89 THE LABILITY OF SKELETAL MAGNESIUM RESERVES. THE INFLUENCE OF RATES OF BONE GROWTH

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It was previously shown [Duckworth *et al.* 1940] that the skeletal Mg of the rat served, in part, as a reserve upon which the animal could draw to remedy soft tissue lack arising from acute dietary deficiency of the element. When Mg was restored to the diet of the deficient rat the skeletal Mg increased at a rate indicating that replenishment of the reserve was occurring. The present communication records attempts at modifying the magnitude and velocity of mobilization from the skeleton. The method of approach was to alter the rate of bone growth by adjustments of the Ca intake of rats receiving a diet acutely deficient in Mg.

We are not aware of any studies having been presented concerning massive liberations of Mg from the skeleton. Other studies in Mg deficiency by various workers are considered in the discussion. The present study is concerned only with Mg deficiency in the young growing animal. It is realized that the reaction of the skeleton to Mg deficiency might be different in the adult in which the skeleton and soft tissues have completed their growth.

Analytical methods

The methods used for analysis were those previously reported [Duckworth *et al.* 1940]. In most cases only total ash and Mg were determined, since it was established that there were no disturbances in the Ca and P contents of the bone ash in Mg deficiency, beyond a slight increase in the percentage of Ca, probably arising from the replacement of Mg by Ca in the bone salt crystal.

Experimental

The feeding and housing methods were those previously described. It is regretted that body-weight records were not kept regularly, but handling of the animals in the hypersensitive stage frequently induces convulsive attacks with a fatal termination. As regularity of survival was essential for the completion of the present study the omission was unavoidable.

Four diets were prepared with different Ca contents. Modification of the Ca content was achieved by altering the amount of CaCO_3 in the salt mixture with corresponding compensatory adjustments in the starch content of the diet.

Table 1. *Ca and P contents of Mg-deficient diets*

Diet	% Ca	% P	Ca : P
A	0.90	0.35	2.6 : 1
B	0.30	0.35	0.9 : 1
C	0.10	0.35	0.3 : 1
D	0.0001	0.35	0.0 : 1

The Mg content was less than 6 p.p.m.

Otherwise the diet was the same as that already recorded [Duckworth *et al.* 1940]. No attempt was made to eliminate from the diet more Ca than was removed from its constituents during their purification to reduce the Mg content.

Judging from the results obtained in other studies at present in progress of the percentages of ash in the bones, and also from a comparison with the records of Brown *et al.* [1932], diets A and B can be considered normal except for the deficiency of Mg. Diet C was deficient in Ca and diet D acutely deficient. Diet B was fed only to a small group of rats for the purpose of comparing the symptoms developed on this diet with those arising on the other diets. The skeletons of the rats on diet B were not analysed.

Nine litters, each of 7 hooded Lister (M and F) rats, were divided into 3 groups of 3 litters. Each group received one of the above diets, A, C or D. The rats were weaned at 23 days of age and transferred to stock diet for 2 days to accustom them to a non-milk regimen before feeding the Mg-deficient diet. At 25 days of age one rat from each litter was killed. A second rat was allowed to continue on the appropriate diet until death occurred. The remaining 5 rats were killed at different times during this survival interval, the killings being arranged to be more frequent during the early phase of the deficiency since it was expected that the greatest changes in the degree of mobilization would occur about this time. Occasionally an animal died before the scheduled time and, in such cases, an interchange was made with the surviving animal due to be killed on that day. A possible bias may have entered the experiment for this reason but it was not apparent from inspection of the results in the few cases in which interchange was necessary.

The skeleton was freed from flesh by the trypsin digestion method of Subrahmanyam *et al.* [1939]. After digestion the skeleton was divided into 4 parts: the long bones, the flat bones, the rib bones and the spinal plus caudal bones. The 4 parts were analysed separately. The paws and sternum were discarded before tryptic digestion. The teeth were also discarded, because in Mg deficiency their behaviour differs from that of the skeleton [Duckworth & Godden, 1940].

Table 2. *The influence of the Ca content of Mg-deficient diets upon the survival of rats*

Diet	Survival periods days	Mean days
A	5, 5, 5, 5, 7, 8, 8	6.1
B	7, 7, 8	7.3
C	9, 10, 11, 11, 12, 12, 13, 16, 16	12.2
D	8, 12, 14, 16, 24, 27	16.8

Results

Symptomatology. The results in Table 2 show the survival periods of rats dying spontaneously of Mg deficiency in the present and other studies in progress. The period of survival was lengthened by a reduction in the Ca intake. The results were erratic and overlapping of groups was extensive. Vasodilatation and hyperaemia of the peripheral vascular system occurred in groups A, B and C. The interval between the introduction to the diet and the onset of vasodilatation was extended and the severity of the condition was rendered less marked by lowering the Ca intakes. Vasodilatation occurred only in some of the rats receiving diet D and even then it was transitory and mild in nature. Assessment of differences is difficult in piebald rats which were used in the present studies

the albino rat is much superior for exact determination of the onset and persistence of vasodilatation. In group A, particularly, oedema of the ears was very marked at the time of vasodilatation. This condition also seemed less intense in groups with lower Ca intakes.

Mobilization of Mg from different parts of the skeleton

Mg appeared to be liberated at about the same rate from all parts of the skeleton in each group. The final percentages of Mg in the ash were slightly higher in the ribs and flat bones of group D rats than in the long bones and spine bones. In agreement with the observations of other workers it was found that the ribs and flat bones were considerably more reduced in ash content than the other parts of the skeleton in group D which was extremely deficient in Ca. Otherwise the changes observed in different fractions corresponded closely with those of the skeleton as a whole and detailed presentation of the results for the different skeletal fractions is therefore unnecessary.

Skeletal ash. The values shown in Fig. 1 for total skeletal ash are the sums of the values for the 4 skeletal fractions analysed. The results are slightly lower than those we have previously recorded for total skeletal ash, owing to the discarding of the sternum, paws and teeth. As was expected, 3 rates of skeletal growth were obtained (Fig. 1). In group A there was rapid deposition of bone ash and in

Fig. 1. The effect of different levels of Ca in Mg-deficient diets on the skeletal ash of rats.

Fig. 2. The effect of different rates of bone growth on Mg liberation from the skeleton.

group C there was a much slower deposition. In group D there was a progressive demineralization of the skeleton, which proceeded more rapidly during the early part of the deficiency.

Skeletal Mg. The liberation of Mg was least in quantity and slowest in velocity in group A (Fig. 2). Considerably more Mg was liberated, and at a more rapid rate, in group C. In group D occurred the mobilization of greatest magnitude and with a rate of liberation slightly greater than that of group C. About one-

fifth of the Mg was liberated in group A rats, about one-third in group C and about two-thirds in group D. Mobilization appeared to cease in groups A and C after about 5 and 9 days respectively, but in group D, in which there was a progressive demineralization of the skeleton, the mobilization, after a rapid initial liberation, continued at a very slow rate.

The percentages of Mg in the ash fell most rapidly in the rats in group A in which bone growth was most active, and least rapidly in group C in which mineralization of the skeleton was proceeding at a slow rate (Fig. 3). In group D the curve for percentage Mg in ash coincided with that of group C for the first half of its course and thereafter assumed a path below the group C curve.

Fig. 3. The percentage of Mg in the skeletal ash of rats receiving Mg-deficient diets with different Ca intakes.

The quantities of skeletal ash and of total skeletal Mg were somewhat variable as between rats of the same age. Differences of up to 100 mg. of ash were found between newly weaned rats of the same body-weight. The percentages of Mg in the ash, especially in weaning animals, were less variable.

None of the above observations appeared to be influenced by the sex of the animal.

DISCUSSION

It seems reasonable to believe that the survival of Mg-deficient rats will depend on two factors; first, the transport of Mg from centres where its concentration can be reduced with little or no impairment of function (e.g. bone salt) to positions where Mg is required to sustain vital processes and, secondly the adjustments that can be made in growth and metabolism to reduce the Mg requirement. Before examining the present data on the skeletal reserve in relation to the rest of the organism consideration of the distribution of Mg in the body is essential.

Of the total body water of the rat about 40% is extracellular [Harrison *et al.* 1936] and of this about one-eighth is plasma water, calculated on the basis of Skelton's [1927] data. About 30% of the plasma Mg is non-ultrafiltrable and

is thought to be in combination with plasma proteins. From this colloidal form Mg can be ionized and it thus serves as a circulatory reserve capable of partially remedying reductions in ultrafiltrable Mg. Peters [1935] considers that interstitial fluid contains only a trace of protein and that the maximum value of 0.5% is probably too high. The other fluids and transudates of the extracellular phase are generally much lower than plasma in protein content and also, in the majority of pleural and ascitic fluids of clinical cases studied by Greene *et al.* [1931], lower in Mg. It follows that, except in the case of plasma, extracellular fluid can carry but a very small reserve of protein-combined Mg. Since alterations in electrolyte levels are rapidly transmitted throughout the extracellular fluid, Mg combined with plasma protein is the main reserve of this phase, though even this is small in relation to the volume of fluid it serves and the amount which can be liberated from it is limited by the conditions of physico-chemical equilibrium obtaining.

Another possible source of Mg ions might be a combination of Mg with the proteins of interstitial tissue. The question whether Mg is bound by the organic matrix of bone is not yet settled. While the majority of workers regard Mg as mainly, if not exclusively, a bone salt component, Klement [1931] holds that it is not. Subrahmanyam *et al.* [1939] showed that when part of the organic matrix is hydrolysed by trypsin only very small quantities of Mg are lost. Further, Na, which is bound by plasma proteins, but to a less extent than Mg [Greene & Power, 1931], is not bound by any tissue except bone [Harrison *et al.* 1936] and most of this is precipitated in the bone salt [Harrison, 1937]. On the basis of the above evidence, admittedly fragmentary, it seems unlikely that interstitial tissue binds significant amounts of Mg.

Erythrocyte Mg was found by Tufts & Greenberg [1937-8, 1] to be gradually reduced in Mg deficiency, but these authors were unable to demonstrate that transport across the membrane had occurred, because of the disturbing influence of the entry into circulation of new erythrocytes low in Mg. The reduction in Mg level will, however, free Mg for use by other tissues and whether this occurs by slow transport across the erythrocyte membrane or by phagocytosis is immaterial from the present standpoint, except in connexion with the velocity of reparation of the deficit. It is generally believed that tissue cell membranes do not permit the loss of Mg, but whether this condition persists in Mg deficiency is not known.

From the foregoing it seems probable that, excluding the bone salt, the organism has very little reserve of Mg except the small amount combined with plasma protein.

Support for the foregoing evaluation of the body's Mg reserves is given by the data on the rate of liberation of skeletal Mg. The rapid release of the element from the skeleton in all groups and the absence of an initial lag suggests that there are no other reserves of significant magnitude and lability.

The large differences in the amounts of Mg liberated from the skeleton in different groups are related to differences in rates of bone growth, the mobilization increasing progressively with decreasing mineralization. During the formation of the bone salt crystal considerable interchanges of ions with those in the fluid phase of the bone occur [Logan, 1940]. While the above results show the rapidity with which Mg ions are lost by bone salt the gain of Mg ions by newly deposited bone salt is equally important. The possibility that Mg ions may even be involved in the initial precipitation as well cannot be excluded. It follows that the curves in Fig. 2 show not gross but net mobilization of Mg, that is, the quantity of Mg liberated for soft tissue use. The value obtained on analysis represents unmobilized Mg remaining in the bone salt initially present plus Mg in the salt deposited during the experimental period. An exception exists in group D

where there was a gradual reduction in the total amount of bone salt so that the analytical value represents more than the gross liberation arising from Mg deficiency, in contrast to the state of affairs met with in groups A and C. The curve of gross liberation would lie between the curve of group D and that of group C, in which there was slight mineralization. Similarly, the velocity of mobilization, or lability, of skeletal Mg is not revealed by analysis. Only the velocity of liberation of the net quantity mobilized is obtained.

This finding that the skeleton is simultaneously a source of, and a competitor for, Mg is in agreement with our previous observations on teeth [Duckworth & Godden, 1940] where it was shown that the incisor of the rat continues to gain Mg throughout the course of the deficiency, although at a subnormal rate.

Tufts & Greenberg [1937-8, 1] state: 'the course of Mg deficiency in the rat conforms to two fairly definite phases, the first being characterized chiefly by vasodilatation, hyperaemia and hyperexcitability, and the second being marked by nutritional failure, cachexia and kidney damage'. The present observations indicate that during the first phase there are soft tissue gains of Mg from the skeleton, while the second phase persists during the period when no further exchanges occur. These workers found that plasma Mg was sharply reduced during the first phase of the deficiency and then returned to normal levels. Thereafter the level gradually declined but the previous minimum was not again reached. The interval required for the plasma Mg to reach its minimum varied directly with the Mg content of the diet. Their finding that the minimum was reached on the third day of deficiency in rats on diets containing less than 10 p.p.m. of Mg coincides closely with the time of almost complete mobilization of skeletal Mg in group A rats. On our diet, as previously shown [Duckworth *et al.* 1940], there is diminution, and finally cessation, of growth and failure of appetite after 3 days. The interrelation between exhaustion of the skeletal Mg reserve, reduction in appetite to the point of cessation of growth and the two-phase nature of the syndrome of the deficiency, is obvious.

From the data given in Figs. 2 and 3 it is clear that during the second phase of the deficiency the gains in Mg of depositing bone salt are at the expense of crystals already deposited since total skeletal Mg remains constant but the percentages of Mg gradually fall. The mechanism of exchange between the crystals is probably by gradual liberation of Mg from the older crystals in response to the slowly falling plasma Mg level. The liberation of these ions into the fluid phase of bone appears to be followed by their immediate combination with the more recently deposited crystals which are, as stated above, actively exchanging ions with the perfusing liquid during the early stages of development of the crystals. Although the percentages of Mg in bone ash ultimately reach approximately the same levels on all diets (but with consistent small differences between diets) we hesitate to suggest that the bone salt Mg exists in two forms: a labile and a bound form. The minimum values obtained indicate, it is felt, that bone salt which is being precipitated from the fluid phase of bone attracts, during its formation, a quantity of Mg proportionate to the Mg level of the fluid. That Mg ions are in any way an essential component of the crystal seems to be ruled out by the work of Logan & Taylor [1937] who obtained, *in vitro*, crystals very similar to bone salt from Mg-free solutions.

The falling level of plasma Mg has so far been considered to be responsible for the outflow of Mg ions from the bone salt. It is a point of considerable interest that, after mobilization is complete, the rise in plasma Mg is not associated with a movement of Mg back to the bone salt. As stated above, evidence has been presented to indicate an interchange of Ca for Mg ions in the

crystal during liberation of Mg. The reverse displacement of Ca ions by Mg ions when plasma Mg rises does not appear to occur. The organism appears to have no mechanism for independent liberation of Ca from bone salt. Our previous studies [Duckworth *et al.* 1940] of the effect of restitution of Mg in the diet after a period of depletion, demonstrated that Mg does not rapidly return to the skeleton. Even after 10 days of normal Mg intake the percentage of Mg in the bone ash had risen considerably but had not attained normal levels. It is tentatively suggested that the return of Mg to the depleted bone salt can only occur during redeposition following resorption. This resorption and redeposition of bone salt affords an opportunity for precipitation of crystals of normal Mg content. Such activities of the skeleton are not measurable by ordinary methods of chemical analysis.

This characteristic of Mg, that it can leave the bone salt independently and in large amounts, does not appear to be possessed by other components of the crystal, nor does it seem possible for toxic ions, such as Pb and F, to leave the crystal independently after deposition. Considering the importance of Mg as an intracellular ion this freedom of movement is probably of great value to the organism.

No mention has been made of a possible involvement of the endocrine system in the liberation of skeletal Mg. There are no records in the literature of large mobilizations, such as have been observed in the present study, after administration of hormone preparations or following the partial or complete ablation of endocrine glands. It is felt, however, that the level of Mg in the fluid phase of bone cannot alone be responsible for the removal of the ion from the bone salt crystal, since both tryptic digestion of bone and the isolation of bone salt by methods based on the complete solution of the organic matrix have little effect upon the Mg content of the bone salt. Greenberg & Mackey [1932] showed that, after parathyroid hormone administration in dogs, serum Mg increased and then returned to normal before the serum Ca elevation began, an observation we have confirmed for the horse (unpublished data). Unfortunately there has not yet been presented proof that the increment observed is of skeletal origin.

The present observations of the effect of Ca on the survival period confirm those of Tufts & Greenberg [1937-8, 1, 2]. The erratic survival periods on all diets suggest that several factors are involved. The action of normal Ca intakes in reducing the survival interval probably does not depend on the failure to liberate more than a relatively small quantity of Mg alone but also upon the so-called 'physiological antagonism' of the two elements. The death of an animal in tetanic convulsions appears to arise directly from a lack of Mg ions since administration of MgSO_4 solutions to animals during a seizure has only once, in our experience, failed to initiate recovery and, if the treatment were continued, survival. This is true not only in rat experiments but also in the naturally occurring Mg deficiency of milk-fed calves. Hitherto workers studying Mg deficiency have emphasized the pathological calcification which occurs throughout the body. The diets used in such experiments were, however, higher in Mg content than ours and permitted longer survivals. In the present study several rats in group A died at 5 days of age. Irving [1940], studying the rats used in the tooth experiment of Duckworth & Godden [1940], failed to detect pathological calcification in the enamel organ before the sixth day of deficiency. No other organ was examined but calcareous deposits might reasonably be expected as early in the enamel organ, with its intense Ca metabolism, as elsewhere.

SUMMARY

The net quantity of Mg liberated from the skeleton and its apparent rate of mobilization were found to be related to the rate of bone growth.

The distribution of Mg in the organism was discussed and the conclusion was drawn that the skeleton contains the body's main reserve of Mg. It was shown to be highly labile.

The two-phase nature of the syndrome of Mg deficiency was shown to be explicable on the basis of mobilization and then cessation of mobilization, of skeletal reserves.

A mechanism for the removal and return of skeletal Mg was suggested.

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