The effect of copper on the mineralization of bones of mice fed on a meat diet

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It has been reported that the bone ash of young mice kept for 6 weeks on a diet composed entirely of muscle meat was markedly reduced; osteoporosis could be demonstrated radiologically as well as histologically (Ilan, Schwartz & Guggenheim, 1962; Ulmansky, 1964). The disorder was completely prevented by the addition of calcium carbonate or, to a lesser degree, by replacement of one-quarter of the meat by beef liver. Since liver contains more copper than muscle, the meat diet was supplemented with 20 mg Cu/kg. This supplement increased considerably the proportion of bone ash. It is noteworthy that Cu exerted this effect on the mineralization of bone when added to a basal diet, i.e. meat, that is poor in calcium and rich in phosphorus. It was thought that this effect of Cu deserved further investigation. Mice were therefore maintained on a meat diet to which Cu had been added. After various times their femurs were analysed for bone ash, calcium, phosphorus and manganese, and the incorporation of radioactive Ca was measured.

METHODS

The study involved 432 male Swiss mice. They came from a stock maintained, without inbreeding, at the Hebrew University for over 15 years. They were 3 weeks old and weighed 10–13 g. They were divided into six groups. The diets consisted of raw lean beef muscle, which was offered either unsupplemented (first group) or supplemented with Cu or Ca. Thus, the diets of groups 2–5 were supplemented with 2·5, 5·0, 10·0 and 20·0 mg Cu (as $CuSO_4$)/kg and that of group 6 with 3·6 g Ca (as $CaCO_3$)/kg. A similar supplement (3·3 g/kg) of Ca as $CaCO_3$ had previously been found to induce normal mineralization of bones in mice fed on this diet (Ilan *et al.* 1962; Ulmansky, 1964). The Ca content of the meat was examined in twelve samples and found to be 95 (standard error: 5·9) mg/kg.

Twenty-four mice of each group, i.e. one-third, were killed after 2 weeks and twenty-four mice each after 4 and 6 weeks. The femurs were cleaned of adherent tissue and weighed. They were then broken, extracted for 6 h with alcohol in a Soxhlet apparatus, dried to constant weight and ashed at 600°. In the ash, Ca (Baron & Bell, 1959), P (Fiske & Subbarow, 1925) and Mn (Gates & Ellis, 1947) were determined. P and Mn were determined in the bones of eighteen mice only from each group. Measured portions of the ash of three pairs of femurs were pooled. The figures shown in the tables therefore indicate means of six samples, each comprising three pairs of femurs.

In six animals of each group the uptake of radioactive Ca by bone was studied. These six mice had received at the end of their periods of observation a subcutaneous injection of $^{45}CaCl_2$. Each mouse was injected with 0·1 μc in 1·0 mg Ca per 10 g body-weight. On the next day the animals were killed, and their femurs were ashed at 600°. The ash was dissolved in 1 N-HCl, and a measured portion was taken for determination of radioactivity. The results are expressed as μg of dose administered per g of fresh bone or per 100 mg of bone Ca.

RESULTS

The results obtained after observation periods of 2, 4 and 6 weeks are presented in Tables 1–3.

Even after 2 weeks the effects of supplementing the meat with Cu were discernible. Weight increase was raised; with 20 mg Cu added/kg meat it almost equalled that of mice maintained on the diet enriched with Ca. Bone weight likewise was higher when the meat diet had been supplemented with Cu. The mean weight of both femurs of mice that had subsisted on the unsupplemented diet was 68 mg. It increased to 84–97 mg when Cu had been added to the diet and to 98 mg on the meat diet supplemented with Ca. Figures for ash, Ca and P generally increased with increasing amounts of Cu added. With the highest Cu supplement, however, ash content slightly decreased, and the percentage of Ca and P in fresh bone likewise declined. The percentages of Ca and P in the bone ash of mice whose diet had been supplemented with Cu were similar to or even slightly higher than those of mice fed on the diet enriched with Ca. Fortification of meat with Cu resulted in a lower incorporation of ^{45}Ca in bone and in bone Ca. Mice that had received the highest Cu supplement did not incorporate significantly more Ca into bone than did mice given the diet enriched with Ca. Addition of Cu to meat increased not only Ca and P in bones, but also Mn. Ash and bone of mice on the Cu-supplemented diet contained significantly more Mn than did those of control mice. Moreover, addition of Cu caused a much greater increase of Mn, both in ash and in fresh bone, than of Ca and P.

The effects of adding small amounts of Cu to meat were more evident when the observation period was prolonged to 4 or 6 weeks (Tables 2 and 3). After 4 weeks, the weight increase of the mice was significantly raised by the addition of only 2·5 mg Cu/kg meat. Moreover, supplementation of meat with Cu induced a considerable weight increase between the 2nd and the 4th week and between the 4th and the 6th week; the growth performance of the mice receiving the highest Cu supplement resembled that of mice given the Ca-enriched diet. On the other hand, the mice kept on the unsupplemented diet almost ceased growing after the 2nd week.

The increases in Ca and P contents of bone ash resulting from supplementation with Cu did not greatly differ after 6 weeks from those observed after 2 and 4 weeks. However, the concentrations of these minerals in fresh bone increased markedly with prolongation of the observation period. These increases were accompanied by lowered incorporation of ^{45}Ca in fresh bone and in bone Ca.

Table 1. *Composition of bones and uptake of* ^{45}*Ca by femurs of mice maintained for 2 weeks on a meat diet, unsupplemented or supplemented with copper or calcium*

	Mean value on diet with supplement (mg/kg meat) of:								
	No supplement	2·5 Cu	5·0 Cu	10·0 Cu	20·0 Cu	3600 Ca	SE of mean	No. of pairs of femurs in each sample	No. of samples in each mean
Weight increase (g)	4·6	6·1	6·9	6·3	7·0	7·8	±1·04	—	24
Weight of femurs (mg)	68	84	97	89	92	98	±5·77	1	24
Ash: mg	13·7	17·3	19·4	23·8	21·1	28·4	±1·98	1	24
g/100 g fresh bone	20·2	20·6	22·0	23·8	22·9	29·0	±0·30	1	24
Ca: g/100 g ash	30·4	35·6	35·7	35·8	36·0	35·1	±0·18	1	24
g/100 g fresh bone	6·1	7·0	7·8	8·5	8·3	10·2	±0·18	1	24
Ca incorporated:									
μg/g fresh bone	37·6	27·8	29·2	28·0	27·5	30·4	±0·12	1	6
mg/100 g bone Ca	62	40	37	34	33	30	±0·34	1	6
P: g/100 g ash	15·7	17·9	18·1	18·1	18·3	17·7	±0·15	3	6
g/100 g fresh bone	3·2	3·6	4·0	4·4	4·2	5·2	±0·11	3	6
Mn: mg/100 g ash	490	710	743	623	830	411	±14	3	6
mg/100 g fresh bone	98	145	163	151	190	119	±14	3	6

Table 2. *Composition of bones and uptake of* ^{45}*Ca by femurs of mice maintained for 4 weeks on a meat diet, unsupplemented or supplemented with copper or calcium*

	Mean value on diet with supplement (mg/kg meat) of:								
	No supplement	2·5 Cu	5·0 Cu	10·0 Cu	20·0 Cu	3600 Ca	SE of mean	No. of pairs of femurs in each sample	No. of samples in each mean
Weight increase (g)	4·4	8·5	8·6	7·8	9·6	9·3	±1·30	—	24
Weight of femurs (mg)	97	114	108	105	125	129	±7·10	1	24
Ash: mg	19·0	22·2	23·9	25·3	31·5	43·5	±2·48	1	24
g/100 g fresh bone	19·6	19·5	22·2	24·1	25·2	33·3	±0·25	1	24
Ca: g/100 g ash	28·9	36·4	37·1	37·0	37·4	37·4	±0·14	1	24
g/100 g fresh bone	5·6	7·0	8·3	9·0	9·4	12·6	±0·10	1	24
Ca incorporated:									
μg/g fresh bone	35·0	27·8	27·8	26·5	26·3	24·7	±0·14	1	6
mg/100 g bone Ca	62	40	34	30	28	20	±0·50	1	6
P: g/100 g ash	15·0	18·7	18·9	18·8	19·0	18·8	±0·11	3	6
g/100 g fresh bone	2·9	3·6	4·2	4·5	4·8	6·4	±0·07	3	6
Mn: mg/100 g ash	474	735	733	686	851	404	±0·18	3	6
mg/100 g fresh bone	92	143	163	167	215	138	±0·13	3	6

Table 3. *Composition of bones and uptake of ^{45}Ca by femurs of mice maintained for 6 weeks on a meat diet, unsupplemented or supplemented with copper or calcium*

	Mean value on diet with supplement (mg/kg meat) of:								
	No supplement	2·5 Cu	5·0 Cu	10·0 Cu	20·0 Cu	3600 Ca	SE OF mean	No. of pairs of femurs in each sample	No. of samples in each mean
Weight increase (g)	5·4	12·3	10·2	11·7	13·1	13·5	±1·40	—	24
Weight of femurs (mg)	98	129	96	104	108	141	±5·71	1	24
Ash: mg	19·8	27·6	24·3	27·9	27·9	44·0	±1·88	1	24
g/100 g fresh bone	20·4	21·4	25·2	26·8	25·8	31·2	±0·29	1	24
Ca: g/100 g ash	32·9	36·3	36·2	36·8	38·3	41·2	±0·32	1	24
g/100 g fresh bone	6·7	7·7	9·1	9·9	9·9	12·9	±0·18	1	24
Ca incorporated:									
μg/g fresh bone	33·1	27·8	26·1	25·0	20·9	21·3	±0·21	1	6
mg/100 g bone Ca	50	36	27	25	21	17	±0·37	1	6
P: g/100 g ash	17·6	10·3	19·6	19·7	20·8	20·9	±0·29	3	6
g/100 g fresh bone	3·5	4·1	4·9	5·3	5·3	6·5	±0·17	3	6
Mn: mg/100 g ash	530	795	801	800	860	475	±14	3	6
mg/100 g fresh bone	109	166	201	210	217	148	±14	3	6

Mn content of ash and fresh bone tended to increase, particularly after 6 weeks.

It therefore appears that Cu added to meat improves mineralization of bone of mice fed upon meat.

DISCUSSION

Our findings indicate that mice maintained on a diet consisting of beef muscle develop a severe bone disorder; bone ash and concentrations of Ca and P in both fresh bone and bone ash decrease, and the incorporation of ^{45}Ca in bones decreases. This disorder is completely prevented by the addition of Ca to the meat. Bone ash and the concentrations of Ca and P in bones and ash of mice subsisting on a meat diet enriched with Ca resemble those found in mice raised on a presumably normal stock diet (Guggenheim & Tal, unpublished).

Adding 2·5–20·0 mg Cu/kg meat considerably improved growth and mineralization of bones. It increased not only the amount of bone ash and the contents of Ca and P in fresh bones and in ash, but also that of another mineral with an affinity for bone tissue and so tending to accumulate in bone, i.e. Mn (Underwood, 1962, p. 189). The effect of Cu was generally greater when the quantity added to meat was increased and when the observation period was prolonged. The amounts of ash, Ca and P in bones as well as the uptake of ^{45}Ca by bones of animals receiving the highest Cu supplement resembled more closely those of mice kept on meat supplemented with Ca than those of mice fed on unsupplemented meat. The improved mineralization of bones of mice maintained on meat enriched with Cu is probably not the result of the weight gain brought about by the supplement. Rats kept on the meat diet for 9 weeks do not increase their weight more when 20 mg Cu/kg of meat are added, though this supplement leads to a significant increase in bone Ca and P and a marked decrease in uptake of injected ^{45}Ca (Tal & Guggenheim, unpublished).

The deleterious effect exerted by a meat diet on the skeleton of kittens (Scott, Greaves & Scott, 1961) and of rats (Moore & Sharman, 1960) has been reported. Growth of rats ceased after a few weeks, and the bones were undersized, had thin walls and a low ash content and were often fractured. The disorder could be prevented by adding adequate amounts of Ca to meat (Moore, Sharman, Constable, Symonds, Martin & Collinson, 1962). Our results indicate that not only Ca is involved. Meat is a poor source of Cu, containing only 1·3 mg/kg (Guggenheim, Ilan, Fostick & Tal, 1963). Rats fed upon meat are, therefore, liable to develop Cu deficiency (Moore, 1962).

Bone defects resulting in spontaneous fractures have been described in sheep and cattle grazing on Cu-deficient pastures (Underwood, 1962, p. 75). Histological studies of dogs made deficient in Cu (Baxter & Van Wyk, 1953; Baxter, Van Wyk & Follis, 1953) indicated that there was an excessive resorption of bone, with decreased deposition of bone matrix. The ash content remained normal, however, probably because the diet supplied sufficient Ca.

Muscle meat is not only poor in Ca and Cu but is rich in Zn (Guggenheim, 1964). Intestinal absorption of Zn is lowered by Ca (Guggenheim, 1964), and its toxic effects are counteracted by Cu (Underwood, 1962, p. 181). Thus, the anaemia developing in mice kept on a meat diet can be prevented by adding Cu or Ca to the meat. Moreover, mice appear to be more susceptible to the toxic action of Zn than are rats (Guggenheim, 1964). Dietary excess of Zn has been shown to affect weight gain in young rats and to decrease bone Ca and P (Stewart & Magee, 1964). The deleterious effect of the meat diet on bones of mice may, therefore, partly result from the excess of Zn present in meat, which can be counteracted by Ca or Cu. Recently, Moore, Constable, Day, Impey & Symonds (1964) reported that the ash content of femurs of young rats fed for 7 weeks upon meat is low. Addition of 5 mg Cu/kg meat increased it only slightly. In the experiments mentioned above (Tal & Guggenheim, unpublished) supplementation of meat with 20 mg Cu/kg did not affect bone ash of young rats after a 4-week period, but had a small though distinct effect after 9 weeks. The different responses of rats and mice to a meat diet may reside in their different susceptibility to the toxic action of Zn.

The meat diet is also characterized by a high content of protein. The interaction of protein and Ca on growth and composition of bones has recently been studied in young rats (El-Maraghi & Stewart, 1963). Bones of rats kept on a high-protein, low-Ca diet had a lower ash content with a lower radiographic density than bones of rats on a low-protein, low-Ca diet. El-Maraghi & Stewart (1963) conclude that 'an osteoporosis-like condition may therefore be produced in growing animals when protein and Ca intakes are unbalanced'. Thus, demineralization of bones of mice subsisting on the unsupplemented meat diet may be aggravated by the gross imbalance of protein and Ca.

SUMMARY

1. Young mice were fed on muscle meat, which was either unsupplemented or supplemented with 2·5, 5·0, 10·0 or 20·0 mg copper or with 3·6 g calcium per kg. After 2, 4 and 6 weeks the composition of the femurs and their uptake of ^{45}Ca were investigated.

2. Mice maintained on the unsupplemented meat diet ceased growing after the 2nd week. The amounts of ash, Ca and P in their bones were low, and incorporation of ^{45}Ca was high. Mice given the Ca-enriched diet had much more ash, Ca and P in their bones and a lower uptake of ^{45}Ca. The differences increased with prolongation of the experimental period.

3. Mice kept on the diet supplemented with Cu increased in weight during the whole experimental period; their femurs were heavier than those of the mice consuming the unsupplemented meat and contained more ash, Ca, P and Mn. Incorporation of ^{45}Ca was lower, suggesting improved mineralization. The effect of Cu generally increased on increasing the quantity added to meat and on prolonging the experimental period.

4. It is concluded that Cu improves mineralization of bones of mice when they subsist on a meat diet.

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REFERENCES

Baron, D. N. & Bell, J. L. (1959). *J. clin. Path.* **12**, 143.
Baxter, J. H. & Van Wyk, J. J. (1953). *Bull. Johns Hopkins Hosp.* **93**, 1.
Baxter, J. H., Van Wyk, J. J. & Follis, R. H. (1953). *Bull. Johns Hopkins Hosp.* **93**, 25.
El-Maraghi, N. R. H. & Stewart, R. J. C. (1963). *Proc. Nutr. Soc.* **22**, xxx.
Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
Gates, E. M. & Ellis, G. H. (1947). *J. biol. Chem.* **168**, 537.
Guggenheim, K. (1964). *Blood*, **20**, 786.
Guggenheim, K., Ilan, J., Fostick, M. & Tal, E. (1963). *J. Nutr.* **79**, 245.
Ilan, J., Schwartz, A. & Guggenheim, K. (1962). *Metabolism*, **11**, 535.
Moore, T. (1962). *Brit. med. J.* i, 689.
Moore, T., Constable, B. J., Day, K. C., Impey, S. G. & Symonds, K. R. (1964). *Brit. J. Nutr.* **18**, 135.
Moore, T. & Sharman, I. M. (1960). *Brit. med. J.* ii, 1704.
Moore, T., Sharman, I. M., Constable, B. J., Symonds, K. R., Martin, P. E. N. & Collinson, E. (1962). *J. Nutr.* **77**, 415.
Scott, P. P., Greaves, J. P. & Scott, M. G. (1961). *Brit. J. Nutr.* **15**, 35.
Stewart, A. K. & Magee, A. C. (1964). *J. Nutr.* **82**, 287.
Ulmansky, M. (1964). *Amer. J. Path.* **44**, 85.
Underwood, E. J. (1962). *Trace Elements in Human and Animal Nutrition*, 2nd ed. New York: Academic Press Inc.

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