On the origin of the increased tissue iron content in graded magnesium deficiency states in the rat

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To investigate the mechanism of tissue Fe accumulation in graded Mg deficiency rats were fed on diets of different Mg contents (70, 110, 208, 330, and 850 mg Mg/kg) for 10, 20, and 30 d during rapid growth. There was no significant impact of Mg deficiency or high luminal Mg concentrations on intestinal ^{59}Fe transfer *in vitro* or *in vivo*. Plasma Mg concentrations and body weight started to decrease after 10 d. Significant haemolytic anaemia was observed after 20 d with siderosis in liver and spleen developing in parallel. Anaemia showed no features of Fe deficiency or inflammation. Comparison between the 70 mg Mg/kg group and animals that received the same quantity of a Mg-adequate diet (850 mg Mg/kg) permitted estimation of quantities of Fe liberated by haemolysis and the increased Fe content in liver and spleen. Both variables showed a high degree of correlation, indicating that the excess of liberated haemoglobin Fe was stored in the tissue. The erythropoietic activity was high during rapid growth, i.e. at days 10 and 20 and decreased significantly after 30 d in all except the most Mg-deficient groups. However, haemolytic anaemia developed because even the high erythropoietic activity in the 70 and 110 mg Mg/kg groups was not sufficient to recycle all haemoglobin Fe liberated by haemolysis. After 30 d of Mg-deficient feeding the erythrocyte Mg content had decreased to 40% of control values. According to the literature Mg-deficient erythrocytes have a decreased survival time which is likely to be the cause of the observed haemolysis.

Magnesium: Haemolysis: Iron: Haemosiderosis

Increased stress susceptibility, histamine reactions, growth reduction and the pathophysiological consequences of a disturbed Ca metabolism are among the sequels of severe Mg deficiency in rats (Günther, 1981). On the other hand, pharmacological Mg doses are applied for suppression of uterine contractility, treatment of pre-eclampsia and certain types of cardiac arrhythmia (Classen *et al.* 1994). The use in the treatment of myocardial infarction is under discussion (Woods & Fletcher, 1994). The cellular Mg content is tightly regulated. With the exception of erythrocytes it changes only slightly even when the extracellular Mg concentration is drastically decreased (Vormann *et al.* 1995). Thus, intracellular effects of Mg deficiency must be mediated indirectly, e.g. by modulation of the cellular Ca content or of catecholamine effects (Günther, 1981). An intracellular mediator is also needed to explain increased hepatic lipid peroxidation in severely Mg-deficient rats. This phenomenon is accompanied by hepatic siderosis and a decreased hepatic vitamin E

content (Günther *et al.* 1992). In rats in different states of Mg deficiency the extent of malondialdehyde production was positively correlated to the Fe content in a variety of tissues (Vormann *et al.* 1995).

As Fe is known to catalyse lipid peroxidation by Fenton chemistry (Crichton, 1991) these findings argue that the stimulation of lipid peroxidation in Mg deficiency is the consequence of increased tissue Fe accumulation. Therefore, the present study focused on the mechanism by which the intracellular Fe content may be modulated in Mg deficiency. It investigated whether there is increased intestinal Fe absorption or an inhibitory effect of high luminal Mg concentrations on Fe absorption which, consequently, would be reduced in Mg deficiency. In addition, the time course and extent of haemolysis and increased erythropoiesis were compared with the time course and extent of tissue Fe accumulation at different degrees of Mg deficiency in order to investigate whether there is a shift of haem Fe into the tissue. Histology was used to determine the location of accumulated Fe in the tissue, the extent of medullar and extramedullar erythropoiesis and the extent of morphological damage. Some of the data were first presented at the spring meeting of the Deutsche Gesellschaft für experimentelle und klinische Pharmakologie und Toxikologie in March 1994.

MATERIALS AND METHODS

Animals and experimental design

The experiments were performed according to the rules of animal care and were approved by the local animal protection committee (Tierschutzkommission der Regierung von Oberbayern: AZ 211 – 2531 – 25/93).

Expt 1. Ninety-six conventionally bred male Wistar rats (Interfauna, Tuttlingen, Germany) were housed in stainless-steel cages, five animals per cage unless stated otherwise (12 h light–12 h dark, 22°, 60 % humidity). The animals were randomly divided into six groups of fifteen animals each. In five groups the rats received, *ad libitum*, diets with different Mg content. In the sixth group the animals were fed on a Mg-adequate diet (850 mg Mg/kg) and were allocated the average amount of food that had been consumed by the most Mg-deficient group (70 mg Mg/kg) on the day before (parallel feeding). The animals in the parallel-feeding group were housed individually to avoid competition for the restricted food allocation. Food consumption was measured daily. Spillage was accounted for by feeding the animals over a big steel plate from which the spilled material was collected and quantified. Food consumption was significantly reduced in the 70 mg Mg/kg group after 20 and 30 d compared with 850 mg Mg/kg *ad libitum* feeding. The reduction amounted to 20–25 % of *ad libitum* intake (Table 1). The food was allocated to the restrictively-fed animals once daily in the mornings. The animals consumed only part of their allocation immediately which showed that they were not in a situation of severe starvation. The animals in the 70 mg Mg/kg group were numbered individually in order to relate their results to those of the corresponding animal in the 850 mg Mg/kg parallel-feeding group with the matching weight at the beginning of the experiment.

Body weights on the day of arrival and at the beginning of the feeding experiment are given in Table 2. There were no significant differences between the animals allocated to the different feeding groups at either of these points in time. Diets for the experiments were prepared by adding $MgCl_2$ to a basal Mg-deficient diet (Ssniff, Soest, Germany; for composition see Table 3) leading to Mg contents of 70, 110, 208, 330 and 850 mg Mg/kg.

Table 1. *Food consumption (g/d per animal) of rats receiving diets containing 70 or 850 mg magnesium/kg during growth*

(Mean values and standard deviations)

Diet...		70 mg Mg/kg		850 mg Mg/kg *ad libitum*	
	n	Mean	SD	Mean	SD
Acclimatization period (7 d)	15	23·1	2·6	22·8	2·0
Days 0–10	5	23·4	2·7	25·3	1·9
Days 10–20	5	20·8	1·7	26·9*	1·3
Days 20–30	5	21·0	2·0	26·1*	1·4

*Mean values were significantly different from those for the 70 mg/kg group, $P < 0{\cdot}05$ (Student's *t* test).

The Mg content of the diets was assessed by atomic absorption spectrophotometry in ashed portions. The Fe content in all diets was 180 mg/kg. During an acclimatization period of 7 d after arrival the animals were fed on the control diet (850 mg Mg/kg). All animals had free access to distilled water. After 10, 20, and 30 d five rats of each feeding group were killed. Six additional rats (three animals/cage) served to provide initial day 0 values for the variables measured. All animals were fasted overnight before slaughter. All animals including the six rats on day 0 were killed by bleeding, under ether anaesthesia.

In all animals of Expt 1 the number of erythrocytes, haemoglobin concentration, packed cell volume, mean corpuscular volume, mean corpuscular haemoglobin, erythrocyte distribution width and the number of reticulocytes, the plasma concentrations of lactate dehydrogenase (*EC* 1.1.1.2; LDH), bilirubin, transferrin, Fe and Mg as well as the Fe content in liver and spleen were determined, as well as the erythrocyte Mg content and the plasma concentrations of urea and creatinine after 30 d. Liver, spleen, kidney and bone marrow were examined histologically after 30 d.

Expt 2. Intestinal Fe absorption was determined in thirteen severely Mg-deficient male Wistar rats (Mg-deficient diet, Ssniff; 70 mg Mg/kg) and in twenty-three Mg-adequate controls (Ssniff; 850 mg Mg/kg). Except for the Mg content both diets had the same nutrient and mineral composition (Table 3). The animals were housed in stainless steel cages as described in Expt 1 and received food and water *ad libitum*. After 30 d the body weights of the animals on the 70 mg Mg/kg diet had increased from 193 (SD 8) g to 346 (SD 18) g; corresponding values for the 850 mg Mg/kg group were 190 (SD 12) g and 462 (SD 26) g. Proximal small intestines were removed under ether anaesthesia to determine the ^{59}Fe-transfer *in vitro*.

Determination of duodenal ^{59}Fe transfer in vitro

One segment of proximal small intestine (approximately 100 mm length, beginning 10 mm distal of the pylorus) was removed from each animal under ether anaesthesia and perfused *in vitro* according to the method of Fisher & Parsons (1949). The method, as modified by ourselves, has been used repeatedly to determine intestinal ^{59}Fe transfer and has been extensively described elsewhere (Schümann *et al.* 1986, 1990*a*,*b*). Conditions of *in vitro* incubation were: perfusion with bicarbonate-buffered Tyrode solution; 37°, pH 7·2; hydrostatic perfusion pressure: 25 cm H_2O; flow rate: 50 ml/min, incubation time: 2 h. During perfusion the active transfer of glucose against a concentration gradient of $> 1:2$

Table 2. *Body weights (g) of rats consuming diets of different magnesium contents during growth*

(Mean values and standard deviations)

Dietary Mg content...		70 mg Mg/kg		110 mg Mg/kg		208 mg Mg/kg		330 mg Mg/kg		850 mg Mg/kg		850 mg Mg/kg (parallel feeding)	
	n	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
On arrival	15	143[a]	8	142[a]	7	144[a]	9	145[a]	7	146[a]	7	142[a]	3
Start of feeding	15	190[a]	8	195[a]	8	194[a]	13	197[a]	10	192[a]	12	191[a]	6
10 d	5	261[a]	11	281[b]	14	284[b]	11	275[ab]	13	282[b]	23	269[ab]	6
20 d	5	323[a]	16	349[ab]	11	366[ab]	34	386[b]	27	386[b]	22	353[ab]	9
30 d	5	343[a]	19	390[ab]	18	415[bc]	25	453[cd]	27	458[c]	37	380[ab]	18

[a,b,c,d]Mean values within a row not sharing a common superscript letter were significantly different, $P < 0{\cdot}05$ (one-way ANOVA).

Table 3. *Composition of the basal magnesium-deficient diet**

	g/kg
Protein	170·0
Fat	53·0
Carbohydrate	606·9
Fibre	29·3
Ash	56·6
Water	99·8

*The diet also contained the following; vitamins (mg/kg): retinyl acetate 5·16, cholecalciferol, 0·013, α-tocopherol 164·0, menadione 10·0, thiamin 20·0, riboflavin 20·3, pyridoxine 15·0, cyanocobalamin 0·04, nicotinic acid 50·0, pantothenic acid 50·0, pteroylmonoglutamic acid 10·0, biotin 0·2, choline 1010, para-aminobenzoic acid 100·0, inositol 111·0, ascorbic acid 20·0; minerals (g/kg): Ca 9·50, P 7·50, Mg 0·07, Na 2·50, K 7·00, S 2·81, Cl 3·66; trace elements (mg/kg): Fe 182·1, Mn 100·8, Zn 30·4, Cu 12·2, I 0·45, Mo 0·20, F 4·19, Se 0·31.

was continuously monitored and served as vitality criterion. The cumulative water transfer was determined gravimetrically and was close to linear during the entire experimental period. Transfer of glucose and water showed no significant differences between Mg-deficient and control segments.

Fe was labelled with $^{59}FeCl_3$ (7·4 kBq/ml) and offered at luminal concentrations of 10 and 100 μmol Fe/l. It was chelated with a twofold molar excess of nitrilotriacetic acid to prevent formation of iron hydroxide. ^{59}Fe radioactivity was determined in a gamma-counter (Contron GAMMAmatic, Contron, Eching, Germany). Intestinal Fe transfer rates were calculated from the ratio between the ^{59}Fe activity in the absorbate and in the perfusate. After an initial lag phase the cumulative ^{59}Fe transfer was linear in all experimental groups. The length of the segments was measured while segments were suspended under a uniform tension of a 6 g weight. To test the impact of excess Mg on intestinal ^{59}Fe transfer 10 mmol Mg/l (as $MgCl_2$) was added to the perfusate at luminal Fe concentrations of 10 μmol/l and 100 μmol/l. The perfusate in control experiments contained 50 μmol Mg/l.

Determination of intestinal ^{59}Fe-transfer in vivo

Proximal jejunal segments from Mg-deficient rats (diet: 70 mg Mg/kg, 30 d) as well as from three Mg-adequate animals (diet: 850 mg/kg, 30 d) were used to cross-check the *in vitro* ^{59}Fe transfer data. For this purpose the animals were anaesthetized (Ketanest: 0·5 mg/kg) and the ligated segment (approximately 100 mm length, starting at the flexura duodenal-jejunalis) was filled with $^{59}FeSO_4$ solution (0·5 ml, 20 mmol/l ascorbate, 5 mmol glucose/l in physiological saline, pH 2, 1 mmol Fe/l, 0·185 MBq ^{59}Fe/ml). The ^{59}Fe radioactivity in the animal was determined in triplicate in a whole-body counter for small animals (ARMAC Model 446, Packard Instruments, Palo Alto, CA, USA). After 30 min the ligated segments were removed from the animal and the ^{59}Fe radioactivity was determined in the carcass. The retained ^{59}Fe activity was related to the length of the ligated segments and to the duration of the experiment. Results were expressed as transfer rates (pmol/cm per min), assuming the same specific activities in the carcass and in the luminal solution.

Determination of haematological and plasma variables; tissue metal content

Heparinized blood was obtained by puncturing the abdominal aorta. Some of the blood was centrifuged (4°, 500 *g*). Plasma was frozen at − 20° to determine Mg, Ca, Zn, and Fe

concentrations by atomic absorption spectrometry (Philips SP9, Philips, Kassel, Germany). Packed cell volume, haemoglobin concentration and the number of erythrocytes were determined by use of a Coulter counter. The plasma concentration of LDH was determined photometrically by use of the pyruvate method. Total bilirubin was determined after reaction with 2,5 dichlorophenyl diazonium (DPA method). This compound binds quantitatively to bilirubin to give azobilirubin which was determined photometrically. Urea and creatinine were quantified by use of the urease (*EC* 3.5.1.5) and pikrat (Jaffe) method according to the manual of the manufacturer (Boehringer, Mannheim, Germany: BM/Hitachi 717 System; wavelength: bilirubin 660–570 nm; LDH 405–340 nm; urease 405–340 nm; creatinine 570–505 nm). Reticulocytes were counted in blood smears stained with Brilliant Cresyl Blue.

Liver, kidney, spleen, and the femur were removed. The wet weight of liver, spleen, and kidney after 30 d did not differ significantly between the 70 mg Mg/kg group and the 850 mg Mg/kg *ad libitum* group ($P > 0{\cdot}05$, Student's t test). The tissues were washed in 250 mM-sucrose, freeze-dried and dry weight was determined. The powdered tissues were ashed in a plasma processor (Model 200 E, Technics, München, Germany), dissolved in 0·1 M-HCl and the tissue Fe content was determined in liver and spleen, after appropriate dilution, by use of atomic absorption spectrometry (Philips SP9, Philips). All reagents used were of analytical grade and purchased from Merck, Darmstadt, Germany and Sigma, München, Germany; ^{59}Fe was supplied by NEN Dreieich, Germany.

Histological examination

For light-microscopic evaluation of liver, spleen and kidney small specimens from all animals after a 30 d feeding period were fixed in a 40 ml/l neutral buffered formalin, while bone marrow specimens were fixed and carefully decalcified according to the method of Schaefer (1995). Paraffin sections (4 μm) were stained with haemotoxylin and eosin, periodic acid–Schiff (PAS) reagent and trichrome. Perls' blue reaction was used for histochemical determination of Fe storage. Calcification in the kidneys was demonstrated by Kossa's stain. Fe storage in the macrophages from the spleen and in Kupffer cells from the liver was semiquantitatively evaluated, as well as erythropoiesis in the bone marrow and extramedullary erythropoiesis in the spleen. These variables were ranked on a scale from $\emptyset$ to $+++$ according to the grading system proposed by Searle *et al.* (1994). This system is based on the ease of observation and the magnification required for light-microscopical examination.

Statistical treatment of data

Significant differences between the mean values of the different groups were analysed by one-way ANOVA (5 % level). The homogeneity of variances was checked for each ANOVA by means of Hartley's test. When significant differences were found, the Scheffé test was used (5 % level) to test which means were significantly different from each other and which formed a homogeneous group. Differences between the transfer variables, in food consumption, and organ weight as well as between urea and creatinine concentrations in the most Mg-deficient and Mg-adequate groups were assessed by means of Student's t test ($P < 0{\cdot}05$).

RESULTS

Graded magnesium deficiency

The plasma Mg concentration was significantly reduced in all groups with less than 850 mg Mg/kg in the diet after 10, 20 and 30 d (see Table 7). After 10 and 20 d body weight increases were significantly lower in the 70 mg Mg/kg group compared with the 850 mg Mg/kg group. After 30 d body weight was significantly decreased in all groups with less than 208 mg Mg/kg. At this point in time the body weight in the 70 mg Mg/kg group was also significantly lower than that of the animals fed on a Mg-adequate diet in parallel, indicating that weight reduction in Mg-deficiency was not exclusively due to a reduced food intake (Table 2). Both variables indicated that the chosen feeding scheme led to different degrees of Mg-deficiency as intended.

Intestinal ^{59}Fe transfer (Expt 2)

There were no significant differences between the ^{59}Fe transfer rates *in vitro* in severely Mg-deficient and Mg-adequate duodenal segments, either at a luminal ^{59}Fe concentration of 10 or of 100 μmol/l (Table 4). Addition of $MgCl_2$ (10 mmol/l) to the luminal perfusion medium had no impact on ^{59}Fe transfer rates. The jejunal ^{59}Fe transfer data *in vivo* support these findings (Table 4).

Haematological findings, tissue iron accumulation and clinical chemistry (Expt 1)

Significant reductions in erythrocytes, haemoglobin concentration, and packed cell volume were observed no earlier than after 30 d (Table 5) when the haematological variables indicative for anaemia were reduced by approximately 10–20 % in the 70 mg Mg/kg group compared with those animals with the same intake of Mg-adequate food (850 mg Mg/kg). Anaemia in Mg deficiency was neither microcytic nor hypochromic (Table 5). Erythrocyte distribution width was significantly increased in the 70 mg Mg/kg group after 30 d. Plasma transferrin concentrations were not significantly reduced in severe Mg deficiency (plasma transferrin concentrations after 30 d: 70 mg Mg/kg 2800 (SD 300) mg/l; 850 mg Mg/kg *ad libitum* 3000 (SD 300) mg/l; other results not shown). Plasma Fe was significantly increased in the 70 mg Mg/kg group after 20 and 30 d. Significant decreases in plasma Fe were seen in the 850 mg Mg/kg group with restricted

Table 4. *Rates of transfer of ^{59}Fe (pmol/cm per min) across intestinal segments from rats fed on magnesium-deficient and magnesium-adequate diets, measured* in vitro *and* in vivo*

(Mean values and standard deviations)

Luminal ^{59}Fe concentration	*n*	Mg-deficient diet		Mg-adequate diet		Mg-adequate diet + 10 mmol Mg/l in perfusate	
		Mean	SD	Mean	SD	Mean	SD
In vitro transfer							
10 μmol/l	5	5·0	2·5	5·4	2·9	6·5	2·8
100 μmol/l	5	37·5	12·5	38·8	17·0	42·1	11·9
In vivo transfer							
1 mmol/l	3	118	17	121	61		

*For details of procedures, see pp. 477–479.

Table 5. *Haematological variables in rats fed on diets containing different levels of magnesium*

(Mean values and standard deviations for five rats per dietary group)

	Dietary magnesium content (mg/kg)													
	70		110		208		330		850		850 (parallel feeding)		Initial values at day 0	
Feeding period	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Erythrocytes (10^{12}/l)														
10 d	$6{\cdot}20^{a,x}$	0·60	$6{\cdot}16^{a,x}$	0·40	$6{\cdot}49^{a,x}$	0·25	$6{\cdot}49^{a,x}$	0·25	$6{\cdot}41^{a,x}$	0·22	$6{\cdot}36^{a,x}$	0·23	$5{\cdot}71^{a}$	0·28
20 d	$6{\cdot}70^{a,x}$	0·44	$6{\cdot}94^{a,y}$	0·20	$7{\cdot}16^{a,y}$	0·27	$7{\cdot}26^{a,y}$	0·28	$7{\cdot}26^{a,y}$	0·27	$6{\cdot}95^{a,y}$	0·32		
30 d	$6{\cdot}32^{a,x}$	0·58	$7{\cdot}02^{ab,y}$	0·46	$7{\cdot}70^{b,z}$	0·36	$7{\cdot}50^{b,y}$	0·12	$7{\cdot}58^{b,z}$	0·16	$7{\cdot}78^{b,z}$	0·13		
Haemoglobin concentration (g/l)														
10 d	$128^{a,x}$	11	$125^{a,x}$	9	$129^{a,x}$	5	$131^{a,x}$	1	$130^{a,x}$	4	$130^{a,x}$	3	120^{a}	2
20 d	$135^{a,x}$	8	$141^{a,y}$	1	$141^{a,y}$	4	$142^{a,y}$	3	$141^{a,y}$	4	$138^{a,y}$	3		
30 d	$132^{a,x}$	4	$139^{ab,y}$	0·8	$142^{ab,y}$	3	$142^{b,y}$	2	$145^{b,y}$	3	$144^{b,z}$	3		
Packed cell volume (l/l)														
10 d	$0{\cdot}37^{ab,x}$	0·03	$0{\cdot}36^{ab,x}$	0·02	$0{\cdot}39^{ab,x}$	0·01	$0{\cdot}40^{b,x}$	0·00	$0{\cdot}38^{ab,x}$	0·01	$0{\cdot}38^{ab,x}$	0·01	$0{\cdot}35^{a}$	0·01
20 d	$0{\cdot}39^{a,x}$	0·02	$0{\cdot}41^{a,y}$	0·01	$0{\cdot}41^{a,xy}$	0·02	$0{\cdot}41^{a,x}$	0·01	$0{\cdot}40^{a,xy}$	0·01	$0{\cdot}39^{a,x}$	0·01		
30 d	$0{\cdot}38^{a,x}$	0·01	$0{\cdot}40^{ab,xy}$	0·03	$0{\cdot}42^{abc,y}$	0·02	$0{\cdot}41^{abc,x}$	0·01	$0{\cdot}42^{bc,y}$	0·01	$0{\cdot}44^{c,y}$	0·01		
Mean corpuscular volume (fl)														
10 d	$60{\cdot}5^{a,x}$	2·5	$58{\cdot}9^{a,x}$	0·9	$59{\cdot}6^{a,y}$	0·9	$60{\cdot}1^{a,y}$	2·6	$59{\cdot}8^{a,y}$	1·6	$59{\cdot}3^{a,y}$	0·9	$62{\cdot}1^{a}$	1·2
20 d	$57{\cdot}9^{a,x}$	1·9	$58{\cdot}5^{a,x}$	2·0	$57{\cdot}2^{a,y}$	2·0	$57{\cdot}0^{a,xy}$	0·9	$55{\cdot}4^{a,x}$	1·8	$56{\cdot}7^{a,x}$	1·4		
30 d	$61{\cdot}0^{b,x}$	5·3	$56{\cdot}7^{ab,x}$	1·2	$53{\cdot}8^{a,x}$	0·8	$54{\cdot}9^{a,x}$	1·3	$56{\cdot}2^{ab,x}$	1·1	$56{\cdot}2^{ab,x}$	1·8		
Mean corpuscular haemoglobin (pg)														
10 d	$20{\cdot}6^{ab,x}$	0·4	$20{\cdot}2^{ab,x}$	0·4	$19{\cdot}9^{ab,y}$	0·5	$19{\cdot}6^{a,y}$	0·5	$20{\cdot}2^{ab,x}$	0·5	$20{\cdot}4^{ab,y}$	0·4	$21{\cdot}0^{b}$	0·8
20 d	$20{\cdot}1^{a,x}$	0·6	$20{\cdot}4^{a,x}$	0·5	$19{\cdot}7^{a,y}$	0·4	$19{\cdot}6^{a,y}$	0·4	$19{\cdot}4^{a,x}$	0·7	$19{\cdot}9^{a,y}$	0·5		
30 d	$21{\cdot}0^{b,x}$	1·4	$19{\cdot}8^{ab,x}$	0·3	$18{\cdot}5^{a,x}$	0·5	$18{\cdot}9^{a,x}$	0·3	$19{\cdot}2^{a,x}$	0·4	$18{\cdot}5^{a,x}$	0·7		
Erythrocyte distribution width (%)														
10 d	$14{\cdot}2^{a,x}$	1·1	$13{\cdot}8^{a,x}$	0·5	$14{\cdot}0^{a,x}$	1·2	$13{\cdot}6^{a,x}$	0·9	$14{\cdot}0^{a,x}$	0·3	$14{\cdot}1^{a,x}$	0·7	$14{\cdot}6^{a}$	0·8
20 d	$13{\cdot}9^{a,x}$	0·9	$14{\cdot}0^{a,x}$	0·6	$13{\cdot}3^{a,x}$	0·4	$13{\cdot}0^{a,x}$	0·4	$13{\cdot}9^{a,x}$	1·0	$13{\cdot}9^{a,x}$	1·2		
30 d	$15{\cdot}2^{b,x}$	1·0	$14{\cdot}2^{ab,x}$	0·9	$14{\cdot}4^{ab,x}$	0·5	$13{\cdot}4^{ab,x}$	0·4	$13{\cdot}5^{ab,x}$	0·8	$13{\cdot}2^{a,x}$	1·1		
Reticulocytes (‰)														
10 d	$77^{a,x}$	26	$78^{a,x}$	21	$100^{a,y}$	38	$100^{a,z}$	18	$117^{a,z}$	16	$86^{a,y}$	23	70^{a}	17
20 d	$67^{a,x}$	27	$68^{a,x}$	9	$77^{a,y}$	12	$82^{a,y}$	9	$94^{a,y}$	28	$76^{a,y}$	17		
30 d	$87^{c,x}$	16	$60^{b,x}$	13	$18^{a,x}$	5	$30^{a,x}$	8	$36^{ab,x}$	11	$29^{a,x}$	11		

[a,b,c]Mean values within a row not sharing a common superscript letter were significantly different, $P < 0{\cdot}05$ (one-way ANOVA).

[x,y,z]Mean values within a column not sharing a common superscript letter were significantly different, $P < 0{\cdot}05$ (one-way ANOVA).

*For details of diets and procedures, see Table 3 and pp. 476–480.

Table 6. *Magnesium content of the erythrocytes of rats fed on diets containing different levels of magnesium for 30 d*

(Mean values and standard deviations for five rats per dietary group)

Dietary magnesium content (mg/kg)	Erythrocyte magnesium (μmol/g dry wt)	
	Mean	SD
70	2·7[a]	0·3
110	3·1[a]	0·9
208	5·8[b]	0·5
330	6·6[bc]	0·5
850	7·1[c]	0·4
850 (parallel feeding)	7·0[c]	0·3

[a,b,c]Mean values not sharing a common superscript letter were significantly different, $P < 0·05$ (ANOVA).

feeding after 10 d which were probably caused by insufficient Fe supply during fast growth due to reduced food intake (see Table 7). The Mg content was determined in erythrocytes after 30 d. It was significantly reduced in groups with less than 330 mg Mg/kg in the diet. In the 70 mg Mg/kg group the values were reduced to about 40% of those found in the 850 mg Mg/kg group that was fed in parallel (Table 6).

Total plasma bilirubin and plasma LDH concentrations indicated a significantly increased degree of haemolysis in the 70 mg Mg/kg group after 20 and 30 d (Table 5). After 30 d the number of reticulocytes increased significantly in severe Mg deficiency (Table 7). Significant increases in the hepatic and splenic tissue Fe content in the 70 mg Mg/kg group were observed no earlier than after 20 and 30 d respectively, i.e. they appeared in parallel to haemolysis (Table 7).

Urea and creatinine showed no significant differences between the 70 mg Mg/kg group and the 850 mg Mg/kg group after 30 d (urea: 294 (SD 58) *v.* 244 (SD 63) mg/l; creatinine: 5·4 (SD 3) *v.* 5·0 (SD 3) mg/l; *n* 5) (Student's *t* test; $P > 0·05$).

Correlation between released haemoglobin iron and increased tissue iron content (Expt 1)

The released haemoglobin Fe quantities and the increases of the tissues Fe content in spleen and liver in the 70 mg Mg/kg group were estimated by comparison with the 850 mg Mg/kg controls that were fed in parallel to the food consumption in the most Mg-deficient group. The body weights of corresponding animals in each group at the beginning of the experiment were similar (Table 2). The animals in both groups ate the same quantity of food with the same Fe content. Intestinal Fe absorption was not significantly different between the two groups (Table 4) and Fe losses in rats are negligible (Kreuzer & Kirchgessner, 1991). Therefore, for the comparisons made in this paragraph corresponding animals in the two groups were assumed to have the same total body Fe content. Haemoglobin-bound Fe was calculated from the individual haemoglobin concentration, assuming a blood volume of 6·7% of the body weight (Whittaker *et al.* 1984) and an Fe content of 3·4 mg/g haemoglobin. The difference in the estimated haemoglobin-bound Fe for each pair of animals from both groups was regarded as haemoglobin Fe released by haemolysis. The differences between the Fe content in the liver and spleen for each pair were correlated with the corresponding value estimated for liberated haemoglobin Fe. As no significant haemolysis or tissue Fe accumulation was seen after 10 d, only the values

Table 7. *Plasma magnesium and iron concentrations, indicators of haemolysis, and iron content in liver and spleen of rats fed on diets containing different levels of magnesium*

(Mean values and standard deviations for five rats per dietary group)

	Dietary magnesium content (mg/kg)													
	70		110		208		330		850		850 (parallel feeding)		Initial values at day 0	
Feeding period	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Plasma magnesium concentration (mmol/l)														
10 d	$0{\cdot}18^{a,y}$	0·06	$0{\cdot}15^{a,x}$	0·03	$0{\cdot}40^{b,x}$	0·06	$0{\cdot}46^{b,x}$	0·06	$0{\cdot}81^{c,y}$	0·09	$0{\cdot}67^{c,y}$	0·05	$0{\cdot}73^{c}$	0·09
20 d	$0{\cdot}09^{a,x}$	0·01	$0{\cdot}12^{a,x}$	0·02	$0{\cdot}32^{b,x}$	0·06	$0{\cdot}41^{b,x}$	0·04	$0{\cdot}57^{c,x}$	0·04	$0{\cdot}60^{c,xy}$	0·05		
30 d	$0{\cdot}08^{a,x}$	0·02	$0{\cdot}12^{a,x}$	0·04	$0{\cdot}31^{b,x}$	0·04	$0{\cdot}50^{b,x}$	0·06	$0{\cdot}59^{c,x}$	0·04	$0{\cdot}57^{c,x}$	0·06		
Plasma iron concentration (μmol/l)														
10 d	$44{\cdot}5^{b,x}$	2·5	$39{\cdot}5^{ab,x}$	2·5	$50{\cdot}5^{b,y}$	1·4	$47{\cdot}7^{b,y}$	0·4	$44{\cdot}2^{b,y}$	2·2	$33{\cdot}3^{a,x}$	3·7	$63{\cdot}8^{c}$	9·3
20 d	$44{\cdot}4^{c,x}$	4·6	$40{\cdot}0^{bc,x}$	6·2	$35{\cdot}3^{abc,x}$	2·8	$37{\cdot}6^{abc,x}$	4·8	$30{\cdot}8^{ab,x}$	2·9	$29{\cdot}2^{a,x}$	3·0		
30 d	$46{\cdot}2^{c,x}$	5·6	$42{\cdot}7^{bc,x}$	3·0	$33{\cdot}0^{a,x}$	2·4	$34{\cdot}4^{ab,x}$	2·0	$35{\cdot}4^{ab,xy}$	2·9	$30{\cdot}0^{a,x}$	5·0		
Lactate dehydrogenase (*EC* 1.1.1.2) (U/l)														
10 d	$65^{a,x}$	20	$58^{a,x}$	6	$57^{a,x}$	18	$69^{a,x}$	18	$66^{a,x}$	20	$79^{a,xy}$	13	62^{a}	9
20 d	$116^{c,y}$	18	$58^{a,x}$	4	$71^{ab,x}$	5	$61^{a,x}$	3	$59^{a,x}$	7	$91^{bc,y}$	20		
30 d	$83^{b,x}$	16	$56^{a,x}$	9	$62^{ab,x}$	3	$56^{a,x}$	8	$52^{a,x}$	4	$64^{ab,x}$	9		
Bilirubin (pg/l)														
10 d	$920^{a,x}$	280	$800^{a,xy}$	100	$1040^{a,y}$	630	$700^{a,y}$	340	$920^{a,y}$	310	$1100^{a,y}$	190	630^{a}	150
20 d	$1480^{c,x}$	500	$960^{bc,y}$	360	$700^{ab,xy}$	410	$320^{ab,xy}$	160	$380^{ab,xy}$	110	$160^{a,x}$	40		
30 d	$1100^{b,x}$	220	$380^{a,x}$	190	$200^{a,x}$	70	$220^{a,x}$	80	$200^{a,x}$	20	$260^{a,x}$	110		
Liver iron content (mmol/kg dry wt)														
10 d	$3{\cdot}9^{a,x}$	0·6	$3{\cdot}3^{a,x}$	0·7	$3{\cdot}2^{a,x}$	0·8	$3{\cdot}3^{a,x}$	0·8	$3{\cdot}2^{a,x}$	0·8	$3{\cdot}0^{a,x}$	0·6	$3{\cdot}8^{a}$	0·7
20 d	$5{\cdot}1^{b,xy}$	1·4	$4{\cdot}0^{ab,xy}$	0·5	$3{\cdot}9^{ab,x}$	0·5	$3{\cdot}0^{a,x}$	0·4	$3{\cdot}1^{a,x}$	0·6	$3{\cdot}2^{a,x}$	0·8		
30 d	$7{\cdot}6^{b,y}$	2·4	$5{\cdot}1^{ab,y}$	0·8	$4{\cdot}0^{a,x}$	0·3	$3{\cdot}5^{a,x}$	1·0	$3{\cdot}1^{a,x}$	0·2	$3{\cdot}4^{a,x}$	0·6		
Spleen iron content (mmol/kg dry wt)														
10 d	$8{\cdot}9^{a,x}$	3·0	$8{\cdot}1^{a,x}$	1·7	$8{\cdot}9^{a,x}$	1·2	$9{\cdot}6^{a,x}$	2·2	$8{\cdot}6^{a,x}$	1·2	$8{\cdot}5^{a,x}$	0·8	$9{\cdot}3^{a}$	0·5
20 d	$34{\cdot}1^{b,y}$	8·8	$18{\cdot}8^{a,y}$	2·5	$19{\cdot}0^{a,y}$	1·0	$16{\cdot}6^{a,y}$	1·2	$12{\cdot}9^{a,x}$	1·2	$14{\cdot}0^{a,y}$	2·1		
30 d	$65{\cdot}1^{b,z}$	11·9	$35{\cdot}0^{a,z}$	9·1	$28{\cdot}7^{a,z}$	4·4	$24{\cdot}1^{a,z}$	5·0	$23{\cdot}1^{a,y}$	5·0	$20{\cdot}1^{a,z}$	2·8		

[a,b,c]Mean values within a row not sharing a common superscript letter were significantly different, $P < 0{\cdot}05$ (one-way ANOVA).
[x,y,z]Mean values within a column not sharing a common superscript letter were significantly different, $P < 0{\cdot}05$ (one-way ANOVA).

after 20 and 30 d were used for the correlation. The correlation coefficients for these organs were 0·83 and 0·82 ($P < 0·01$; results not shown).

Histological findings (Expt 1)

Light microscopy revealed no morphological alterations of the hepatic parenchyma. Fe storage in the liver was restricted to the Kupffer cells. Kupffer-cell siderosis was found almost exclusively in the 70 mg Mg/kg and 110 mg Mg/kg groups and occurred in parallel with marked siderosis of macrophages in the spleen (Table 8). Due to the fast growth rates the bone marrow showed high erythropoietic activity in all groups accompanied by negative Perls' blue reaction, indicating a lack of stainable Fe in macrophages from the bone marrow. Although extramedullary haematopoiesis was observed in the spleen in all animals, the 70 mg Mg/kg group showed the highest erythropoietic activity (Table 8). In the young control animals which were slaughtered at the beginning of the feeding period high extramedullary erythropoiesis went along with a lack of siderosis in the spleen.

Renal tubular calcifications as a characteristic lesion in Mg deficiency were found in almost all animals of the 70, 110, and 208 mg Mg/kg groups. Only one in five rats in the 330 and two in five animals in the 850 mg Mg/kg groups showed intratubular concretions (Table 8). The extent of these changes correlated with the extent of Mg deficiency and was observed even in the 850 mg Mg/kg *ad libitum* group, though not, however, in the 850 mg Mg/kg group with restricted feeding.

DISCUSSION

The quantitative correlation and the simultaneous appearance of haemolysis and siderosis hint that an incomplete erythropoietic utilization and, hence, a disturbed redistribution of Fe from haemolysed erythrocytes may be the cause of tissue Fe accumulation in Mg deficiency. The authors are well aware that correlation cannot prove a causal relationship. However, it seems worthwhile to draft a hypothesis for the mechanisms leading to tissue siderosis in Mg deficiency based on the presented findings and on the available data from the literature.

There are three possible ways in which Fe can accumulate in the body: first, a reduction in Fe excretion can be assumed to lead to increased body Fe retention. This possibility can be discounted, however. As daily Fe losses in mammals make up less than 0·1% of total body Fe (Lynch, 1984), a further reduction cannot account for the substantial increases in tissue Fe that were found after 20 and 30 d. Second, the increased tissue Fe load in Mg deficiency may be due to increased intestinal Fe absorption or, third, to an altered distribution of total body Fe.

Intestinal iron absorption

The state of Fe repletion is geared to the demand by adaptation of intestinal Fe absorption (McCance & Widdowson, 1937). An increased intestinal absorption capacity for Fe serves to normalize the tissue Fe content in Fe deficiency and is assumed to cause Fe overload in hereditary haemochromatosis (Lambard *et al.* 1990). Thus, increased Fe absorption is a possible cause of Fe overload in the tissue. Intestinal Fe absorption increases most markedly in Fe-deficient segments from the proximal small intestine (Schümann *et al.* 1990*a*) which makes such segments first choice for Fe transfer experiments. In Mg deficiency intestinal Fe absorption was not significantly increased, either *in vitro* or *in vivo*,

Table 8. *Semiquantitative evaluation of histological findings in rats after 30 d feeding on a magnesium-deficient diet**

	Siderosis in hepatic Kupffer cells			Siderosis in spleen				Extramedullar erythropoiesis in the spleen				Tubular calcification in the kidney			
Dietary magnesium content	Ø	+	++	Ø	+	++	+++	Ø	+	++	+++	Ø	+	++	+++
70 mg/kg	0	3	2	0	0	0	5	0	0	0	5	0	1	2	2
110 mg/kg	3	2	0	0	1	4	0	0	0	2	3	1	0	3	1
208 mg/kg	5	0	0	0	3	2	0	0	1	3	1	0	4	1	0
330 mg/kg	4	1	0	0	4	1	0	0	0	2	3	4	1	0	0
850 mg/kg	5	0	0	0	4	1	0	0	0	2	3	3	2	0	0
850 mg/kg (parallel feeding)	5	0	0	0	4	1	0	0	2	3	0	5	0	0	0
Initial values on day 0	6	0	0	6	0	0	0	0	0	0	6	6	0	0	0

Ø, No indication of siderosis or extramedullar erythropoiesis; +, mild extent of siderosis or extramedullar erythropoiesis; ++, moderate siderosis or extramedullar erythropoiesis; +++, marked siderosis or extramedullar erythropoiesis.

*For details of diets and procedures, see Table 3 and pp. 476–477 and p. 480.

either at high or at low luminal Fe concentrations. Also, there was no reduction of intestinal ^{59}Fe mucosa-to-serosa transfer at high luminal Mg concentrations. The ^{59}Fe transfer rates were in the same order of magnitude as the control values determined with corresponding methods in earlier experiments (Schümann *et al.* 1990*a*). Mg had no appreciable effect on the $^{59}Fe^{3+}$ uptake into the mucosa of hypoxic mice while it reduced the $^{59}Fe^{3+}$ uptake in control mice (Raja *et al.* 1987). To our knowledge no information is available on the impact of high Mg concentrations on ^{59}Fe mucosa-to-serosa transfer. Our present results indicate that intestinal Fe absorption does not increase in Mg deficiency due to lack of inhibition by high luminal Mg concentrations.

Redistribution of iron from haemolysed erythrocytes

As decreased Fe losses and increased Fe absorption are ruled out as possible causes, the mechanism to be considered is a changed distribution of body Fe. In neoplastic diseases and in chronic inflammation Fe accumulates in the reticuloendothelial system (RES) which decreases the plasma Fe concentration (Bothwell *et al.* 1979). In severe Mg deficiency (70 and 110 mg Mg/kg after 20 and 30 d), however, the plasma Fe conCentration was increased. Thus, tissue Fe accumulation in Mg deficiency cannot be caused by this mechanism. Also, anaemia in Mg deficiency was neither microcytic nor hypochromic which shows that it was not due to decreased availability of Fe. This is supported by the unchanged plasma transferrin concentrations in Mg deficiency indicating ample availability of Fe in the livers.

In normal states of Fe repletion approximately 60–70 % of body Fe is contained in haemoglobin while another 20 % is stored in the tissue (Lynch, 1984). These values show that the only compartment sufficiently large to serve as a source for substantial increases of tissue Fe stores is the haemoglobin Fe pool. Plasma Mg was significantly decreased as early as after 10 d. Haemolysis was retarded in comparison with this effect. Excessive amounts of haemoglobin Fe were liberated in the 70 mg Mg/kg and 110 mg Mg/kg groups no earlier than after 20 and 30 d of Mg-deficient feeding. Haemolysis developed in parallel to the tissue Fe increases in liver and spleen. These two organs were chosen because they reach the highest Fe content in Mg deficiency (Kimura & Itokawa, 1989). The simultaneous appearance of haemolysis and tissue Fe accumulation suggests that the excess tissue Fe may, indeed, derive from haemolysed erythrocytes. This conclusion is supported by the high correlation of the estimated increases in tissue Fe content with the estimated Fe quantities liberated from haemolysed erythrocytes.

At the end of their life cycle erythrocytes are phagocytosed. Haemoglobin Fe is taken up by the reticuloendothelial system and is rechannelled into erythropoiesis (Bothwell *et al.* 1979). Thus, increased haemolysis should be compensated by increased erythropoiesis. Therefore, erythropoietic activity was quantified by reticulocyte counts and by semiquantitative evaluation of extramedullar erythopoiesis. There was no indication of insufficient erythropoiesis due to inaccessibility of Fe to the erythron: plasma Fe concentrations were high, anaemia was not microcytic or hypochromic and plasma transferrin concentrations were not increased. Fast growth increases the demand for erythrocytes which, in turn, leads to an increased erythropoietic activity. Indeed, high reticulocyte counts were found during the animal's fast growth periods, i.e. in the controls on day 0 as well as after 10 and 20 d. Correspondingly, reticulocytosis was most marked in the Mg-adequate controls (850 mg Mg/kg) which grew the fastest. The expected stimulation of erythropoiesis after 20 d was entirely obscured by the growth effect, indicating that a reticulocyte count of 80–120‰ represents a maximum of erythropoietic

activity that cannot be surpassed under these conditions. After 30 d reticulocytosis had significantly decreased in the Mg-adequate controls and this was accompanied by a reduced erythrocyte distribution width. However, it remained high in the 70 mg Mg/kg and 110 mg Mg/kg groups which showed marked haemolysis. In the 70 mg Mg/kg group reticulocytosis reached the same order of magnitude as during fast growth which seems to be the maximum that can be reached by this type of stimulus. These findings are supported by the semiquantitative evaluation of the extramedullar erythropoiesis in the spleen which was at maximum in all control animals on day 0 as well as in the 70 mg Mg/kg group after 30 d. However, the estimation of the liberated haemoglobin Fe in the 70 mg Mg/kg group shows that this level of erythropoiesis is insufficient to cope with haemolysis. The excess Fe which cannot be rechannelled into erythropoiesis is mopped up by the tissue.

Histological findings

As in other haemolytic states Fe accumulates in the cells of the hepatic and splenic RES. Besides the fact that aged erythrocytes are dissembled preferentially in the spleen the strongly developed RES in liver and spleen may explain why Fe concentrations are highest in these two organs (Kimura & Itokawa, 1989). In the present experiments hepatic and splenic Fe was found exclusively in reticuloendothelial cells. This seems to be a very successful protection of the parenchymal cells. The lack of stainable Fe may explain why no morphological alterations were detectable in hepatocytes. Still, alanine aminotransferase (*EC* 2.6.1.2) and aspartate aminotransferase (*EC* 2.6.1.1), both of which are specific indicators of a parenchymal liver damage, were observed to increase by approximately 30 % in severe Mg deficiency. These findings from the same experiments have been published elsewhere (Vormann *et al.* 1995). Although these increases were significant they were small compared with those of the hepatic malondialdehyde content which indicated a tripling of lipid peroxidation (Vormann *et al.* 1995). The diversion of Fe away from the hepatocytes into the Kupffer cells may explain the discrepancy between the increased lipid peroxidation and the lack of corresponding indicators for parenchymal damage.

Tubular calcifications in the kidney of Mg-deficient rats have been described earlier (Rob *et al.* 1993). Although 500 mg Mg/kg was described to be an adequate Mg supply for rats (Cook, 1973) the observation of tubular calcification in the kidney shows that this is not the case during fast growth. This interpretation is underlined by the lack of calcification in the slower growing 850 mg Mg/kg group with restricted feeding. Concentrations of urea and creatinine in the plasma, however, were not significantly increased. Thus, uraemia as a cause of haemolysis can be excluded.

Speculation on possible causes of haemolysis in magnesium deficiency

The Mg content of erythrocytes corresponds to the plasma Mg at the time of their synthesis (Günther & Vormann, 1985). The life span of rat erythrocytes is approximately 60 d. If haemolysis is assumed to be related to the erythrocyte's Mg content this explains the retardation of haemolysis compared with the decreases in plasma Mg concentrations.

From the results presented here we cannot derive any conclusions regarding the molecular mechanisms which mediate an increase in haemolysis. However, possible explanations can be derived from the literature. For example, the shortened erythrocyte survival is accompanied by decreased erythrocyte contents of pyrophosphatase (*EC* 3.6.1.9) (Elin *et al.* 1971) and by a decreased glucose utilization in erythrocytes leading to decreased ATP and 2,3-diphosphoglycerate concentrations (Oken *et al.* 1971). Recently, binding of free Fe to divalent cation binding sites in the erythrocyte (band 3 protein) has

been shown to induce formation of a senescent antigen which may induce destruction of the erythrocyte by subsequent binding of immunoglobulin G (Signorini *et al.* 1995). Mg is the main divalent cation within the erythrocyte that may compete with Fe for the band 3 binding sites. In the present experiments the Mg content in the erythrocytes was reduced to less than half of the control value after 30 d. This changes the ratio between free intracellular Fe and Mg and should induce increased Fe binding to the band 3 protein and increased formation of the senescent antigen. In consequence, erythrocyte survival should be shortened in Mg deficiency and haemolysis should increase, which is what happened in the present study. It may even explain the retardation of haemolysis as compared with decreases in plasma Mg concentrations.

In summary, the following hypothesis may explain the increased tissue Fe accumulation in Mg deficiency: severe Mg deficiency leads to formation of Mg-deficient erythrocytes with a decreased survival time resulting in haemolysis. The released Fe that exceeds the erythropoietic capacity is mopped up by the tissue, leading to haemosiderosis.

The increased tissue Fe content in Mg deficiency leads to increased lipid peroxidation (Günther *et al.* 1992) without increased dietary Fe supply, which may be of interest in the ongoing discussion on the potential harm of high oral Fe supply. However, haemolysis, which seems to be a prerequisite to increase the tissue Fe content in Mg deficiency occurs only at plasma Mg concentrations below 0·25 mmol/l (Vormann *et al.* 1995). Such values are rarely reached in humans except under medication e.g. with ciclosporine (Rob *et al.* 1993) or diuretics. Provided that there are no dramatic differences between species, therefore, increased tissue Fe accumulation and the described pathophysiological consequences should rarely occur in humans. This may be different, however, when Mg deficiency is accompanied by other factors that stimulate haemolysis or lipid peroxidation.

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