17. THE EFFECT OF ACUTE MAGNESIUM DEFICIENCY ON BONE FORMATION IN RATS

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THE large skeletal requirements for Ca and P and the importance of correct amounts and balance of these elements in diets have resulted in much greater attention being given to their metabolism than to the metabolism of Mg. The amount of Mg in bone is only one-fortieth to one-fiftieth of that of Ca and this low proportion has tended to direct attention away from Mg when consideration is given to the composition and formation of bone salt. Within the last few years increasing attention has been given to Mg and improvements in analytical technique and in knowledge of the preparation of synthetic diets have been responsible for certain advances.

The crystallographic studies of Gruner *et al.* [1937] on enamel and dentine have indicated that atomic replacements can take place in such crystal structures. If their conclusions can be extended to bone salt, the manner in which this material can serve as a reserve of mobilizable Mg could be explained as an exchange or replacement process. That bone salt does act as such a reserve is not yet certain. While some of the evidence favours such a view, contradictions exist. The extent to which bone salt can act as a Mg reserve, that is to say the extent to which replacement can occur in cases where the organism is compelled to draw on the reserve owing to a dietary deficiency of Mg, is so far unknown. The relation between Mg and bone formation, as distinct from its probable presence in the bone salt molecule, has been investigated only to a slight extent and, beyond the accelerating effect of Mg upon the hydrolytic action of phosphatase *in vitro*, very little has been established.

The present communication is concerned with a portion of this problem and studies in acute deficiency have been the method of approach.

Some aspects of Mg metabolism have been reviewed elsewhere by one of us [Duckworth, 1938-9] and only those certain studies of bone formation on acutely deficient diets will be referred to during the discussion of the present results.

EXPERIMENTAL

Preparation of the Mg-deficient diet. One of the principal difficulties in this investigation has been the preparation of a diet which, while adequate in respect of all other known dietary factors, should have a minimal Mg content. This procedure has involved processes of purification designed to remove Mg from all the components of the diet; difficulties arose particularly with respect to adequate supplies of the vitamin B complex. Sources of this vitamin are frequently found to contain relatively large amounts of Mg and it was feared that any process for removal of the Mg might result in a loss of vitamin B potency. Accordingly, two sources of vitamin B complex were used, namely, yeast and liver extract, and as an added safeguard the extracts were prepared

and the Mg was removed by two somewhat different processes. The purified vitamin B extracts were fed at a fairly high level to ensure completeness of the diet and this resulted in a somewhat higher Mg content than would otherwise have been necessary. The complete Mg-deficient diet was found by spectrographic analysis¹ to contain not more than 0.0006 % Mg (i.e. 6 parts per million).

Preparation and purification of yeast extract. To 300 g. dried brewer's yeast (obtained from the Pharmaco-Chemical Products Co., Ltd., Burton-on-Trent), 1200 ml. of 50 % C_2H_5OH were added and the mixture was vigorously shaken for 15 min. and filtered on a Büchner funnel. The yeast was extracted twice more in the same manner except that 900 ml. of alcohol were used in each case. The three filtrates were combined and the volume measured (generally about 2700 ml.). About 15 g. of finely ground $(NH_4)_2HPO_4$ were added to the yeast extract and the mixture shaken until the salt was completely dissolved. Concentrated ammonia was then slowly added at the rate of 30 ml. for each 100 ml. of extract; during the addition the extract was vigorously shaken. The extract was left overnight in the refrigerator. The following day the extract was filtered through a double filter using suction. The filtrate was placed in large flat porcelain trays in a cold draught tunnel and left until the volume was reduced to about 350 ml. During this evaporation all the free ammonia and the alcohol were driven off. The whole of the yeast extract concentrate prepared in this way was incorporated in each 2 kg. of diet.

Purification of liver extract. 120 g. of liver extract (Boots) and 7.2 g. $(NH_4)_2HPO_4$ were dissolved in distilled water and roughly neutralized with NaOH. The volume was adjusted to 480 ml. and 120 ml. NH_4OH (sp. gr. 0.88) were added with constant stirring. The liquid was left overnight in the refrigerator and filtered by suction the following day. The filtrate was exposed on flat porcelain trays in the cold draught tunnel until all the ammonia had been driven off. The extract was then collected, the trays were rinsed with distilled water and the whole made up to 400 ml.

Preparation of inorganic constituents. KCl and KH_2PO_4 were purified by crystallization from boiling saturated aqueous solutions made slightly acid with HCl. NaCl was purified by precipitation from saturated aqueous solution by HCl. $NaHCO_3$ was purified by precipitation from warm saturated aqueous solution by C_2H_5OH . $CaCO_3$ was prepared by double precipitation from dilute $CaCl_2$ solution by $(NH_4)_2CO_3$. It was found that these salts, even when purchased in the most highly purified forms, contained sufficient Mg to increase markedly the Mg content of the diet.

Purification of casein. 1 kg. of casein in small portions at a time was ground with 0.2 % acetic acid until the whole was suspended in a volume of 10 l. in a 12 l. bottle. The bottle was shaken from time to time and the following day the casein was collected by filtering through a linen bag. The last portions of the washing liquor were removed in a hand press. The second washing was with distilled water, the casein being as before ground down in a mortar. The washings were carried out for 10 days, 5 acetic acid and 5 distilled water washings in all being used alternately. The casein was then suspended in 5 l. of 70 % C_2H_5OH for 24 hr. and, after filtering, in the same volume of 90 % C_2H_5OH for a similar period. Finally the casein was dried in the cold draught tunnel and then in an electric oven at 65°.

Purification of starch. Starch, as commercially obtained, frequently contains a large amount of grit. This was removed by suspending 500 g. of starch in

¹ We wish to acknowledge our indebtedness to Dr R. L. Mitchell, of the Macaulay Institute, who performed this analysis.

2 l. of 10 % HCl in a tall cylinder and allowing to stand for 5 min. The suspension was then siphoned off to within 3 in. of the bottom of the cylinder, the remainder, which contained most of the grit, being discarded. The starch was left for 24 hr. in the acid solution with occasional shaking, filtered by suction and washed with large volumes of distilled water. It was then suspended twice more in acid and twice washed on the funnel. After the third day's washing it was dried in the cold draught tunnel and finally in the oven at 65°. The sample was then pulverized.

Paper pulp. As a source of roughage paper pulp was added to the diet. Crude compressed pulp was ground in a mill, suspended in 2.5 % HCl for 24 hr. and then washed by suction with distilled water. This washing was repeated twice and the pulp finally dried.

Butterfat. Butter from a constant source was purified as required. Freshly made butter was melted and filtered through glass wool to remove curd and then through filter paper. The filtered fat was shaken in a separating funnel with an equal volume of hot 2 % HCl and the aqueous layer discarded. The washing was repeated twice. The fat was then washed 3 times with equal volumes of hot distilled water. The complete diet, with the exception of the sources of vitamin B complex, is given in Table 1.

Table 1. *Composition of diet*

Casein	...	...	400 g.
Starch	...	...	1020 g.
Butterfat	...	...	180 ml.
CaCO ₃	...	...	30 g.
KCl	...	...	20 g.
NaHCO ₃	...	...	14 g.
KH ₂ PO ₄	...	...	28 g.
NaCl	...	...	6 g.
MnSO ₄ ·4H ₂ O	...	...	0.2 g.
CuSO ₄ ·5H ₂ O	...	...	0.2 g.
KI	...	...	0.02 g.
Radiostoleum	...	...	2 ml.

Preparation of the diet. The starch, casein and inorganic salts (except those of the three trace elements) were thoroughly mixed. The radiostoleum was dissolved in the melted butterfat and the fat poured into the dry materials; the whole was well mixed until all the fat was blended into the mixture. To this was then added the yeast extract to which had been added the trace elements dissolved in a few ml. of water. After complete mixing the diet was weighed, placed in the cold draught tunnel until the weight was reduced to 2 kg. and then stored in the refrigerator.

The daily ration was prepared by weighing 5 g. of paper pulp and adding to this 10 ml. of the liver extract, 1 ml. of wheat germ oil and a sufficient amount of distilled water so that when 100 g. of the dry diet were added the whole formed a stiff dough. In cases where a Mg-adequate diet was needed 10 ml. of a 7 % MgSO₄·7H₂O solution were also added.

Lister hooded rats were used in Exp. 1 and albino rats in Exps. 2-4. All rats were weaned at 25 days of age and transferred to their experimental diets, only those of good weight and appearance being used.

All animals were housed in galvanized iron cages with screen bottoms and no bedding was used. Rats on the deficient diet were housed individually after the 4th day on the diet to avoid cannibalism which otherwise had been found to occur. To avoid precipitating seizures in rats on deficient diets, all the rats were kept in an almost sound-proof, constant temperature (25°) room. Food and water were offered *ad lib*. All animals, other than those dying in tetany, were killed by coal gas.

Analytical methods

After slaughter the skeleton was isolated quantitatively according to the method of Subrahmanyam *et al.* [1939]. All analyses were performed on a solution of the bone ash in HCl.

Calcium. The estimation was carried out by a slight modification of the method of Clark & Collip [1925], the supernatant washing liquors being siphoned off to prevent the loss of floating particles of Ca oxalate.

Magnesium. The estimation was performed according to the method of Godden & Duckworth [1935].

Phosphorus. The estimation was performed by the method of Fiske & Subbarow [1925].

Exp. 1. Comparison between the synthetic diet supplemented with Mg and the stock diet of the colony

Before proceeding with the actual trials it was necessary to ensure that the diet, as prepared above, when supplemented with nothing but a pure Mg salt, should be adequate for normal growth and reproduction. Proof of this was obtained in the following experiment. Six pairs of female litter-mates were divided into two groups, one of each pair being allotted to each group. Group 1 received the Mg-deficient diet supplemented with Mg (as its sulphate) and glass-distilled water. Group 2 received the stock diet of the Institute colony (details of which have been given by Thomson [1936]) and skimmed milk, tap-water and greenstuff *ad lib.*

Fig. 1. A. Comparison of growth rates of two groups of rats, one receiving the deficient diet supplemented with Mg and the other receiving the stock diet of the main colony. B. Growth rates of rats receiving the Mg-deficient diet supplemented with Mg (as its sulphate) and glass-distilled water until death occurred and of rats given the Mg-adequate diet after 6 days on the deficient diet.

The growth data for these two groups of rats are given in Fig. 1 A. It will be clearly seen that the rats on the synthetic diet which had been supplemented

with nothing but pure Mg sulphate grew normally and had as good a rate of growth as those on the stock diet.

Breeding tests were carried out on the two groups of females, starting at 105 days of age and using the same stock males. Whilst the group on the stock diet bred normally, the does on the synthetic diet plus Mg but without the wheat germ oil did not breed until tocopherol acetate was administered for 10 days. These results indicate that the diet, as shown in Table 1 but without the wheat germ oil, was entirely adequate for normal growth when supplemented with pure MgSO_4 but was deficient in vitamin E. For this latter reason wheat germ oil, in the amount shown, was incorporated in the diet for the remaining experiments.

Exp. 2. The effects of Mg deficiency on the growth and calcification of the skeleton and on the composition of the bone ash

Seven pairs of female and three pairs of male litter-mates of similar weaning weights were allotted, one of each pair to the deficient diet group and the other to the adequate diet group. Each pair continued on experiment until the rat on the deficient diet died, when the corresponding litter-mate on the adequate diet was killed. Such deaths as occurred under observation were typical of Mg deficiency; animals found dead had, as judged by the position and posture of the cadaver, died similarly.

After death the left femur, tibia, fibula, radius, ulna, humerus and scapula were dissected free from adhering tissue and analysed separately. The remainder of the skeleton was cleaned according to the technique recorded above. The separate analysis of the left leg bones was carried out to obtain data for the amount of organic matter in the skeletons; these cannot be obtained from trypsin-cleaned skeletons owing to partial hydrolysis of the organic matrix by the trypsin.

In this and the two subsequent experiments it was found that in all cases the rats on the synthetic diet deficient in Mg failed to grow normally and after about 4 days there was cessation of growth and, in animals with longer survival periods, a tendency to lose weight. Restitution of Mg to the diet after 6 days on the deficient diet produced almost normal rates of growth. These are illustrated in Fig. 1B where a curve showing the average gains from weaning weight during the period on the experimental diets is given. This curve has been constructed from the combined data of Exps. 2-4.

There was less growth of skeleton in deficient animals which survived for longer periods on the diet than in their litter-mate controls. Owing to the normal variation between litter-mates the small differences between animals with short survival periods cannot be regarded as significant. In all cases there was a poorer degree of calcification of the organic matrix in the skeletons of rats fed on the deficient diet than in controls and this difference was statistically significant. The reduction in calcification bore no relation to survival period.

The amount of Mg in the skeletons of rats fed on the deficient diet was in all cases less than that of litter-mate controls. In the two animals which survived the longest (D 9 and D 10) the values were minimal (see Table 2).

The percentage of Ca in the skeletal ash was, with one exception, always somewhat higher in the deficient rat than in its litter-mate control; the mean difference was statistically significant. There was no significant difference in the percentage of P in the skeletal ash of the two groups. The percentage of Mg in the ash was lower with increasing survival on the deficient diet and was generally about half that of the corresponding control animal (see Table 2).

Table 2. *Exp. 2. Ca, P and Mg contents of the total skeletal ash and the percentage ash in the leg bones of rats receiving diets deficient or adequate in Mg*

Rat no.	Days on diet	Wt. skeletal ash mg.	Total mg. in ash of			% in ash of			Leg bones	
			Ca	P	Mg	Ca	P	Mg	Wt., dry fat-free mg.	% ash
D 1	6	1608	609	293	9.2	37.8	18.2	0.57	371	53.5
D 2	9	1622	618	290	8.3	38.1	17.9	0.51	343	52.6
D 3	9	1630	631	293	11.9	38.7	18.0	0.73	379	53.0
D 4	9	1761	699	320	9.1	39.7	18.2	0.52	407	52.7
D 5	9	1836	681	324	9.1	37.0	17.7	0.50	436	53.0
D 6	10	1684	661	307	8.4	39.3	18.2	0.50	379	54.2
D 7	10	1846	718	336	9.0	38.9	18.2	0.49	415	56.9
D 8	11	1900	731	338	9.4	38.5	17.8	0.49	416	56.9
D 9	16	1615	635	296	6.4	39.3	18.3	0.39	346	54.3
D 10	23	1915	756	341	6.6	39.5	17.8	0.34	452	55.2
Average						38.7	18.0			54.2
S.D.						±0.8	±0.2			±1.5
A 1	6	1552	585	288	14.1	37.6	18.6	0.91	322	56.7
A 2	9	1585	592	287	16.1	37.3	18.1	1.01	344	56.4
A 3	9	1784	686	320	15.2	38.4	17.9	0.85	399	55.8
A 4	9	1979	744	354	18.2	37.6	17.9	0.92	428	57.4
A 5	9	2041	765	369	18.4	37.5	18.1	0.90	429	56.6
A 6	10	1946	751	356	17.1	38.6	18.3	0.88	411	57.3
A 7	10	2007	776	361	17.8	38.7	18.0	0.89	348	57.6
A 8	11	2045	771	376	18.8	37.7	18.4	0.98	416	57.1
A 9	16	2579	978	471	21.3	37.9	18.3	0.83	497	59.6
A 10	23	3261	1246	582	24.3	38.2	17.8	0.74	756	57.9
Average						38.0	18.1			57.2
S.D.						±0.5	±0.2			±1.0

Rats on deficient diet numbered D 1 to D 10; corresponding litter-mates on adequate diet numbered A 1 to A 10.

Exp. 3. The effect of Mg deficiency and subsequent restitution of Mg in the diet on the composition of femora of rats

Five litter-mates of both sexes and of closely similar body weight were selected from each of 8 litters. The five chosen from each litter were allotted one to each of the groups described below, the distribution being arranged so that each group contained 4 male and 4 female rats. The groups were treated as follows:

- Group 1. Rats were killed at the time of weaning (25 days).
- Group 2. Rats received the deficient diet for 6 days and were then killed.
- Group 3. Rats received the adequate diet for 6 days and were then killed.
- Group 4. Rats received the deficient diet for 6 days after which they received the adequate diet for 10 days; they were then killed.
- Group 5. Rats received the adequate diet for 16 days and were then killed.

At death the femora were dissected free from all adhering flesh. After alcohol and ether extraction the eight femora from each group were bulked and the analyses were carried out. The results are shown in Table 3.

There was very little difference between the dry fat-free weight of the bulked femora of the 6-day deficient and of the 6-day adequate groups. Unexpectedly, a greater difference appeared between Group 4 (6-days deficient followed by 10-days adequate) and Group 5 (16-days adequate). As was found in Exp. 2, there was a lower percentage of calcification in the deficient animals than in the adequate controls. Since the weights of dry fat-free bone were the same this

Table 3. *Exp. 3. The ash and Ca, Mg and P contents of the bulked femora of groups of rats receiving diets deficient and/or adequate in Mg*

Group	Wt., dry fat-free mg.	Wt. of ash mg.	% of ash	Total mg. in ash of			% in ash of		
				Ca	P	Mg.	Ca	P	Mg
1. Weaning controls	536	264	49.2	97.2	48.4	2.82	36.8	18.3	1.07
2. 6 days deficient	822	415	50.5	161.4	74.1	1.95	38.9	17.8	0.47
3. 6 days adequate	826	433	51.8	165.6	79.0	3.79	38.2	18.2	0.88
4. 6 days deficient and 10 days adequate	1290	713	55.2	279.5	129.8	5.76	39.2	18.2	0.81
5. 16 days adequate	1356	755	55.7	290.6	135.0	6.52	38.5	17.9	0.86

indicated an increased growth of organic matrix during the first 6 days. The difference in the percentages of ash was somewhat reduced after 10 days on the adequate diet after the deficiency period, as shown by the data for Groups 4 and 5, thus indicating an improvement in the degree of calcification of the matrix.

Group 2 showed deposition of Ca and P when compared with the weaning value, Group 1. The Mg values indicated that during the 6-day deficiency period there was marked mobilization of Mg (0.87 mg.) and the difference between Groups 2 and 3 was 1.84 mg. During the 10-day period of adequacy, the amount of Mg deposited in Group 5 was 2.73 mg. more than that in Group 3. Group 4, during a similar period, deposited 3.81 mg. more than its parallel control Group 2, although the deposition of organic matrix and bone salt was less than in the case of Group 5. Group 1 showed a Ca : Mg ratio of 34.5, Group 2 of 82.8, Group 3 of 43.7, Group 4 of 48.5 and Group 5 of 44.6. The large reduction in total bone Mg in Group 2 was not made up in 10 days on the supplemented diet, as indicated by the results for Group 4. The Ca : Mg ratio of the amounts of Ca and Mg deposited during the last 10 of the 16 days on the adequate diet (i.e. Group 5 values minus Group 3 values) was 45.8. On the basis of this same ratio it may be calculated from the amount of Ca deposited by the animals of Group 4 that they should have deposited 2.58 mg. Mg during the 10 days of adequate diet, and this amount added to the value 1.95 for the amount of Mg in Group 2 gives the figure 4.53 mg. Mg for the total amount which might be expected at the end of 16 days in Group 4 animals. This is 1.23 mg. less than the amount actually found in Group 4, this 1.23 mg. representing the extra Mg deposited by Group 4 over and above that deposited by the controls. It is realized that such a calculation is open to the criticism that the Group 4 bone development lagged slightly behind the Group 5 development but the difference cannot vitiate the conclusion that the changes were indicative of the replenishment of a reserve. This is discussed below.

*Exp. 4. The effect of small variations in the Mg content of
Mg-deficient diets on the composition of skeletal ash*

Four female rats from each of 6 litters were divided among 6 groups. The division was such that one rat from each litter was allotted to Group 1 and another to Group 6. The remaining two rats were placed one in each of 2 of the 4 intermediary groups. The groups were as follows:

Group 1.	Deficient diet	(Total Mg, 6 p.p.m.)
Group 2.	„ + 3 p.p.m. Mg	(Total Mg, 9 p.p.m.)
Group 3.	„ + 6 p.p.m. Mg	(Total Mg, 12 p.p.m.)
Group 4.	„ + 9 p.p.m. Mg	(Total Mg, 15 p.p.m.)
Group 5.	„ + 12 p.p.m. Mg	(Total Mg, 18 p.p.m.)
Group 6.	„ + 700 p.p.m. Mg	

Group 6, by this addition of Mg, became the adequate diet as in all the preceding experiments.

All rats were killed after 6 days on the diets. All the rats of each litter were placed together and subjected to an auditory stimulus to precipitate a convulsive seizure except such animals as spontaneously reacted to handling alone. The results of the stimulation are included in Table IV.

Table 4. *Exp. 4. Skeletal ash and Ca, P and Mg of the ash of rats receiving diets adequate and deficient in Mg*

Rat no.	Mg in 100 g. diet mg.	Skeletal ash mg.	Total mg. in ash of			% in ash of		
			Ca	Mg	P	Ca	Mg	P
1-L 1*	0.6	1499	580	9.0	281	39.0	0.60	18.9
1-L 2†	0.6	1297	493	7.5	242	38.0	0.58	18.6
1-L 3	0.6	1298	493	7.5	247	38.0	0.58	19.0
1-L 4*	0.6	1395	530	8.7	248	38.0	0.62	17.8
1-L 5*	0.6	1406	530	8.2	256	37.7	0.59	18.2
1-L 6†	0.6	1151	434	6.2	201	37.7	0.54	17.4
2-L 1†	0.9	1615	623	9.3	296	38.6	0.58	18.3
2-L 2*	0.9	1334	509	7.8	250	38.2	0.58	18.7
2-L 3†	0.9	1348	509	8.2	256	37.8	0.60	19.0
3-L 1*	1.2	1439	561	8.6	269	39.0	0.60	18.7
2-L 4	1.2	1383	530	8.2	247	38.3	0.60	17.9
2-L 5	1.2	1532	581	8.9	274	37.9	0.58	17.9
3-L 2†	1.5	1320	501	7.8	252	38.0	0.59	19.1
3-L 4†	1.5	1437	543	7.6	256	37.8	0.53	17.8
2-L 6*	1.5	1540	589	8.2	267	38.2	0.53	17.3
3-L 3	1.8	1360	509	7.9	254	37.4	0.58	18.7
3-L 5	1.8	1431	543	7.8	253	37.9	0.54	17.7
3-L 6*	1.8	1473	556	7.8	256	37.7	0.53	17.4
4-L 1	70.0	1594	604	15.0	301	37.9	0.94	18.9
4-L 2	70.0	1485	552	15.2	279	37.2	1.02	18.8
4-L 3	70.0	1331	497	13.2	252	37.3	0.99	18.9
4-L 4	70.0	1537	573	13.3	280	37.3	0.86	18.2
4-L 5	70.0	1565	585	13.5	282	37.4	0.86	18.0
4-L 6	70.0	1561	581	13.8	274	37.2	0.88	17.6

In the serial numbers the first digit refers to the numbers of the rat within the litter and the second digit to the number of the litter.

* Seizure induced by auditory stimulus.

† Seizure occurred spontaneously.

Those not marked did not react to prolonged stimulus.

Spontaneous seizures were not restricted to animals on the lowest Mg intake (6 p.p.m.), in fact one animal in this group (1-L 3) did not react to prolonged stimulation. Again, while no spontaneous seizures occurred in the groups receiving 12 and 18 p.p.m. (and only in one case in each group could seizures be induced by auditory stimulus), the intermediary group, receiving 15 p.p.m. Mg, contained two cases of spontaneous and one case of induced seizures. This variability in response suggests that the individual susceptibility of the animal overshadows the effect of variations in Mg intake at low levels.

The data in this experiment confirm those of previous ones. Although Groups 2-5 contained too few rats for a comparison of the averages with that of Group 6, it was noted that deficient rats in any one litter had higher percentages of Ca in the bone ash than the corresponding litter-mates fed on the adequate diet.

The point of greatest interest is, of course, the amount of skeletal Mg. Inspection shows that the rats in Groups 1-5 inclusive had about half the

skeletal Mg of rats in Group 6. Although Group 5 received 3 times the Mg intake of Group 1 the total skeletal Mg values and the percentages of Mg were about the same. Differences of the order used in this experiment were without effect on skeletal Mg.

DISCUSSION

Orent *et al.* [1934] reported experiments on bone growth in rats receiving a diet containing only 0.00018% Mg (1.8 p.p.m.). They stated that there was a marked acceleration in bone growth caused mainly by excessive deposition of Ca. This acceleration was found to occur during the first 5 days on the diet; the subsequent course of bone growth did not differ from that of the control animals although the initial difference was maintained. It was also found that in spite of the low intake of Mg the bone Mg increased during the experimental period, although at a rate less than that of the control animals.

It is felt that this accumulation of Ca in the bone is remarkable since the increase in the percentage of P was not proportional and large depositions of Ca without a corresponding increase in anion are difficult to understand unless a fundamental change in the composition of bone is postulated. Further, the accumulation of Mg in the skeleton during a period when practically no Mg was contributed by the diet seems to us biologically unlikely since it involves a gain in Mg by a principally structural organ at the expense of functional organs. This is even more remarkable when it is considered that these authors had previously reported [Kruse *et al.* 1932] that their animals gained weight on the deficient diet; the amount of Mg available per unit weight of tissue would thus have been even further reduced. Also, this conception that Mg moves to the skeleton in spite of almost complete absence of Mg from the diet and in the face of increasing weight of the soft tissues leads to the conclusion that the Mg of the soft tissues has at least the partial function of a reserve upon which the skeleton could draw to remedy transitory insufficiencies. Such a view seems improbable.

Finally, the data presented are open to certain criticisms. The bone ash analyses show erratic and, in some cases, extremely low Ca : P ratios. The ratio, for example, of the control animals at weaning was given as 1.01 whereas normal animals generally approximate to 2.00. A further criticism is that the sum of the oxides of Ca, Mg and P ($\text{CaO} + \text{MgO} + \text{P}_2\text{O}_5$) generally accounts for less of the ash than is reported by other workers, the oxides accounting for only 83% of the ash in the case of the weaning control animals instead of the usually accepted amount of about 96% [e.g. Hammett, 1925, 1, 2, 3]. Also, the values given for the Mg content of the bone ash of weaning controls (which must be considered as basic to any investigation of reserve mobilization) were only two-thirds of those recorded by Hammett. Our data are in agreement with those of Hammett.

In the present experiments the symptomatology of the deficiency was similar to that reported by other workers on Mg deficiency. Peripheral vasodilatation was observed early in the experiment, its maximum severity being reached by the 4th day and thereafter gradually diminishing until it had completely disappeared by the end of the 5th day. Kruse *et al.* [1932] found that the first appearance of vasodilatation occurred between the 3rd and 5th days and increased in intensity until the 12th day, after which it gradually disappeared. These authors also found that the 11th day was the earliest upon which convulsions occurred, the average duration of deficiency before the first seizure being 18 days. In our case we found that convulsions either occurred

spontaneously or, generally, could be induced from the 6th day onwards. Very few animals survived beyond 12–15 days although occasional subjects lived beyond 20 days. The additional symptoms observed in cases with longer survival periods were very similar to those reported by Tufts & Greenberg [1937–8, 1], namely, ringed-eye dermatitis, roughness of the coat and, in one case surviving 23 days, oedema. In general appearance and response to restitution of Mg in the diet, our rats were identical with those photographically recorded by these workers.

Since the rats in the present investigation had a much shorter survival period than those in the researches of Kruse *et al.* [1932], and of Tufts & Greenberg [1937–8, 1] the explanation which might immediately suggest itself is that there was an additional deficiency to that of Mg. This, we feel, is unlikely because (1) rats fed on the diet supplemented with Mg showed normal growth rates, and (2) the symptomatology did not differ from that found by other workers. The explanation for the difference between a complete Mg deficiency syndrome requiring 6–9 days and one requiring 4–5 weeks must be sought elsewhere and this, we suggest, is in the Ca : P ratio of our diet as compared with that used by other workers, together with the low level of Mg intake which we attained.

The outstanding metabolic change in animals on Mg-deficient diets is the disturbance in Ca metabolism; this has been reported both in rats [Tufts & Greenberg, 1937–8, 1, 2] and in calves [Moore *et al.* 1938]. There is an abnormal deposition of Ca in the soft tissues and, consequently, any factor which would accelerate or intensify disturbances in Ca metabolism might reasonably be expected to increase either the severity or rapidity of onset of the syndrome of Mg deficiency. Thus, Tufts & Greenberg [1937–8, 1, 2] found convulsions occurring earlier on a diet containing 1.19 % Ca, 0.60 % P and 0.005 % Mg than on a diet containing 0.88 % Ca, 0.68 % P and only 0.002 % Mg.¹ Throughout their experiments these authors found that an increase in the Ca intake increased the requirements of Mg for survival and normal development. In our case the intakes were 0.60 % Ca, 0.30 % P and only 0.0006 % Mg. On account of the above observations together with the recognized influence of the ratio Ca : P upon the metabolism of these two elements we feel that the contraction of the syndrome period which we found is best interpreted as an effect of a higher Ca : P ratio together with a low Mg intake.

The effect of Mg deficiency on the growth of the skeleton was slight during the first 6 days of deficiency. There was, however, a reduction in the ratio of inorganic matter to organic matrix during this period (Exp. 3) but the condition did not become progressively worse with longer periods on the deficient diet (Exp. 2). Judging from the total skeletal ash and from the reduction which occurred in food consumption this cessation of growth and maintenance of the slightly imperfect degree of calcification might have been caused by inanition. The animals with longer survival periods had lower amounts of skeletal ash than their litter-mates on the adequate diet. This observation is contrary to that of Orent *et al.* [1934] who found that Mg deficiency increased bone growth and also increased the percentage of ash in bone.

Mean values for the percentage of Ca in skeletal ash showed that throughout all experiments the Mg-deficient animals had higher values than adequately fed

¹ Tufts & Greenberg do not give a complete analysis of their diet. The above values are calculated from the composition of the salt mixture used. The amounts of Ca and P contributed by their other dietary constituents would not, however, be sufficient seriously to affect the above values.

litter-mates. In only one individual case was this otherwise. The mean differences were slight (0.5-0.7 %) but significant. Since this occurred concomitantly with a reduction in the amount of Mg in the ash it might be suggested that a replacement of mobilized Mg by Ca took place, especially since the amounts were closely similar when compared on the basis of equivalents. While this may be correct, and, in view of the studies of Gruner *et al.* [1937] it seems probable, it cannot be regarded as completely established by the data since the differences involved were close to the analytical error of the method in the case of Ca. Also, restitution of Mg in the diet in Exp. 3 did not abolish the difference in the percentages of Ca in the bone ash; but again the difference lay close to the analytical error of the method used. Incidentally the increase in the percentage of Ca was only one-quarter of that reported by Orent *et al.* [1934].

About one-third of the skeletal Mg was lost from the skeleton during the first 6 days on the deficient diet. Among the animals surviving 11 days or less on the deficient diet (Exp. 2) the differences in the total skeletal Mg bore no relation to the length of the survival period. There was a tendency for gradual reduction in the percentage of Mg in the skeletal ash, but such a reduction cannot be interpreted as evidence of slight continued mobilization since it could, and probably did, arise through small depositions of Mg-low, or even Mg-free, bone salt. The animals with the longest survival periods (D 9, 16 days; D 10, 23 days) showed the lowest total skeletal Mg, both values being well below those of animals dying earlier on the diet. From these data, together with the data for the first 6 days in Exp. 3, the conclusion can be drawn that about one-third of the skeletal Mg can be easily mobilized; whether the remainder can be mobilized in periods of longer survival is not yet clear. Unless it is argued that cessation of growth reduced the demand of the soft tissues for Mg after the first 6 days on the deficient diet, the conclusion can be drawn that a difference in the availability of the two fractions of skeletal Mg points to a difference in the forms in which they occur in bone.

That a portion at least of bone Mg functions as a reserve is illustrated by the results of Exp. 3. Restitution of Mg to the diet resulted in a deposition of Mg in relation to Ca considerably beyond that required for the formation of normal bone salt. The increment deposited above that required for the newly formed bone salt appears therefore to have fulfilled the role of replenishment of a partially exhausted reserve.

It will be seen from the above that our results are contrary to those of Orent *et al.* [1934]. Their observation that the skeleton not only does not act as a reserve for Mg but that actual deposition of Mg occurs in acute Mg deficiency is not supported. Our present view is, to some extent, supported by the work of Tufts & Greenberg [1937-8, 1, 2]. These workers found that the Mg of soft tissues was only slightly reduced in acute Mg deficiency, that total body Mg was usually not reduced and that a fairly good body growth was maintained up to the time of hyperirritability. Their data do not permit of recalculation, but the inference from their observations is that the Mg of the soft tissues, under conditions of constant total body Mg, must have been maintained at the expense of the skeleton. These and our own results do not support the view of Orent *et al.* [1934] that "bone has a stronger attractive force than any other tissue for Mg".

A further observation of Orent *et al.* [1934] was that skeletal Mg was, in part, mobilized during convulsions and that this movement was sufficiently rapid to be completed within 2 min. Exp. 4 offered an opportunity to confirm the observation. We were unable to find any difference between the Mg content of the skeletons of animals dying in convulsions, spontaneous or induced, and that

of animals in which convulsions could not be induced. In order to avoid as far as possible any effect being produced by coal gas the animals which did not develop convulsions were gassed and decapitated as soon as they were unconscious. The mean amount of Mg mobilized was about 0.04 mg. for the hind legs alone and this is equivalent to a mobilization for the whole skeleton of about 3 mg. Mg. The increases which these workers found in blood Mg at the time of seizure were slight and, even if the interstitial fluid is taken into account as well, the changes only account for a very small fraction of the bone Mg said to have been mobilized.

The variability in the response to auditory stimuli on the 6th day of deficiency on the part of the deficient rats in Exp. 4 suggests that the incidence of tetany in Mg deficiency is not referable simply to disturbance in body fluid Mg. The differences observed ranged from spontaneous fatal seizure to complete failure to react to prolonged stimulation. Greenberg & Tufts [1938] found that injection of Mg several hours before stimulation did not prevent seizures; they showed in further studies that a mid-brain lesion was involved. Our results agree better with such a view than with the conception that the tetany of Mg deficiency is the outcome of disturbances in tissue fluid composition arising from the deficiency.

In the present experiments it has been noted that rats surviving for longer periods have more fragile bones than normal rats of the same age or, in fact, of much younger rats which have been reared on adequate rations. In this observation we confirm Lavollay [1936], who observed the same increase in fragility under conditions of Mg deficiency. He was, however, unable to find any evidence of abnormality on roentgenographic examination. In addition to the fragility we noticed that the ashes of bones presented an unusual appearance in that there was extensive "pitting". Since the degree of calcification of the organic portion of the bone is only slightly affected in Mg deficiency it seems probable that the fragility arises from other causes. In preliminary studies of the histology of the bones we have obtained some evidence of disordered growth of the organic matrix of the shaft of the leg bones and it seems possible that this may explain the increased fragility observed.

SUMMARY

A method of preparing a Mg-deficient diet is described.

It has been shown that the skeleton can, under conditions of acute Mg deficiency at least, function as a reserve of this element upon which the rat can make large drafts. This effect is reversible as shown by experiments in which Mg was restored to the diet.

REFERENCES

- Clark & Collip (1925). *J. biol. Chem.* **63**, 461.
- Duckworth (1938-9). *Nutr. Abstr. Rev.* **8**, 841.
- Fiske & Subbarow (1925). *J. biol. Chem.* **66**, 375.
- Godden & Duckworth (1935). *Biochem. J.* **29**, 445.
- Greenberg & Tufts (1938). *Amer. J. Physiol.* **121**, 416.
- Gruner, McConnell & Armstrong (1937). *J. biol. Chem.* **121**, 771.
- Hammett (1925, 1). *J. biol. Chem.* **64**, 409.
- (1925, 2). *J. biol. Chem.* **64**, 685.
- (1925, 3). *J. biol. Chem.* **64**, 693.
- Kruse, Orent & McCollum (1932). *J. biol. Chem.* **96**, 519.
- Lavollay (1936). *Actualités Scientifiques et Industrielles*, no. 420.
- Moore, Hallman & Sholl (1938). *Arch. Path. Lab. Med.* **26**, 820.
- Orent, Kruse & McCollum (1934). *J. biol. Chem.* **106**, 573.
- Subrahmanyam, Duckworth & Godden (1939). *Biochem. J.* **33**, 1421.
- Thomson (1936). *J. Hyg., Camb.*, **36**, 24.
- Tufts & Greenberg (1937-8, 1). *J. biol. Chem.* **122**, 693.
- — (1937-8, 2). *J. biol. Chem.* **122**, 715.