a reduced plasma Ca level. Berg *et al.* (1979) claim that a diet high in P leads to a reduced vitamin D synthesis (25 OH vitamin D_3), which contributes to the lowering of plasma Ca levels by reducing Ca absorption in the intestine and reabsorption in the kidney. The low plasma Ca concentration will induce PTH secretion, which aims to restore Ca levels. One of the PTH effects is to activate vitamin D, which counteracts the negative effect of a high dietary P level on vitamin D synthesis.

It can be concluded, on the basis of the results from mineral metabolism, kidney calcification and bone development, that at a dietary Ca level of 0·5 %, increasing dietary P levels lead to more kidney calcification in growing male New Zealand White rabbits. At the dietary Ca level of 0·5 % and the dietary P concentration of 0·1, nephrocalcinosis was only present at a low frequency and severity in the kidney medulla, and no adverse effects on bone density and mineralisation occurred under the present experimental conditions. As the minimum P requirement of 0·2 % (National Research Council, 1977) coincides with kidney calcifications in the cortex and medulla, it is advised that this P level should be regarded as a maximum level instead. Depending on the form of phosphate used, bioavailability could be different and therefore caution is warranted in using dietary P levels of 0·1 and 0·2 %. As the present study only investigated a relatively short period of the rabbits' growing life, long-term studies that include reproduction will be needed before more general conclusions on the rabbits' P requirement can be made. In rats it has been shown that a reduced dietary P level (0·2 instead of 0·4 %) will delay bone mineralisation in following generations (Ritskes-Hoitinga *et al.* 1993). In addition to the chemical analyses of bone mineralisation, it is also advised to perform bone-structure analyses.

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