The effect of moderately and severely restricted dietary magnesium intakes on bone composition and bone metabolism in the rat

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Forty 3-week-old male rats, Wistar strain, average weight 59 g, were randomized by weight into five groups of eight rats each. Three groups were fed *ad libitum* on a semi-purified diet containing (per kg) 400 (adequate), 200 (moderately Mg-restricted) or 20 (severely Mg-restricted) mg Mg for 3 weeks while two groups were pair-fed with the Mg-adequate diet in the same quantities as those consumed by the two Mg-restricted groups respectively. While weight gains and food conversion efficiency values for the Mg-restricted groups were similar to those of the corresponding pair-fed control groups, serum and kidney Mg, and femoral dry weight were reduced by 70, 7 and 9 % respectively in the severely Mg-restricted group and were unaffected in the moderately Mg-restricted group. Significant reductions were observed in urinary pyridinoline (Pyr) (by 44 and 34 %) and deoxypyridinoline (Dpyr) levels (by 40 and 33 %) (markers of bone resorption), serum osteocalcin levels (by 46 and 28 %) (marker of bone formation), femoral Mg levels (by 52 and 14 %) and osteocalcin mRNA levels (by 46 and 22 %) compared with the corresponding pair-fed controls, in the severely and moderately Mg-restricted groups respectively, and these reductions, except for those in urinary Pyr and Dpyr, were more marked in the severely Mg-restricted group. Femoral Ca and P concentrations were unaffected by dietary Mg restriction. These results show that not only severe but also moderate dietary restriction of Mg over 21 d results in qualitative changes in bone (i.e. reduced Mg concentration) as well as in aberrant bone turnover in young growing rats (i.e. severely depressed rates of bone formation and bone resorption), which may impair bone development and bone strength.

Magnesium: Biochemical markers: Bone

Mg plays a major role in bone and mineral homeostasis and can also directly affect bone cell function as well as influence hydroxyapatite crystal formation and growth (Cohen, 1988). Mg deficiency has been suggested as a possible risk factor for osteoporosis in man (Institute of Medicine, 1997; Rude, 1998). Several studies have reported significant reductions in serum Mg and bone Mg content in postmenopausal women with osteoporosis (Manicourt *et al.* 1981; Cohen, 1988; Reginster *et al.* 1989; Cohen & Laor, 1990; Stendig-Lindberg *et al.* 1993). Recently, elderly women who consumed less than 187 mg Mg/d were found to have a significantly lower bone mineral density than women whose average dietary Mg intake was more than 187 mg/d (Tucker *et al.* 1995).

While Mg deficiency sufficiently severe to produce clinical symptoms is very rare in human subjects (Héroux *et al.* 1975), there is evidence that many individuals in Western countries have intakes of Mg which are significantly below the recommended levels (Gregory *et al.* 1990; Van Dokkum, 1995; Cleveland *et al.* 1996). This raises the possibility that

a state of mild deficiency might result from suboptimal intakes of Mg with consequences for bone health. However, to date little research emphasis has been placed on this issue.

It is known that several aberrations in bone mineral homeostasis and bone metabolism result as a consequence of severe Mg deficiency in experimental rats. These include reduced bone growth and bone volume (Wallach, 1990; Carpenter *et al.* 1992), and increased skeletal fragility (Lai *et al.* 1975; Kenney *et al.* 1994), together with increased (Jones *et al.* 1980; Kenney *et al.* 1994; Planells *et al.* 1995), or unchanged (Clark & Belanger, 1967) bone Ca content. Furthermore, there have been a number of reports of an association between severe Mg-deficiency and abnormal bone formation (Jones *et al.* 1980; Boskey *et al.* 1992; Carpenter *et al.* 1992). For example, Carpenter *et al.* (1992) showed that feeding a Mg-deficient diet (20 mg/kg diet) for 12 d to young growing rats led to a 38 % reduction in serum osteocalcin, a marker of bone formation, compared with that of Mg-replete animals. Furthermore, Boskey *et al.* (1992) found that the osteocalcin content of the metaphyseal bone

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Abbreviations: Dpyr, deoxypyridinoline; PCR, polymerase chain reaction; Pyr, pyridinoline.
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of severely Mg-deficient animals was significantly less than that of Mg-replete animals.

Reduced urinary excretion of hydroxyproline, a marker of bone resorption, has also been associated with severe Mg deficiency in rats (MacManus & Heaton, 1969; Rayssiguier & Larvor, 1978). For example, MacManus & Heaton (1969) showed that rats fed on a Mg-deficient diet (3 mg/kg diet) had significantly lower rates of hydroxyproline excretion compared with rats fed on a Mg-adequate diet (780 mg/kg), suggesting that severe Mg deficiency affects the rate of bone resorption. There has been no study of the effect of Mg deficiency on the rate of bone resorption, as assessed by the urinary excretion of pyridinium crosslinks which are regarded as more specific markers of bone resorption in rats (Black *et al.* 1989; Egger *et al.* 1994).

However, much of the knowledge concerning the effect of Mg deficiency on bone is based primarily on observations on growing animals fed on diets which were severely restricted in Mg, i.e. containing between 3 and 100 mg Mg/kg diet (MacManus & Heaton, 1969; Lai *et al.* 1975; Jones *et al.* 1980; Boskey *et al.* 1992; Carpenter *et al.* 1992; Kenney *et al.* 1994; Vormann *et al.* 1997). There is very little, if any, information on the effect of moderate Mg depletion on either bone composition or bone metabolism in the rat. Reduced levels of Mg in bone have been reported in rats fed on a moderately restricted intake of Mg (120–200 mg Mg/kg diet) for periods of 70–500 d (Héroux *et al.* 1975; Lerma *et al.* 1993).

Thus, the aim of the present study was to investigate the effects of moderate and severe restriction of dietary Mg on bone composition and on bone metabolism in a rat model.

Materials and methods

Preparation of rat diets

The AIN-76 purified diet (American Institute of Nutrition, 1977) was used in the present study (Table 1).

Experimental design

Forty male rats, 3 weeks old, Wistar strain (average weight 58·8 g), obtained from the Biological Services Unit, University College, Cork, were randomized by weight into five groups of eight rats each. Three groups were fed *ad libitum* on semi-purified diets (AIN-76) containing (per kg) 400 (adequate), 200 (moderately restricted) or 20 mg (severely restricted) Mg for 21 d while two groups were pair-fed the Mg-adequate diet in the same quantities consumed by the groups fed on the moderately and severely Mg-restricted diets respectively. Rats were housed individually in metabolism cages with a grid-floor and a facility for separate collection of faeces and urine. Feed was provided at 17.00 hours each day and all animals were given distilled water *ad libitum* for the duration of the study. Rats were weighed weekly and examined daily for general condition and symptoms associated with Mg deficiency. Urine samples (24 h) were collected for each animal during the last 3 d of the study in vessels covered with aluminium foil to prevent degradation by light of the pyridinium crosslinks. The urine samples for each animal were pooled and the volumes

Table 1. Composition of the modified AIN-76 diet (American Institute of Nutrition, 1977)

Ingredient*	Content (g/kg)
Casein	200·0
DL-Methionine	3·0
Maize starch	150·0
Sucrose	499·34
Fibre	50·0
Maize oil	50·0
AIN mineral mix‡	35·0
AIN vitamin mix§	10·0
Magnesium oxide	0·66†
Choline bitartrate	2·0

* Sources of ingredients: casein (sodium caseinate, Kerrymore Milk Products Ltd, Listowel, Co. Kerry, Ireland); DL-methionine (Rhone Poulenc, Animal Nutrition, Commentry, France); maize starch (Cagill, Bergen op, Zoom, The Netherlands); sucrose (Irish Sugar plc, Sugar Division, Athy Road, Carlow, Ireland); fibre (Avicel microcrystalline cellulose, N.F., FMC International, Food and Pharmaceutical Products Division, Little Island, Cork, Ireland); maize oil (St Bernard's brand, Dunnes Stores Ltd, 67 Stephen Street, Upper Dublin 8, Ireland); choline bitartrate (Brown and Gilmore, Carrigaline East, Co. Cork, Ireland).
† Representing diet containing 400 mg Mg/kg; level of addition was reduced appropriately and replaced with sucrose for the moderately and severely Mg-restricted diets.
‡ Contained (g/kg): potassium dihydrogen phosphate 376, dipotassium hydrogen phosphate 160, sodium chloride 74, manganous carbonate 3·5, ferric citrate 6, zinc carbonate 1·6, cupric carbonate 0·3, potassium iodate 0·01, sodium selenite 0·01, chromium potassium sulfate 0·55, sucrose 378.
§ Contained (/kg): nicotinic acid 3 g, calcium pantothenate 1·6 g, riboflavin 600 mg, thiamin-HCl 600 mg, pyridoxine-HCl 700 mg, pteroylmonoglutamic acid 200 mg, biotin 20 mg, cyanocobalamin 1 mg, cholecalciferol 2·5 mg, menaquinone 5·0 mg, retinyl palmitate 120 mg, DL-α tocopheryl acetate 5000 mg.

recorded. Portions of the pooled urine samples were acidified with 12 M-HCl (225 μl/100 ml urine) and stored at −20° until required for analysis.

After 21 d on the respective diets, all animals were anaesthetized with diethyl ether and blood was drawn from the heart into vacutainer tubes, processed to serum, and immediately stored at −70° until required. Final body weights were recorded and femora, livers and kidneys (from the right side of each animal) were harvested. Kidneys and livers were cleaned of adhering tissue, weighed and stored at −20° until required for mineral analysis. Femora were cleaned of adhering soft tissue, the distal epiphyses removed, and the metaphyses were freed of bone marrow and blood. The left femoral metaphyses were immediately placed in aluminium foil and immersed in liquid N₂ and were subsequently stored at −80° until required for mRNA analysis. The right femoral metaphyses were dried overnight at 110°, weighed and stored in sealed containers until required for mineral analysis.

Experimental techniques

Urinary pyridinoline and deoxypyridinoline. Pooled urine samples for each animal were analysed in duplicate using a three-step procedure. Urine was first hydrolysed with an equal volume of 12 M-HCl at 110° for 18 h, the crosslinks were then extracted by CF1 cellulose chromatography with the use of an internal standard (acetylated pyridinoline, MetraBiosystems Ltd, Wheatley, Oxon., UK) and were measured using a reversed-phase HPLC method with fluorescence detection (Colwell *et al.* 1993). The acetylated

pyridinoline was used in accordance with the method as described by Calabresi *et al.* (1994) and Robins *et al.* (1994). The crosslinks contents of urine samples were quantitated by external standardization using a commercially available pyridinoline (Pyr)–deoxypyridinoline (Dpyr) HPLC calibrator (MetraBiosystems Ltd). The intra-assay CV for Pyr and Dpyr measured as the variation between ten chromatograms obtained between column regenerations as described by Colwell *et al.* (1993) were 6 % and 9 % respectively. The inter-assay CV for Pyr and Dpyr were 7 % and 9 % respectively.

Femoral phosphorus and femoral, liver, kidney and urinary calcium and magnesium levels. Weighed femoral metaphyses (dried), kidneys and portions (about 1 g) from each liver were digested in 10 ml of $16 M-HNO_3–12 M-HClO_4$ (2 : 1, v/v) on a hot plate (S & J Juniper & Co., Harlow, Essex, UK) until the sample colour resembled that of the reagent blank. Ca and Mg were analysed in duplicate in femoral, kidney and liver digests and in urine by atomic absorption spectrophotometry (Pye-Unicam Atomic Absorption Spectrophotometer, Model SP9; Pye Unicam, Cambridge, Cambs., UK) after appropriate dilution with $LaCl_3$ solution (5 g/l, BDH Ltd, Poole, Dorset, UK). A range of Ca and Mg standards was used to obtain Ca and Mg calibration curves. The intra- and inter-assay CV for Ca were 2·8 % and 7·8 %, and for Mg were 3·2 % and 8·8 % respectively. P was determined in the femoral digests by the method of Weissman & Pileggi (1974). The intra- and inter-assay CV for P were 4·2 % and 6·1 % respectively.

Serum calcium and magnesium. Both Ca and Mg were analysed in duplicate in serum samples according to previously described methods (Trudeau & Freier, 1967; Pesce & Kaplan, 1987). The intra- and inter-assay CV for Ca were 3·1 % and 5·6 %, and for Mg were 2·8 % and 4·2 % respectively.

Serum osteocalcin. Serum osteocalcin concentrations were measured in duplicate using a recently developed ELISA (Biomedical Technologies Inc., Stoughton, MA, USA). The intra- and inter-assay CV were 4·0 and 6·2 % respectively.

Reverse transcription polymerase chain reaction analysis for femoral osteocalcin mRNA. RNA was isolated and analysed in the left femoral metaphysis from each rat within a group (*n* 8) as described previously (Fleet & Hock, 1994). Four pooled total RNA samples from each group were prepared by combining portions of RNA from pairs of animals within a group and 1 μg of each pooled total RNA sample was made into cDNA by a reverse transcription reaction as described by Fleet & Hock (1994). The cDNA solution (containing 0·1 μg equivalent RNA) was then amplified by polymerase chain reaction (PCR) for twenty-five cycles for both osteocalcin and glyceraldehyde phosphate dehydrogenase (*EC* 1.2.1.12) as described by Fleet & Hock (1994). A PCR blank consisting of PCR reaction cocktail and water in place of the RNA sample was included during each amplification; under no circumstance did PCR product bands appear in these control samples. PCR products were electrophoretically run on 20 g/l agarose gels containing ethidium bromide. Gels were visualized under u.v. light and photographed. Following electrophoresis, a ratio between the two PCR products

(i.e. osteocalcin : glyceraldehyde phosphate dehydrogenase) within a cDNA sample was then determined by densitometry. The ratios between the two PCR products in the five dietary groups were compared statistically and the mean ratios in the Mg-restricted groups were expressed as a percentage of the ratios obtained in the respective pair-fed control groups (which were arbitrarily set at 100 %).

Statistical methods

Data are presented as means with their standard errors. All data were subjected to one-way ANOVA, with variation attributed to dietary Mg (Snedecor & Cochran, 1967). To follow up the ANOVA, all pairs of means were compared by the method of least significant difference (Snedecor & Cochran, 1967).

Results

Dietary restriction of Mg to 200 and 20 mg/kg led to reduced food intake which was more marked for the group fed on the diet containing 20 mg Mg/kg (Table 2). This resulted in a slower growth rate which was significant by week 2 in the severely restricted group and by week 3 in the moderately restricted group (Fig. 1). However, weight gain and food conversion efficiency of the Mg-restricted groups were similar to those of the corresponding controls which were pair-fed with the Mg-adequate (400 mg/kg) diet (Table 2).

The rats in the severely restricted group had visible skin sores and were more irritable than rats in the corresponding pair-fed group by day 7 of the study. The rats in the moderately restricted group did not present with these visible symptoms of Mg deficiency at any stage of the study.

The influence of dietary Mg concentration on kidney and liver Ca and Mg concentrations is shown in Table 3. Severe, but not moderate, dietary Mg restriction increased Ca concentration and reduced Mg concentration in the kidney. Neither liver Ca nor Mg concentration was affected by the restriction of dietary Mg.

Serum Mg concentration was greatly reduced (compared with the corresponding pair-fed controls) in the group fed on the severely restricted diet (20 mg Mg/kg) but was unaffected in the group fed on the moderately restricted diet (200 mg Mg/kg) (Table 4). Serum Mg level was unaffected by the restricted intake of the Mg-adequate diet in the pair-fed control groups compared with the *ad libitum*-fed Mg-adequate group.

Urinary Pyr and Dpyr and serum osteocalcin levels were significantly reduced (compared with the corresponding pair-fed controls) in both the severely restricted group and the moderately restricted group (Table 4). For serum osteocalcin, but not urinary Pyr or Dpyr, the reduction was more marked in the severely restricted group than in the moderately restricted group. However, these markers were unaffected by the restricted intake of the Mg-adequate diet in the pair-fed control groups compared with the *ad libitum*-fed Mg-adequate group.

Expression of the osteocalcin gene in the femurs of the severely restricted and moderately restricted groups was down-regulated compared with that in the corresponding

Table 2. Effect of moderately and severely restricted dietary magnesium intakes on food intake, weight gain and food conversion efficiency in young male rats*

(Mean values with their standard errors)

Group	Dietary Mg (mg/kg)	n	Food intake (g/21 d)		Weight gain (g/21 d)		Food conversion efficiency†	
			Mean	SE	Mean	SE	Mean	SE
Severely Mg-deficient	20	8	198[c]	4	46.7[c]	5.1	0.23[b]	0.02
Severely Mg-deficient, PF	400	8	204[c]	5	48.9[c]	5.2	0.24[b]	0.02
Moderately Mg-deficient	200	8	269[b]	3	86.2[b]	6.0	0.32[a]	0.02
Moderately Mg-deficient, PF	400	8	269[b]	3	86.6[b]	7.1	0.32[a]	0.02
Adequate Mg	400	8	310[a]	3	105.8[a]	7.6	0.34[a]	0.02
ANOVA (one-way), P value			< 0.001		< 0.001		< 0.001	

PF, pair-fed to the corresponding Mg-deficient group.
[a,b,c] Mean values within a column with different superscript letters were significantly different, $P < 0.05$ (ANOVA followed by least significant difference test).
* For details of diets and procedures, see Table 1 and pp. 64–65.
† Calculated as weight gain (g)/food intake (g) over 21 d.

pair-fed control groups (Fig. 2). This was more marked in the severely restricted group (46 % reduction) than in the moderately restricted group (22 % reduction). Expression of the osteocalcin gene was unaffected by the restricted intake of the Mg-adequate diet in the pair-fed control groups compared with the *ad libitum*-fed Mg-adequate group.

Femoral dry weight was reduced in the severely restricted group, but not in the moderately restricted group, compared with the corresponding pair-fed control groups (Table 5). However, femoral dry weights were unaffected

by the restricted intake of the Mg-adequate diet in the pair-fed control groups compared with the *ad libitum*-fed Mg-adequate group.

Femoral Mg concentration was reduced in both the severely restricted and moderately restricted groups compared with the pair-fed controls and this was more marked for the severely restricted group (Table 5). However, femoral Mg was unaffected by the restricted intake of the Mg-adequate diet in the pair-fed groups compared with the *ad libitum*-fed Mg-adequate group. Neither femoral Ca nor
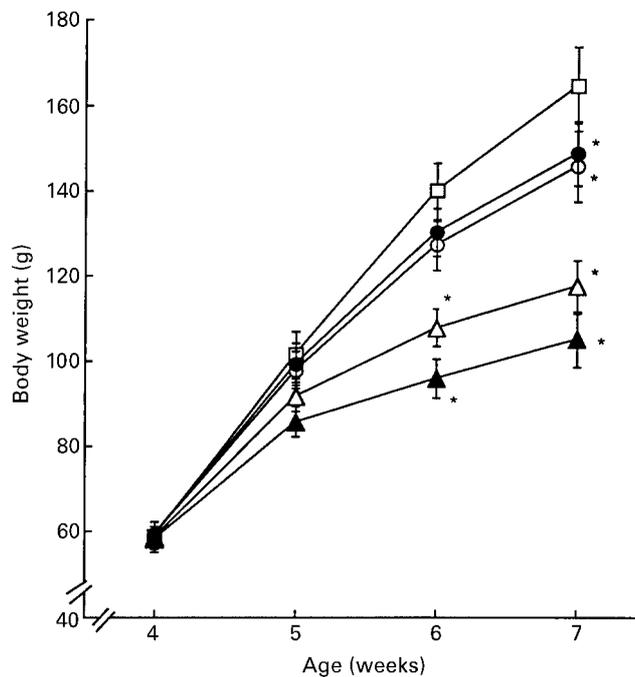

Fig. 1. Body weights over time in rats fed on diets containing different levels of magnesium. Groups were: (□), adequate magnesium diet; (●), moderately magnesium-deficient diet; (○), adequate magnesium diet but pair-fed to the moderately deficient group; (▲), severely magnesium-deficient diet; (△), adequate magnesium diet but pair-fed to the severely-deficient group. Values are means for eight rats, with their standard errors represented by vertical bars. Mean values were significantly different from those of the adequate magnesium group: *$P < 0.05$ (ANOVA followed by least significant difference test). For details of diets, see Table 1.

Table 3. Effect of moderately and severely restricted dietary magnesium intakes on concentrations of calcium and magnesium in kidney and liver in young male rats*

(Mean values with their standard errors)

| | | | Kidney | | | | Liver | | | |
| | | | Ca (μg/g)† | | Mg (μg/g)† | | Ca (μg/g)† | | Mg (μg/g)† | |
Group	Dietary Mg (mg/kg)	n	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Severely Mg-deficient	20	8	482[b]	16	154[b]	1	83·2[a]	3·1	171[a]	5
Severely Mg-deficient, PF	400	8	122[a]	16	166[a]	3	80·0[a]	4·6	175[a]	2
Moderately Mg-deficient	200	8	103[a]	9	167[a]	4	73·2[a]	4·2	160[a]	5
Moderately Mg-deficient, PF	400	8	103[a]	11	171[a]	4	80·8[a]	4·8	166[a]	4
Adequate Mg	400	8	97[a]	6	170[a]	3	88·9[a]	4·9	169[a]	3
ANOVA (one-way), P value			< 0·001		< 0·01		0·20		0·35	

PF, pair-fed to the corresponding Mg-deficient group.
[a,b] Mean values within a column with different superscript letters were significantly different, $P < 0.05$ (ANOVA followed by least significant difference test).
* For details of diets and procedures, see Table 1 and pp. 64–65.
† Expressed on a wet weight basis.

P concentration was affected by the restriction of dietary Mg (Table 5).

Discussion

It is well established that in an experiment in which food is given *ad libitum* the animals on a Mg-deficient diet reduce their food intake and the control animals consume far more. This can cause difficulties in attributing changes in animals entirely to Mg intake, as there will be multiple nutritional deficiencies as a result of reduced food consumption. The present study used a pair-feeding paradigm and therefore the differences and similarities in bone-related variables between animals of the Mg-restricted groups and those of the respective pair-fed control groups are emphasized as valid comparisons attributable to Mg intake alone.

In the present study, moderate dietary restriction of Mg (200 mg/kg diet) reduced femur Mg concentration, but had no effect on the Mg concentration in serum, kidney or liver, suggesting that bone is particularly sensitive to dietary Mg

restriction. This is in agreement with the findings of other studies which have examined the effect of moderate Mg deprivation on femoral Mg concentration in the rat (Héroux *et al.* 1975; Lerma *et al.* 1993). Similarly, severe dietary restriction of Mg (20 mg/kg diet) greatly reduced rat femur Mg concentration in the present study. This is in agreement with other studies which have examined the influence of severe dietary Mg deprivation on femoral Mg concentration (Jones *et al.* 1980; Welsh & Weaver, 1988; Kenney *et al.* 1994; Vormann *et al.* 1997).

Bone has been suggested as being one of the tissues most affected by Mg deficiency as it is one of the major storage sites for Mg in the body (Anast & Gardener, 1981; Wallach, 1990). Mg in bone is found mainly either absorbed on the surface of the apatite crystallites or in the crystal lattice where it replaces Ca (Dallemagne & Fabry, 1956; Alfrey *et al.* 1974). The surface-limited Mg in bone is readily exchangeable and rapidly available during Mg depletion (Neuman & Mulryan, 1971). It has been reported that rat femur containing a normal concentration of Ca but a

Table 4. Effect of moderately and severely restricted dietary magnesium intakes on serum calcium, magnesium and osteocalcin levels and urinary pyridinoline (Pyr) and deoxypyridinoline (Dpyr) concentrations in young male rats*

(Mean values with their standard errors)

| | | | Serum | | | | | | Urine | | | |
| | | | Ca (mg/l) | | Mg (mg/l) | | Osteocalcin (μg/l) | | Pyr (nmol/d) | | Dpyr (nmol/d) | |
Group	Dietary Mg (mg/kg)	n	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Severely Mg-deficient	20	8	124[a]	4	7·3[b]	0·4	46·9[c]	3·7	12·7[b]	1·7	17·7[b]	3·4
Severely Mg-deficient, PF	400	8	108[a]	5	23·9[a]	1·2	87·4[a]	7·4	22·8[a]	2·5	29·4[a]	3·2
Moderately Mg-deficient	200	8	111[a]	6	21·3[a]	1·9	62·9[b]	2·2	15·2[b]	1·8	19·8[b]	3·7
Moderately Mg-deficient, PF	400	8	123[a]	4	23·9[a]	1·4	87·4[a]	6·2	23·0[a]	1·9	29·5[a]	1·9
Adequate Mg	400	8	116[a]	7	25·8[a]	1·4	93·6[a]	7·4	27·8[a]	1·8	37·5[a]	3·7
ANOVA (one-way), P value			0·14		< 0·001		< 0·001		< 0·001		< 0·001	

PF, pair-fed to the corresponding Mg-deficient group.
[a,b,c] Mean values within a column with different superscript letters were significantly different, $P < 0.05$ (ANOVA followed by least significant difference test).
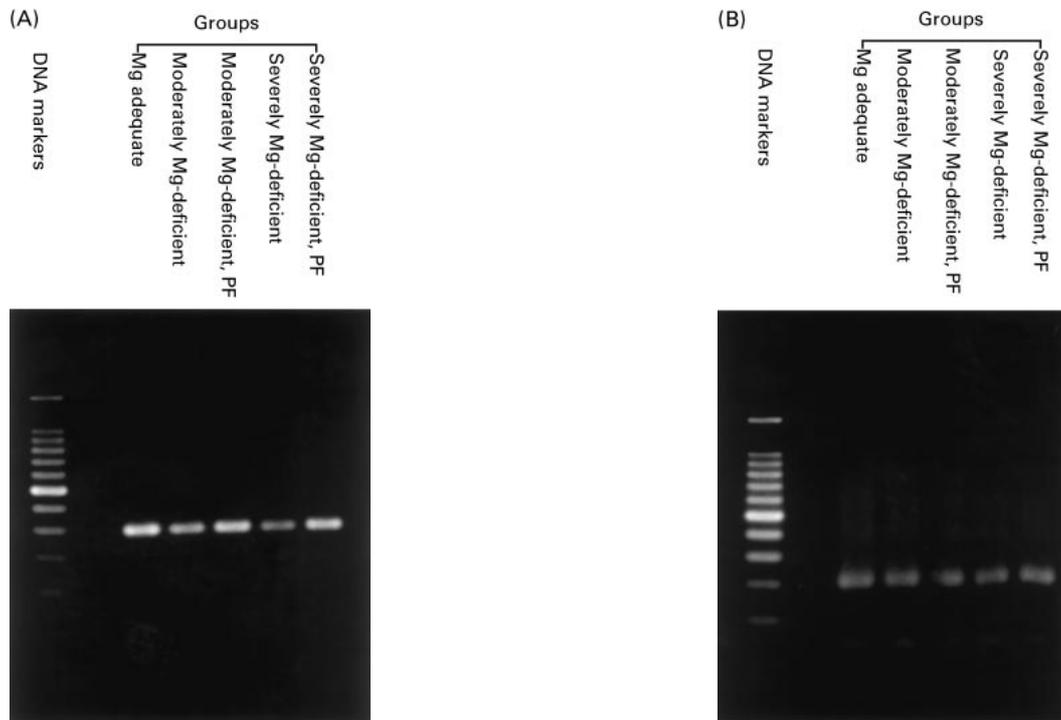* For details of diets and procedures, see Table 1 and pp. 64–65.

Fig. 2. (A) Osteocalcin mRNA and (B) glyceraldehyde phosphate dehydrogenase (GADPH) mRNA levels in the femurs of rats fed on severely magnesium-deficient, moderately magnesium-deficient or magnesium-adequate diets, or pair-fed (PF) with the magnesium-adequate diet to the magnesium-deficient groups. Total RNA was isolated from the femoral metaphyses of rats (*n* 8) in each of the five dietary groups. cDNA was prepared from total pooled RNA and subsequently analysed for osteocalcin and GADPH mRNA by reverse transcription polymerase chain reaction using twenty-five cycles of amplification (for details, see p. 65). DNA size markers: 1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 base pairs from top to bottom. Osteocalcin and GADPH polymerase chain reaction product sizes were 293 and 194 base pairs respectively.

reduced Mg concentration had significantly reduced strength (Kenney *et al.* 1994).

In the present study, moderate Mg restriction (200 mg/kg diet) had no effect on femur dry weight. In contrast, severe Mg restriction (20 mg/kg diet) reduced femur dry weight. This is in agreement with the findings of Kenney *et al.* (1994) which showed that rats receiving 50 mg Mg/kg diet

for 32–42 d had lower femoral dry weights compared with animals receiving adequate Mg intakes (500 mg Mg/kg diet). In the present study, neither moderate nor severe Mg restriction affected the Ca or P content of femur. This is in agreement with the findings of other studies which examined the influence of dietary Mg deprivation on femoral concentration of Ca (Héroux *et al.* 1975; Jones *et al.* 1980;

Table 5. Effect of moderately and severely restricted dietary magnesium intakes on femur dry weight and concentrations of calcium, magnesium and phosphorus in young male rats*

(Mean values with their standard errors)

Group	Dietary Mg (mg/kg)	*n*	Dry wt (mg) Mean	SE	Ca (mg/g dry wt) Mean	SE	Mg (mg/g dry wt) Mean	SE	P (mg/g dry wt) Mean	SE
Severely Mg-deficient	20	8	182[b]	6	213[a]	6	1·72[c]	0·09	74·0[a]	1·6
Severely Mg-deficient, PF	400	8	201[a]	6	219[a]	3	3·57[a]	0·05	79·7[a]	1·6
Moderately Mg-deficient	200	8	202[a]	6	218[a]	3	3·16[b]	0·06	76·5[a]	2·5
Moderately Mg-deficient, PF	400	8	214[a]	8	219[a]	4	3·68[a]	0·05	76·8[a]	1·4
Adequate Mg	400	8	209[a]	4	223[a]	4	3·70[a]	0·05	76·1[a]	1·4
ANOVA (one-way), *P* value			<0·01		0·85		<0·001		0·20	

PF, pair-fed to the corresponding Mg-deficient group.
[a,b,c] Mean values within a column with different superscript letters were significantly different, $P < 0.05$ (ANOVA followed by least significant difference test).
* For details of diets and procedures, see Table 1 and pp. 64–65.

Boskey *et al.* 1992; Kenney *et al.* 1994) and P (Welsh & Weaver, 1988; Kimura & Itokawa, 1989) in rats.

Both moderate and severe dietary restriction of Mg lowered serum levels of osteocalcin, a marker of bone formation. The reduction in serum osteocalcin, however, was much less marked in moderately restricted rats (28 %) than in severely restricted rats (54 %). These findings are in agreement with the findings of Carpenter *et al.* (1992) which showed that severe Mg deficiency (feeding 20 mg Mg/kg diet for 12 d) in young growing rats led to 38 % lower serum osteocalcin levels compared with that of Mg-replete animals (400 mg Mg/kg diet). Furthermore, Carpenter *et al.* (1992) showed that the effect of Mg deprivation on osteocalcin occurred relatively rapidly as significant differences were apparent 2 d after implementation of the Mg-deficient diet and preceded changes in parathyroid hormone, 1,25-dihydroxyvitamin D, or serum Ca, supporting the notion that Mg deprivation has a direct effect on the osteoblast. Boskey *et al.* (1992) found that the osteocalcin content of the metaphyseal bone of severely Mg-deficient animals (fed with 20 mg Mg/kg diet for 17 d) was significantly less than that of Mg-replete animals (fed with 650 mg Mg/kg diet). Alkaline phosphatase (*EC* 3.1.3.1) activity, another biochemical marker of bone formation, has also been reported to be reduced in bone (Heaton, 1965; Elin, 1969; Lai *et al.* 1975) and serum (Synder & Tweedy, 1942; Heaton, 1965; Elin, 1969) in severely Mg-deficient rats. The effect of moderate Mg restriction on serum osteocalcin or on other markers of bone formation in the rat has not been reported elsewhere in the literature.

In the present study there was a reduced expression of osteocalcin mRNA in femora of both moderately and severely Mg-restricted animals compared with the corresponding pair-fed control groups which seemed to be dose-related and were consistent with the serum findings. Reduced expression of osteocalcin mRNA in osteoblasts in rat calvaria has also been demonstrated by Carpenter *et al.* (1992) but only in severe Mg deficiency. Although an effect on the osteocalcin secretory process cannot be excluded, this evidence suggests that the regulation of osteocalcin synthesis is altered at the transcriptional level during Mg deprivation and that this, at least in part, accounts for the changes described in the circulating levels of the protein. The role of osteocalcin in bone is unclear. Until recently, osteocalcin was believed to have a positive role in bone mineralization, as it has a very strong ability to bind hydroxyapatite (Power & Fottrell, 1991); however, recent evidence from osteocalcin gene knockout studies indicates that it may act as a negative regulator of bone formation (Ducy *et al*. 1996). Therefore, it is unclear if the reduced synthesis of osteocalcin resulting from Mg deprivation (both moderate and severe) in the present study adversely affected the mineralization process or whether it merely reflected either reduced osteoblast numbers or reduced osteoblastic activity or both, as suggested by Robins & New (1997). Reduced osteoblast numbers and reduced osteoblastic activity have been suggested to have a role, at least in part, in the decreased rate of bone formation observed in Mg-deficient rats (Jones *et al.* 1980; Carpenter *et al*. 1992).

Both moderate and severe dietary restriction of Mg reduced the rate of bone resorption in the present study, as measured by the urinary excretion of pyridinium crosslinks which are regarded as specific markers of bone resorption in rats (Black *et al.* 1989; Egger *et al.* 1994). This is in agreement with the findings of earlier studies which reported reduced excretion of urinary hydroxyproline in severe Mg deficiency in young rats (MacManus & Heaton, 1969; Rayssiguier & Larvor, 1978). In addition, the release of ^{45}Ca from labelled rat bone, both *in vitro* and *in vivo* (an index of bone resorption), has also been shown to be reduced by severe Mg deficiency (MacManus & Heaton, 1969; Larvor & Labat, 1978). The effect of moderate Mg restriction on the rate of bone resorption in the rat has not been reported elsewhere in the literature. It was notable that in the present study moderate restriction of Mg intake reduced the excretion of the pyridinium crosslinks to the same degree as did severe Mg restriction.

The mechanism by which Mg deficiency caused a reduction in the rate of bone resorption remains unclear. One possible explanation is that it could be due to a reduction in serum parathyroid hormone levels, common in Mg-deficient rats (Rayssiguier *et al.* 1982; Anast & Forte, 1983). An alternative explanation may be a reduced sensitivity to parathyroid hormone of Mg-deficient bone, as suggested by some researchers (MacManus *et al.* 1971; Rayssiguier & Larvor, 1978; Jones *et al.* 1980). However, Kenney *et al.* (1994) have argued against this since it has been shown that infusion of parathyroid hormone into parathyroidectomized, Mg-deficient rats brings about expected responses of bone (Hahn *et al.* 1972).

In addition to its effects on bone composition and bone metabolism in the present study, dietary Mg restriction produced several other characteristic signs of Mg depletion. For example, animals fed on the severely Mg-restricted diet (20 mg/kg diet) showed signs that are characteristic of gross Mg deficiency, including skin sores and irritability, and reduced food intake with consequent reduced weight gain (Walser, 1967; Jones *et al.* 1980; Lerma *et al.* 1993; Kimura *et al.* 1996). Furthermore, Mg concentration was reduced in serum and kidney as well as in femur, indicative of body Mg depletion. These findings are in agreement with those of many other studies on severe dietary restriction of Mg (Lai *et al*. 1975; Jones *et al.* 1980; Boskey *et al.* 1992; Kenney *et al.* 1994; Kimura *et al.* 1996; Vormann *et al.* 1997). In addition, there was an increased concentration of Ca in kidneys, a well-established sign of severe Mg deficiency which is believed to be due to the formation of calcium phosphate and calcium oxalate in renal tubules (Bunce & King, 1978; Koh *et al.* 1989). Moderate dietary restriction of Mg (200 mg/kg diet) also resulted in signs of Mg deficiency, i.e. reduced food intake and weight gain, although these were much less marked than for the severely restricted group.

In conclusion, the findings of the present study suggest that not only severe but also moderate dietary restriction of Mg over 21 d resulted in qualitative changes in bone (i.e. reduced Mg concentrations) as well as in aberrant bone turnover in young growing rats (i.e. severely depressed rates of bone formation and bone resorption). Aberrant bone turnover could impair bone development and reduced Mg concentration in bone may have implications for bone

strength and increased risk of bone fragility (Kenney *et al.* 1994). These effects of moderate Mg restriction in rats may be of relevance to human populations in Western countries where it has been shown that many individuals have intakes of Mg which are significantly below the recommended levels (Gregory *et al.* 1990; Van Dokkum, 1995; Cleveland *et al.* 1996). Therefore, the evidence given here together with data from epidemiological studies which suggest a link between low Mg intake and osteoporosis (see reviews by Sojka & Weaver, 1995; Institute of Medicine, 1997; Rude, 1998) indicate a need for investigation of the role of Mg in bone metabolism in man.

Acknowledgement

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