

## Inhibitory effect of high protein intake on nephrocalcinogenesis in female rats

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Increased intakes of protein have been shown to reduce kidney calcification (nephrocalcinosis) in female rats. Two questions were addressed in the present study. First, can protein-induced inhibition of nephrocalcinosis be demonstrated when the diets used are balanced for calcium, magnesium and phosphorus in the added protein? Second, can the protein effect be explained by the frequently observed magnesiuria after giving high-protein diets? Nephrocalcinosis was induced in female rats by giving purified diets containing 151 g casein/kg and either an increased concentration of P (6 v. 2 g/kg) or a decreased concentration of Mg (0.1 v. 0.4 g/kg). To these diets 151 g ovalbumin/kg was added at the expense of glucose, and the diets were balanced for Ca, Mg and P in ovalbumin. The diets were given for 29 d. In rats fed on the diet containing 151 g protein/kg, an increased intake of P or a decreased intake of Mg caused nephrocalcinosis as measured chemically by analysis of kidney Ca as well as histologically by scoring kidney sections stained according to Von Kossa's method. The addition of ovalbumin to the diet prevented the induction of nephrocalcinosis. High P intake and low Mg intake with the low-protein diets induced enhanced loss of albumin in urine, suggesting that nephrocalcinosis caused kidney damage. Increased protein intake with a non-calcinogenic diet also caused increased albumin excretion in urine. Irrespective of the composition of the background diet, increased protein intake caused increased urinary excretion of Mg. When all dietary groups were considered, differences in nephrocalcinosis and urinary Mg output were not proportionally related.

**Nephrocalcinosis: Phosphorus: Magnesium: Protein: Rat**

Nephrocalcinosis refers to the deposition of calcium phosphates in the corticomedullary region of the kidney. In general, the primary lesion is an intratubular formation of calcium phosphate precipitates. Diets with either high concentrations of phosphorus or low concentrations of magnesium are known to promote nephrocalcinosis in the rat (Hellerstein *et al.* 1957; Bunce *et al.* 1965; Du Bruyn, 1972; Parker, 1985; Hoek *et al.* 1988; Mars *et al.* 1988). Decreasing the intake of P or increasing that of Mg not only prevents nephrocalcinosis but also causes magnesiuria (Goulding & Malthus, 1969; Bunce & Bloomer, 1972; Draper *et al.* 1972; Hitchman *et al.* 1979; Harwood, 1982; Al-Modhefer *et al.* 1986). Possibly there is a relationship between urinary Mg excretion and nephrocalcinogenesis. This is supported by *in vitro* observations that Mg inhibits the precipitation of calcium phosphates (Boulet *et al.* 1962).

High-protein diets have been shown to induce magnesiuria (Schneider & Menden, 1988). Thus, it could be suggested that diets rich in protein inhibit nephrocalcinogenesis. Indeed, evidence has been reported that high protein intakes reduce the degree of nephrocalcinosis in rats (Eklund *et al.* 1973; Schneider & Menden, 1988). However, the protein preparations

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used in these studies were not pure, and the experimental diets were not balanced for components other than protein in the protein preparations, such as Ca, Mg or P. This prompted us to re-address the question whether the amount of protein in the diet affects nephrocalcinosis in female rats, or rather whether high protein intakes can counteract nephrocalcinogenesis induced by either high P or low Mg concentrations in the diet. In addition, we tested our hypothesis that protein effects on nephrocalcinosis are related to protein-induced magnesuria.

#### MATERIALS AND METHODS

##### *Animals, housing and diets*

Two experiments of the same design were performed. The interval between the two experiments was about 12 weeks. Outbred, female Wistar (Cpb: WU) rats, aged 7–8 weeks, were used throughout. They had been fed on a commercial pelleted diet (RMHB; Hope Farms, Woerden). All rats received a purified, pre-experimental diet and demineralized water *ad lib*. The pre-experimental diet contained (g/kg): 151 protein 0.4, Mg and 2 P (Table 1). The pre-experimental period was inserted before the experimental period in order to eliminate possible effects of the commercial diet and to allow the rats to adjust to a purified diet. After the pre-experimental period of 10 d (day 0), the rats were divided into four groups of either six (Expt 1) or eight rats each (Expt 2). The groups were formed so that their body-weight distributions were similar. Expt 1 was performed to see whether high protein intake counteracts P-induced nephrocalcinosis. In Expt 1, one group remained on the pre-experimental diet and the other three groups were switched to purified diets containing 0.4 g Mg/kg and different amounts of P (2 or 6 g/kg) and protein (151 or 302 g/kg). Expt 2 was carried out to check, in part, the reproducibility of Expt 1 and to find out whether high protein intake counteracts nephrocalcinosis as induced by Mg restriction. In Expt 2, all groups were transferred from the pre-experimental diet to a diet containing 6 g P/kg and different amounts of Mg (0.4 or 0.1 g/kg) and protein (151 or 302 g/kg). All diets contained 5 g Ca/kg. The experimental period lasted 29 d.

Table 1 shows the composition of the diets. Extra protein was added in the form of ovalbumin at the expense of glucose, and the diets were balanced for Ca, Mg and P in the protein preparation. The calculated amounts of Ca, Mg, P and protein in the diets agreed well with the analysed concentrations (Table 1). All diets were in powdered form and stored at 4° until used. Before feeding, the meal was mixed with demineralized water in the proportion 5:1 (w/w). The animals were fed *ad lib*. and had free access to demineralized water. The compositions of the pre-experimental diet and the diets containing 6 g P/kg and 0.4 g Mg/kg were identical for Expts 1 and 2. Separate batches of diets were made for the two experiments.

During the experimental period (days 0–29) the rats were housed individually in metabolism cages which were located in a room with controlled temperature (20–22°), humidity (55–65%) and lighting (12 h light–dark cycle with light from 06.00–18.00 hours).

##### *Collection of samples and chemical analyses*

From days 13 to 15 (period 1) and days 27 to 29 (period 2), urine and faeces from each rat were collected quantitatively. Excreta were collected daily and stored at 4°. After complete collection, part of the urine was acidified to pH 1–2 with 6 M-hydrochloric acid and centrifuged (1200 g, 10 min). The supernatant fraction was frozen at –20° until analysed for Ca, Mg and P. Faeces were freeze-dried, homogenized and weighed. A sample of 150 mg was ashed at 500° for 17 h and then dissolved in 6 M-HCl. Feed samples were

Table 1. *Composition of diets (g/kg)*

Expt...	Diet*					
	1/2†	1	1/2	1/2	2	2
Protein ...	151	302	151	302	151	302
Phosphorus...	2	2	6	6	6	6
Magnesium...	0.4	0.4	0.4	0.4	0.1	0.1
Ingredient						
Ovalbumin‡	—	151.0	—	151.0	—	151.0
Glucose	709.3	559.26	695.9	545.86	696.88	546.98
CaCO <sub>3</sub>	12.4	12.24	12.4	12.24	12.4	12.24
NaH <sub>2</sub> PO <sub>4</sub> ·2H <sub>2</sub> O	5.0	4.2	25.2	24.4	25.2	24.4
Na <sub>2</sub> CO <sub>3</sub>	10.2	10.48	3.4	3.68	3.4	3.68
MgCO <sub>3</sub>	1.4	1.12	1.4	1.12	0.42	—
Constant components§	261.7	261.7	261.7	261.7	261.7	261.7
Chemical analysis						
Calcium	4.7/4.0	4.8	4.7/4.2	4.7/4.2	4.2	4.1
Magnesium	0.4/0.3	0.4	0.4/0.3	0.4/0.4	0.1	0.1
Phosphorus	2.2/1.8	2.0	5.6/5.4	5.7/5.0	5.0	4.8
Crude protein	140/138	260	139/134	260/254	135	260

\* Dietary variables refer to the calculated amounts of protein, P and Mg.

† Pre-experimental diet for both experiments.

‡ Chemical analysis showed that the ovalbumin preparations contained (g/kg for Expts 1 and 2 respectively) 0.44, 0.42 Ca; 0.54, 0.80 Mg; 1.06, 0.98 P.

§ Constant components consisted of (g/kg diet): casein 151, maize oil 25, coconut fat 25, cellulose 30, KCl 1, KHCO<sub>3</sub> 7.7, mineral premix 10, vitamin premix 12. The compositions of the mineral and vitamin premixes have been described by Hoek *et al.* (1988).

processed as faeces samples. Ca and Mg in urine, faeces and feed samples were determined by atomic absorption spectroscopy (Hoek *et al.* 1988; Mars *et al.* 1988). P was analysed according to Taussky & Shorr (1953). Non-acidified urine samples were frozen at  $-20^{\circ}$  until analysed for albumin, lysozyme, urea and creatinine. Albumin in urine was determined by radial immunodiffusion (Mancini *et al.* 1965). Lysozyme in urine was detected by the agar plate diffusion method of Osserman & Lawlor (1966).

At the end of the experiment (day 29) between 09.00 and 13.00 hours, the non-starved rats were anaesthetized with diethyl ether, and blood was obtained by orbital puncture into heparinized tubes. The anaesthetized rats were killed by decapitation (Expt 1) or exposure to carbon dioxide (Expt 2). Different methods of euthanasia were used in the two experiments for practical reasons. The kidneys from each rat were collected and after capsules had been removed they were weighed. The right kidneys were used for chemical analysis of Ca, Mg and P as described for faeces and feed samples. The left kidneys were fixed in formalin and stained by Von Kossa's method (Mallory, 1961). The severity of nephrocalcinosis was graded on a scale from 0 (no Ca deposits) to 3 (severe calcinosis). To aid in scoring, four reference slides were used. The kidneys were scored in random order by three persons who were blinded to treatment modality. The score of each rat was the average score of the three assessors.

Plasma and urinary urea were determined with a test combination (Urea UV, MA-KIT 10 ROCHE; Roche Diagnostics, Basel, Switzerland). Creatinine in plasma and urine was also determined with a test combination (UNI-KIT II ROCHE; Roche Diagnostics). Both analyses were performed with the use of a COBAS-BIO autoanalyser (Hoffman-La Roche BV, Mijdrecht).

*Statistical analyses*

Analysis of variance was performed to disclose protein, P and Mg effects, and P  $\times$  protein and Mg  $\times$  protein interactions. Scores of nephrocalcinosis were not distributed normally, and the Kruskal–Wallis rank statistics test was used to compare group means.

## RESULTS

*Growth and feed intake*

Rats fed on the high-protein (302 g/kg) diets showed significantly lower feed intakes and lower rates of body-weight gain (Table 2). In Expt 2, the amount of protein in the diet did not significantly affect body-weight gain but did influence feed intake (Table 3). The discrepancy between the effect of high protein intake on body-weight gain in Expts 1 and 2 may relate to the fact that in Expt 1 the group mean initial body-weights of the low- and high-protein groups were different. The lower initial body-weight of the high-protein group may have caused an overestimation of the observed depressing effect of high protein intake on weight gain. P intake (2 v. 6 g/kg) did not significantly affect feed intake or body-weight gain. Body-weight gain was significantly depressed in rats fed on the low-Mg diets, while their feed intake was only slightly reduced (Table 3).

*Effects of P intake at two dietary protein levels*

An increase in P intake caused a decreased urinary output of Ca and Mg and an increase of faecal excretion of Mg (Table 2). In rats fed on the high-P diets, urinary and faecal excretion and retention of P were significantly increased when compared with rats fed on the low-P diets.

In rats fed on the low-protein (151 g/kg) diets, an increase in dietary P level from 2 to 6 g/kg induced increased kidney concentrations of Ca, P and Mg. In rats fed on the high-protein diets, an increased intake of P did not influence mineral concentrations of kidney. In rats fed on the low-protein diet, but not in those fed on the high-protein diet, an increased intake of P caused an increase in severity of nephrocalcinosis as determined histologically.

In rats fed on the low-protein diets, an increased P intake tended to stimulate urinary excretion of albumin. Creatinine clearance, urea and creatinine in urine and plasma were not influenced by the P content of the diet.

*Effects of Mg intake at two dietary protein levels*

A decrease in Mg intake reduced Mg excretion in urine and faeces, lowered urinary Ca excretion, but did not systematically influence P excretion.

In rats fed on the low-protein diets, a decrease in Mg intake resulted in a significant increase in kidney weight. When the diet contained 151 g protein/kg, a decrease in dietary Mg caused significantly increased concentrations in kidney of Ca, Mg and P and caused increased incidence and severity of nephrocalcinosis as assessed histologically. This Mg effect was not seen with the high-protein diet.

In rats fed on the low-protein diet, a decrease in Mg intake tended to cause an increase in urinary output of albumin. This Mg effect was not seen in rats fed on the high-protein diet. Lowering the Mg content of the diet increased the urea content in plasma. Urinary creatinine was not significantly influenced by the amounts of Mg in the diet. Creatinine clearance tended to be increased by low Mg intake.

Table 2. *Expt 1. Effects of protein and phosphorus intakes on growth and feed intakes, mineral balance and kidney function in rats*

(Means with pooled standard errors for six rats per dietary group)

	Diet*				Pooled SE	Statistical significance†
	151	302	151	302		
Protein (g/kg)...	151	302	151	302		
P (g/kg)...	2	2	6	6		
Magnesium (g/kg)...	0.4	0.4	0.4	0.4		
<b>Body-wt (g)</b>						
Initial	191.1	186.8	193.6	178.2	5.5	
Final	224.7	212.4	232.4	206.8	5.2	
Wt gain (g/d)	1.2	0.9	1.4	1.0	0.1	Prot
Feed intake (g/d)	16.8	15.4	17.5	15.5	0.3	Prot
<b>Mineral balance, period 1 (mg/d)</b>						
<b>Intake</b>						
Calcium	73.6	70.7	74.1	66.9	2.7	
Mg	6.3	5.9	6.5	5.7	0.2	Prot
P	31.3	29.5	96.7	85.4	2.7	Prot,P
<b>Urinary output</b>						
Ca	0.4	1.1	0.2	0.3	0.1	Prot,P,Prot × P
Mg	1.6	1.8	0.5	0.7	0.1	P
P	3.6	3.3	40.4	35.7	1.7	P
<b>Faecal output</b>						
Ca	50.1	45.1	48.3	44.4	3.2	
Mg	2.7	2.2	4.0	3.3	0.2	Prot,P
P	15.7	14.3	29.9	23.8	1.3	Prot,P
<b>Retention</b>						
Ca	23.1	24.5	25.6	22.2	3.5	
Mg	2.0	2.0	1.8	1.7	0.2	
P	12.1	11.9	26.5	26.0	2.5	P
<b>Mineral balance, period 2 (mg/d)</b>						
<b>Intake</b>						
Ca	76.2	72.3	77.2	69.3	2.1	Prot
Mg	6.5	6.0	6.7	5.9	0.2	Prot
P	32.4	30.2	100.7	88.5	1.7	Prot,P,Prot × P
<b>Urinary output</b>						
Ca	0.6	1.7	0.3	0.5	0.1	Prot,P,Prot × P
Mg	1.4	1.9	0.6	0.7	0.2	Prot,P
P	3.1	2.9	38.9	37.6	1.3	P
<b>Faecal output</b>						
Ca	53.6	49.6	52.5	45.1	2.2	Prot
Mg	3.0	2.3	4.3	3.7	0.2	Prot,P
P	18.6	16.1	31.5	27.3	1.2	Prot,P
<b>Retention</b>						
Ca	22.0	21.1	24.4	23.7	1.9	
Mg	2.1	1.8	1.9	1.5	0.2	Prot
P	10.8	11.2	30.3	23.6	1.1	Prot,P,Prot × P
<b>Kidney variables</b>						
Relative kidney wt (g/kg body-wt)	3.4	3.8	3.6	3.9	0.2	Prot
Dry wt (mg)	182	190	195	190	6	
Ca (g/kg)	0.36	0.32	10.42	0.20	0.2	Prot,P,Prot × P
Mg (g/kg)	1.03	1.02	1.21	1.06	0.01	P
P (g/kg)	13.0	12.8	17.3	13.4	0.1	P
<b>Nephrocalcinosis</b>						
Incidence	0/6	0/6	4/6	0/6		
Severity‡	0.0 <sup>a</sup>	0.0 <sup>a</sup>	2.0 <sup>b</sup>	0.0 <sup>a</sup>		

Table 2. (cont.)

	Diet*				Pooled SE	Statistical significance†
	151	302	151	302		
Protein (g/kg)...	151	302	151	302		
P (g/kg)...	2	2	6	6		
Magnesium (g/kg)...	0.4	0.4	0.4	0.4		
	Urinary output					
Production (ml/d)						
Period: 1	11.8	12.4	9.6	11.4	4.1	
2	11.1	14.4	9.9	12.0	5.1	
pH						
Period: 1	8.7	8.7	7.3	7.6	0.2	P
2	9.1	8.7	7.7	7.5	0.1	Prot,P
Albumin (mg/d)						
Period: 1	0.58	1.10	0.86	1.00	0.16	
2	0.61	1.30	0.87	1.22	0.21	Prot
Lysozyme (incidence)						
Period: 1	1/6	6/6	3/6	6/6		
2	0/6	6/6	2/6	6/6		
Urea (g/kg body-wt per d)						
Period: 1	2.36	4.58	2.16	4.74	0.17	Prot
2	2.26	4.59	2.13	4.50	0.19	Prot
Creatinine (mg/kg body-wt per d)						
Period: 1	36.3	39.8	33.9	39.1	1.4	Prot
2	38.5	38.5	34.7	36.1	2.0	
	Plasma variables					
Urea (mM)	6.6	8.1	6.7	8.8	0.3	Prot
Creatinine ( $\mu$ M)	30.4	34.0	30.0	31.5	2.1	
Creatinine clearance (ml/min per kg body-wt)	7.9	7.0	7.1	7.2	0.5	

\* Dietary variables refer to the calculated amounts of protein, P and Mg.

† Statistical significance ( $P < 0.05$ ): Prot, effect of amount of protein; P, effect of amount of P; Prot  $\times$  P, effect of interaction.

‡ On a 0-3 scale.

<sup>a,b</sup> Group means with different superscript letters were significantly different ( $P < 0.05$ ).

#### *Effects of protein intake*

An increase in protein intake, at either low or high dietary P concentrations, significantly stimulated the excretion of Ca in urine and tended to increase urinary excretion of Mg (Table 2). This protein effect was less pronounced if the background diet contained 6 g P/kg instead of 2 g P/kg. The high-protein diets significantly lowered the excretion of Mg in faeces. In Expt 2, an increase in protein intake significantly increased urinary excretion of Mg (Table 3). Urinary Ca excretion was elevated in rats fed on the high-protein diets only during the first collection period (Table 3).

When rats were fed on the high-P diets, containing 6 g P/kg and 0.4 g Mg/kg, an increase in dietary protein concentration significantly reduced kidney levels of Ca (Tables 2 and 3). The increase in kidney weight caused by Mg restriction was counteracted by increasing the protein concentration of the diet (Table 3). In rats fed on the high-Mg diets, an increase in dietary protein tended to increase kidney weight. Nephrocalcinosis induced by decreased Mg intake, as assessed either chemically or histologically, was nullified by increasing the amount of protein in the diet.

Table 3. *Expt 2. Effects of protein and magnesium intakes on growth and feed intakes, mineral balance and kidney function in rats*

(Means with pooled standard errors for eight rats per dietary group)

	Diet*				Pooled SE	Statistical significance†
	151	302	151	302		
Protein (g/kg)...	151	302	151	302		
P (g/kg)...	6	6	6	6		
Magnesium (g/kg)...	0.4	0.4	0.1	0.1		
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Body-wt (g)						
Initial	164.9	164.5	164.9	164.1	4.7	
Final	201.7	201.6	199.5	193.5	6.9	
Wt gain (g/d)	1.3	1.3	1.2	1.0	0.1	Mg
Feed intake (g/d)	15.4	14.7	15.0	14.3	0.5	Prot
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Mineral balance, period 1 (mg/d)						
Intake						
Calcium	61.1	61.2	61.8	56.4	2.2	
Mg	4.9	5.1	1.9	1.9	0.2	Mg
P	79.3	73.3	74.5	63.4	2.8	Prot,Mg
Urinary output						
Ca	0.2	0.3	0.1	0.2	0.0	Prot,Mg
Mg	0.4	0.5	0.1	0.3	0.0	Prot,Mg
P	32.5	33.3	34.8	35.0	1.6	
Faecal output						
Ca	43.6	42.0	37.4	39.1	2.6	
Mg	3.6	3.2	1.5	1.4	0.2	Mg
P	26.6	24.5	23.3	21.8	1.6	Mg
Retention						
Ca	17.3	19.0	24.3	17.1	2.3	
Mg	0.9	1.5	0.3	0.3	0.1	Prot,Mg,Prot × Mg
P	20.2	15.4	16.3	8.6	1.5	Prot,Mg
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Mineral balance, period 2 (mg/d)						
Intake						
Ca	63.7	62.3	63.3	61.3	2.9	
Mg	5.1	5.2	2.0	2.1	0.2	Mg
P	82.7	74.6	76.1	71.0	3.5	
Urinary output						
Ca	0.3	0.3	0.2	0.2	0.0	Mg
Mg	0.3	0.6	0.1	0.2	0.0	Prot,Mg
P	31.8	32.3	34.1	33.2	1.7	
Faecal output						
Ca	45.8	41.2	40.8	40.3	2.4	
Mg	3.9	3.2	1.6	1.5	0.2	Prot,Mg
P	29.0	24.9	25.3	23.7	1.6	
Retention						
Ca	18.3	20.8	22.3	20.8	2.1	
Mg	0.9	1.5	0.3	0.4	0.1	Prot,Mg
P	22.0	17.4	16.8	14.1	1.8	Mg
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Kidney variables						
Relative kidney wt (g/kg body-wt)	3.7	4.0	4.7	4.3	0.2	Mg,Prot × Mg
Dry wt (mg)	166	175	188	179	7	
Ca (g/kg)	1.05	0.50	20.65	0.71	0.25	Prot,Mg,Prot × Mg
Mg (g/kg)	0.83	0.86	0.98	0.81	0.01	Prot,Prot × Mg
P (g/kg)	12.6	12.4	24.2	12.4	0.1	Prot,Mg,Prot × Mg
Nephrocalcinosis						
Incidence	3/8	1/8	8/8	1/8		
Severity‡	0.4 <sup>b</sup>	0.0 <sup>a</sup>	2.6 <sup>c</sup>	0.0 <sup>a</sup>		

Table 3. (cont.)

	Diet*				Pooled SE	Statistical significance†
	151	302	151	302		
Protein (g/kg)...	151	302	151	302		
P (g/kg)...	6	6	6	6		
Magnesium (g/kg)...	0.4	0.4	0.1	0.1		
Urinary output						
Production (ml/d)						
Period: 1	15.0	10.8	15.3	16.2	8.3	
2	10.9	10.7	14.2	15.4	5.6	Mg
pH						
Period: 1	8.0	7.5	7.6	7.7	0.1	Prot, Prot × Mg
2	7.8	7.5	7.6	7.8	0.1	Prot × Mg
Albumin (mg/d)						
Period: 1	0.48	0.87	0.94	0.78	0.25	
2	0.77	1.08	0.92	0.94	0.32	
Lysozyme (incidence)						
Period: 1	0/8	2/8	0/8	1/8		
2	2/8	3/8	0/8	2/8		
Urea (g/kg body-wt per d)						
Period: 1	2.13	4.44	2.14	4.53	0.12	Prot
2	2.09	4.33	2.14	4.34	0.14	Prot
Creatinine (mg/kg body-wt per d)						
Period: 1	39.1	40.2	39.1	41.3	1.6	
2	33.8	37.2	37.4	37.5	1.4	
Plasma variables						
Urea (mM)	5.7	7.8	7.4	8.2	0.3	Prot, Mg
Creatinine ( $\mu$ M)	35.9	38.9	36.8	32.0	1.8	Prot × Mg
Creatinine clearance (ml/min per kg body-wt)	5.8	6.1	6.3	7.4	0.6	

\* Dietary variables refer to the calculated amounts of protein, P and Mg.

† Statistical significance ( $P < 0.05$ ): Prot, effect of amount of protein; Mg, effect of amount of Mg; Prot × Mg, effect of interaction.

‡ On a 0-3 scale.

<sup>a, b, c</sup> Group means with different superscript letters were significantly different ( $P < 0.05$ ).

An increase in protein intake with low- or high-P diets containing 0.4 g Mg/kg caused enhanced albumin loss in urine, but this effect did not reach statistical significance (Tables 2 and 3). The incidence of lysozyme-positive urine was on average higher in rats fed on the high-protein diets compared with low-protein diets. Increasing the concentration of protein in the diet significantly increased urea output in urine and its concentration in plasma. Urinary creatinine and creatinine clearance were not significantly influenced by the amount of protein in the diet.

#### DISCUSSION

An increase in the concentration of P in the diet from 2 to 6 g/kg or a decrease in Mg concentration from 0.4 to 0.1 g/kg, with a dietary background of 151 g protein/kg diet, caused nephrocalcinosis as based on increased Ca concentrations in kidney and the formation of Von Kossa-positive material in kidney sections. In rats fed on similar diets, the degree of nephrocalcinosis differed markedly in Expts 1 and 2. There was a 10-fold difference between experiments in kidney Ca in rats fed on the diet with 151 g protein/kg



containing 6 g P/kg and 0.4 g Mg/kg (Tables 2 and 3). In our earlier studies (Hoek *et al.* 1988; Mars *et al.* 1988; Ritskes-Hoitinga *et al.* 1989) it was also found that in rats fed on nephrocalcinogenic diets the absolute concentrations of kidney Ca are poorly reproducible. However, dietary effects within experiments have proved quite reproducible. Likewise, in the present study the anti-nephrocalcinogenic effect of high protein intake was seen in rats fed on diets containing 6 g P/kg and 0.4 g Mg/kg both in Expt 1 (Table 2) and Expt 2 (Table 3). It would appear that fluctuations in intangible factors determine the absolute rather than relative sensitivity of rats to nephrocalcinogenic diets. Thus, it can be concluded from the present study that an increase in dietary protein concentration from 151 to 302 g/kg completely counteracted nephrocalcinogenesis induced by either increased P or decreased Mg intake. It is unlikely that this protein effect refers to a specific characteristic of ovalbumin. There is evidence that increased intakes of casein and soya-bean protein also reduce nephrocalcinosis (Eklund *et al.* 1973; Kaunitz & Johnson, 1976; Schneider & Menden, 1988), but the diets used were not balanced for minerals in the proteins.

One objective of the present study was to determine whether the inhibitory effect of high protein intake on nephrocalcinosis is related to protein-induced magnesuria. In order to reduce the risk of finding a spurious relationship, nephrocalcinosis was induced by two different dietary changes, that is by P loading in Expt 1 and by Mg restriction in Expt 2. The protein-induced inhibition of nephrocalcinogenesis was paralleled by increased urinary excretion of Mg in Expts 1 and 2. Only in the first balance period of Expt 1 did the protein effect not reach statistical significance. Thus, the present study would support a cause-and-effect relationship between nephrocalcinogenesis and urinary Mg excretion, if any. Other present observations speak against such a causal relationship. In keeping with earlier studies (Hoek *et al.* 1988; Mars *et al.* 1988), an increase in P intake in rats fed on a diet containing 151 g protein/kg lowered Mg excretion in urine. This P effect was of the same order of magnitude in rats fed on a diet containing 302 g protein/kg, but the degree of nephrocalcinosis was not influenced in this situation (Table 2). The same conclusion would be reached when urinary Mg concentrations are considered (cf. Table 2). A decrease in Mg intake by rats fed on the low-protein diet resulted in marked nephrocalcinosis associated with a decrease in Mg excretion in urine (Table 3). Lowering of the Mg concentration in the high-protein diet reduced urinary Mg excretion but did not produce nephrocalcinosis. Thus, urinary Mg concentrations and nephrocalcinosis appear not to be proportionally related. It is also questionable whether the two variables are causally associated.

With diets containing 151 g protein/kg, an increase in P intake or decrease in Mg intake resulted in increased group mean urinary excretion of albumin (Tables 2 and 3). As suggested earlier (Ritskes-Hoitinga *et al.* 1989), this could be related to nephrocalcinosis-induced kidney damage. Albumin excretion is assumed to reflect glomerular leakage or defective re-absorption in the proximal tubules, or both (Massry & Glasscock, 1983). Massive nephrocalcinosis induced by decreased Mg intake with the diet containing 151 g protein/kg was associated with increased group mean plasma concentrations of urea (Table 3). This would also point to kidney damage as a result of nephrocalcinosis. In keeping with the proposition that nephrocalcinosis causes kidney damage, Kang *et al.* (1979) found increased urinary *N*-acetyl- $\beta$ -glucosaminidase (*EC* 3.2.1.30) activity in rats with nephrocalcinosis.

Since high protein intakes reduce nephrocalcinosis, it would be anticipated that high protein intakes lower albumin excretion in urine. However, such an effect was not found. High protein intake itself (Expt 1) might cause kidney damage, resulting in increased albumin excretion (Rosenberg *et al.* 1988). This would be supported by the observed protein-induced increase of urinary excretion of lysozyme in Expt 1. However, urinary lysozyme could not be detected in Expt 2. Lysozyme excretion points to damage of the

proximal tubulus (Balazs & Roepke, 1966; Harrison *et al.* 1973). The protein-induced increased urinary urea excretion and increased concentrations of urea in plasma are most likely related to enhanced protein catabolism rather than kidney damage (Rosenberg *et al.* 1988). There was no systematic effect on creatinine clearance, implying that the glomerular filtration rate was not influenced by feeding the high-protein diets. In contrast, Bouby *et al.* (1988) found a significant increase in creatinine clearance, glomerular filtration rate and kidney mass in male rats after feeding for 6 weeks a diet containing 320 g casein/kg, compared with a diet containing 100 g casein/kg. The diets were balanced for P content, but not for Ca and Mg. The fact that we used rats of another sex and strain and a shorter feeding period may explain the discrepancy between the outcome of the study of Bouby *et al.* (1988) and that of the present study. Stonard *et al.* (1984) did not measure a different inulin clearance after feeding two protein levels (300 *v.* 180 g/kg). Thus, the amount of protein in the diet may not consistently alter the glomerular filtration rate in rats.

In the present study, the addition of extra protein to the non-nephrocalcinogenic basal diets caused an increased relative kidney weight. This is in accordance with the findings of Bouby *et al.* (1988). Nephrocalcinosis also caused an increased kidney weight which is due not only to the Ca deposits, but also to hypertrophy (Ritskes-Hoitinga *et al.* 1989). The addition of protein to nephrocalcinogenic diets did not clearly affect kidney weight. This suggests that under this condition the protein-induced increase in kidney weight and the decrease in kidney weight associated with diminished nephrocalcinosis are of the same order of magnitude. The hypertrophy on high-protein diets is characterized by a preferential increase in the thickness of the inner stripe of the outer medulla, coinciding with marked hypertrophy of the thick ascending limb of Henle's loop (Bouby *et al.* 1988). Possibly the hypertrophy of the thick ascending limb on a high-protein diet relates to decreased sensitivity towards the development of kidney calcification.

In summary, increased intake of protein very effectively counteracted nephrocalcinogenesis in female rats induced by the feeding of either high-P or low-Mg diets. The metabolic basis for this protein effect is not yet clear. Further work on this issue is relevant as it will broaden our basic knowledge of interactions between diet composition and nephrocalcinogenesis, which can occur in various animal species, including man. Ultimately, this knowledge may assist in treating or preventing kidney calcification, or both.

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