The manganese and copper of chick bone

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Both manganese and copper are known to influence the metabolism of bone in the chick (Wilgus, Norris & Heuser, 1937; Gallagher, 1957), as well as in other species (Underwood, 1962), but very little is known regarding their distribution and mode of action in bone, nor is it known whether the amounts present can be mobilized in times of dietary shortage. The object of the work now described was to study variations in the Mn and Cu contents of bone and to ascertain their relationship with the mineral and organic components. Earlier, Fore & Morton (1952) had reported on the distribution of Mn in the ox femur.

EXPERIMENTAL

Bones of day-old and 3-week-old chicks

The leg bones, including articular cartilages, of twenty-four day-old chicks (twelve male and twelve female) were dissected free of soft tissue, dried and extracted with an ethyl alcohol–benzene (2:1, v/v) mixture in the Soxhlet apparatus. Bones of the left legs were each cut into three pieces: the proximal and distal ends, each about 20% of the total length, and the shaft. The ends of left leg bones from six chicks were pooled for analysis, as were the corresponding shafts.

From six 3-week-old chicks that had been fed on a control diet the bones, including articular cartilages, of one leg of each were prepared, as described above, in a dry, fat-free state. Bones of two birds were pooled for analysis and tibia shafts and ends were analysed separately. For comparison with the values for day-old chicks, results are given here for whole leg bone and for tibia shafts and ends.

Extraction of bone with trisodium ethylene diamine tetraacetate (EDTA) or with ethylene diamine hydrate (ED)

Extraction of bone with EDTA removes almost all the mineral component and leaves most of the organic component, whereas ED has the reverse effect; thus analysis of the extracted material for Mn and Cu may provide evidence for the association of these elements with either the mineral or organic component.

The left and right femurs of six $8\frac{1}{2}$-week-old male birds that had been fed on a control diet were prepared in a dry, fat-free state as described earlier. The right femur from each bird was broken into three or four pieces and placed in a 6% (w/v) aqueous solution of EDTA. The solution was changed after 2 days and the extraction

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was allowed to proceed, with occasional agitation of the solution, for a further $1\frac{1}{2}$ days. The softened pieces of tissue were washed thoroughly with water and dried for analysis.

The left and right tibias of the same birds were also prepared in a dry, fat-free state. Each right bone was broken into a few pieces of convenient size and then extracted in a Soxhlet apparatus with ED for about 5 h. To induce a fairly rapid rate of syphoning, about every 20 min, it was necessary to lag the Soxhlet apparatus with cotton-wool. The brittle pieces of tissue remaining after extraction were washed very carefully with water and dried for analysis.

The left femurs and tibias were analysed untreated as controls, and the Mn and Cu contents were used, with the dry fat-free bone weights of the corresponding right bones, to calculate weights of Mn and Cu for the right bones before treatment with either EDTA or ED.

Bones of the right legs of twenty-four $4\frac{1}{2}$-week-old male chicks that had been fed on diets of different Mn content were used in a second series of extractions with EDTA and ED. Three diets were given: basal, containing 5μg Mn/g, basal plus 5μg Mn/g (total 10μg/g) and basal plus 25μg Mn/g (total 30μg/g). Four birds were taken at random from each group to provide bones for EDTA extraction and a further four from each group to provide bones for ED extraction. The three bones (femur, tibia and metatarsal) from each bird were sawn longitudinally, one set of halves being analysed as a control to the other set that was extracted and then analysed. Sawn halves of bones without the saw dust did not differ significantly in Mn and Cu contents from comparable unsawn bones.

Mn and Cu contents of the untreated halves were used, as described above, to calculate 'untreated' weights of Mn and Cu of the halves that were extracted.

All fat-free bones or parts of bones or extracted 'bones' were dried, weighed and ashed overnight at 600°, and the weight of ash was determined. The ash was taken up with a sufficient excess of HCl to give an approximately normal solution, and Mn and Cu were determined by the methods of Yuen (1958) and Abbott & Polhill (1954) respectively.

RESULTS

Bones of day-old and 3-week-old chicks

Mn and Cu contents, and percentages of ash, of whole leg bones and of ends and shafts are given in Table 1. The bones of male and female day-old chicks were analysed separately, but there was no sex difference.

The mean Mn content of whole leg bones of day-old chicks was considerably smaller than the mean Cu content and, notwithstanding the much greater variability among Cu than among Mn values, the difference between Mn and Cu contents was clearly significant. In 3-week-old chicks the difference was smaller and reversed, the Mn content being always greater than that of Cu.

The distribution of Mn between the shafts and ends of the bones was similar at both ages, there being a greater concentration of Mn in shafts than in the ends. But the distribution of Cu was markedly different: at day-old the concentration in the ends was

approximately twice that in the shafts, and at 3 weeks there was no consistent difference between ends and shafts.

The percentages of ash followed the expected pattern, but they are given for comparison with Mn and Cu concentrations. The shafts of the bones had higher Mn

Table 1. *Manganese, copper and ash contents of whole bones and of parts of bones of day-old and 3-week-old chicks*

	Mn (μg/g dry, fat-free bones)			Cu (μg/g dry, fat-free bones)			Percentage of ash		
	Whole leg bones	Shafts	Ends	Whole leg bones	Shafts	Ends	Whole leg bones	Shafts	Ends
Day-old chicks	2·56	3·77	3·00	12·45	6·13	12·60	32·3	51·7	22·3
	(3)	(6)	(6)	(3)	(6)	(6)	(3)	(6)	(6)
	3·42			9·32			34·4		
	2·91	3·64	—	11·69	10·69	20·55	33·5	50·3	23·3
	2·86			10·22			34·7		
	2·41	2·48	2·28	8·98	3·89	7·76	33·0	51·4	21·6
	3·06			5·15			32·8		
	3·74	3·40	2·08	12·37	5·56	8·39	34·0	50·1	22·9
Mean	2·93	—	—	9·61	—	—	33·6	—	—
3-week-old chicks*	4·33	6·45	4·21	3·10	4·27	3·66	44·1	59·3	35·1
	(2)	(2)	(2)	(2)	(2)	(2)	(2)	(2)	(2)
	3·21	3·56	2·87	2·32	2·30	2·89	40·9	55·3	29·9
	3·49	4·03	2·71	2·19	2·20	2·17	44·7	59·2	34·4
Mean	3·68	—	—	2·54	—	—	43·2	—	—

Figures in parentheses are the nos. of birds pooled for analysis.
* Values for shafts and ends of 3-week-old chicks are for tibia only.

Table 2. *Influence of extraction with trisodium ethylene diamine tetraacetate or with ethylene diamine hydrate on the manganese and copper contents and on the gross composition of bone of 8½-week-old chicks*

(Mean values with their standard errors for six birds)

	Trisodium ethylene diamine tetraacetate*			Ethylene diamine hydrate†		
	Untreated‡	Treated	Change (%)	Untreated‡	Treated	Change (%)
Mn: μg/g	4·10 ± 0·27	—	—	3·84 ± 0·28	—	—
μg	7·16 ± 0·52	0·93 ± 0·13	−86·8 ± 2·7	8·17 ± 0·61	7·13 ± 0·64	−12·9 ± 4·2
Cu: μg/g	1·54 ± 0·16	—	—	1·73 ± 0·30	—	—
μg	2·72 ± 0·36	1·24 ± 0·12	−45·8 ± 6·6	3·79 ± 0·84	0·58 ± 0·10	−82·0 ± 4·0
Ash: %	59·1 ± 0·4	—	—	62·2 ± 0·3	—	—
g	1·03 ± 0·04	0·08 ± 0·02	−92·7 ± 1·5	1·33 ± 0·08	1·30 ± 0·08	−2·4 ± 1·7
Organic matter (g)	0·72 ± 0·03	0·57 ± 0·04	−20·8 ± 4·2	0·81 ± 0·06	0·13 ± 0·01	−83·6 ± 0·7

* Right femur treated with EDTA.
† Right tibia treated with ED.
‡ Concentration was obtained by analysis of corresponding left bone. Weight is estimate for the right bone before treatment, obtained by calculation from the concentration in the left bone and the weight of the untreated right bone.

contents and higher ash contents than the corresponding ends, suggesting a close association of Mn with the mineral fraction of bone. This suggestion is supported also by the tendency for the bones of 3-week old birds to have higher ash and Mn contents than those of day-old birds. High Cu concentrations were found in the bones of day-old chicks, particularly in the ends, which were regions of very low ash content and therefore of high organic matter content, but this apparent relationship between Cu and the organic fraction of bone was not observed on comparing the composition of the shafts with that of ends of bones of 3-week-old chicks.

Table 3. *Influence of extraction with trisodium ethylene diamine tetraacetate or with ethylene diamine hydrate on the manganese and copper contents, and on the gross composition of bone of 4½-week-old chicks fed on diets of different Mn content*

(Mean values with their standard errors for four birds)

	Trisodium ethylene diamine tetraacetate			Ethylene diamine hydrate		
	Untreated*	Treated	Change (%)	Untreated*	Treated	Change (%)
	25 μg Mn/g added to diet					
Mn: μg/g	2·28 ± 0·15	—	—	3·17 ± 0·31	—	—
μg	4·63 ± 0·22	1·12 ± 0·11	−75·5 ± 2·7	6·44 ± 0·71	6·21 ± 0·07	−0·2 ± 9·9
Cu: μg/g	2·36 ± 0·40	—	—	1·69 ± 0·10	—	—
μg	4·77 ± 0·75	4·79 ± 0·44	+7·0 ± 16·4	3·44 ± 0·29	1·12 ± 0·21	−66·8 ± 6·5
Ash: %	42·6 ± 0·9	—	—	43·2 ± 1·4	—	—
g	0·87 ± 0·03	0·14 ± 0·03	−84·6 ± 2·9	0·88 ± 0·04	0·79 ± 0·04	−9·6 ± 2·1
Organic matter (g)	1·17 ± 0·04	1·05 ± 0·19	−10·3 ± 2·9	1·15 ± 0·03	0·07 ± 0·01	−94·1 ± 0·7
	5 μg Mn/g added to diet					
Mn: μg/g	1·19 ± 0·23	—	—	0·89 ± 0·01	—	—
μg	2·29 ± 0·67	0·81 ± 0·04	−58·0 ± 7·8	1·99 ± 0·08	1·93 ± 0·12	−3·25 ± 4·1
Cu: μg/g	2·42 ± 0·20	—	—	1·39 ± 0·21	—	—
μg	4·51 ± 0·64	4·81 ± 0·27	+14·5 ± 24·1	3·08 ± 0·37	1·16 ± 0·07	−60·8 ± 5·2
Ash: %	43·9 ± 1·3	—	—	43·7 ± 1·5	—	—
g	0·82 ± 0·11	0·12 ± 0·03	−86·0 ± 2·2	0·98 ± 0·07	0·90 ± 0·08	−8·8 ± 3·1
Organic matter (g)	1·06 ± 0·14	0·93 ± 0·13	−7·2 ± 1·7	1·26 ± 0·03	0·08 ± 0·01	−93·5 ± 0·9
	0 μg Mn/g added to diet					
Mn: μg/g	0·93 ± 0·83	—	—	0·68 ± 1·20	—	—
μg	1·82 ± 0·18	0·72 ± 0·11	−59·0 ± 7·1	1·33 ± 0·29	1·02 ± 0·18	−21·8 ± 2·5
Cu: μg/g	2·87 ± 0·42	—	—	2·14 ± 0·25	—	—
μg	4·94 ± 0·72	5·28 ± 0·54	+11·2 ± 16·5	4·18 ± 0·49	0·98 ± 0·10	−75·0 ± 7·9
Ash: %	45·1 ± 1·0	—	—	43·1 ± 1·3	—	—
g	0·88 ± 0·07	0·13 ± 0·04	−85·7 ± 4·1	0·84 ± 0·05	0·78 ± 0·05	−7·2 ± 2·2
Organic matter (g)	1·08 ± 0·09	0·98 ± 0·08	−9·3 ± 0·03	1·10 ± 0·03	0·06 ± 0·01	−92·6 ± 2·4

* Concentrations were obtained by analysis of untreated halves of the bones. Weights are estimates for the treated halves, obtained by calculation from the concentrations in the untreated halves, and the weights of bone before treatment, of treated halves.

Extraction of bones with EDTA or with ED

The Mn and Cu contents and the gross composition of normal bone after extraction with EDTA and ED, together with corresponding values for untreated bone are given in Table 2.

EDTA removed a very large proportion of the total Mn (86·8%) and of ash (92·7%) and a much smaller proportion of the Cu (45·8%) and organic matter (20·8%). ED had very nearly the reverse effect; very little Mn (12·9%) and ash (2·4%) was removed, but large proportions of Cu (82·0%) and organic matter (83·6%) were extracted by this substance.

Results for bones obtained from birds fed on diets of different Mn content are given in Table 3. In general they resemble those in Table 2, that is EDTA removed

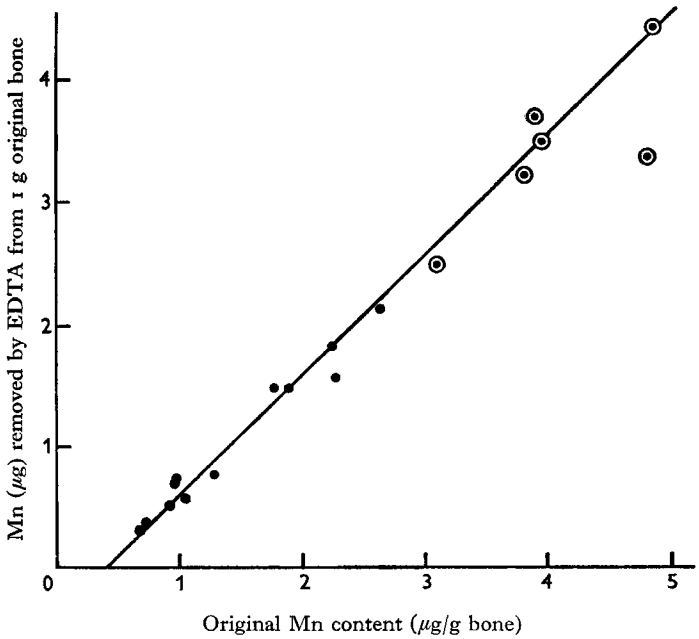

Fig. 1. Relationship between the original manganese content of bone and amounts extracted by trisodium ethylene diamine tetraacetate. ⊙, values for six 8½-week-old birds given a control diet (Table 2); ●, values for twelve 4½-week-old birds given diets of different Mn content (Table 3).

large proportions of both Mn and mineral matter and small proportions of Cu and organic matter, and ED had approximately the reverse effect. However, the magnitude of the effect of EDTA on Mn depended on the Mn content of the original bone. From the bones of high Mn content EDTA removed 75·5%, 3·51 of the 4·63μg Mn originally present, but from the bones of low Mn content 59·0%, 1·10 of the 1·82μg Mn originally present. This relationship between original content of Mn and amounts removed and remaining is illustrated in Fig. 1 with the values of Mn calculated to 1 g of original bone rather than total weights for the whole sample, as given in Table 3. Values for the six birds of the first series (Table 2) are also plotted: these all had higher Mn contents than those of the second series (Table 3). From all the values plotted, a highly significant correlation between the initial Mn content and the amount removed by EDTA was found ($r = 0{\cdot}996$, $P < 0{\cdot}001$), representing the line $y = 0{\cdot}98x - 0{\cdot}43$, where x is the original Mn content and y the amount removed, both

as μg/g original bone. The quantity remaining after extraction was fairly constant, about 0·5μg, a feature that is evident from the above equation.

DISCUSSION

The findings presented here indicate that in normal bone most of the Mn is associated with mineral matter. This view is supported by the observation that (*a*) the concentration of Mn and of ash was greater in the shafts of the long bones than in the ends, (*b*) the concentrations of both were greater in mature than in immature bone, and (*c*) treatment with EDTA extracted most of the Mn and a smaller proportion of Cu along with the mineral matter. This third point is clarified by reference to stability constants; i.e. the values for the affinity of the ligands, EDTA and ED, to the metals (Chemical Society, 1957). The stability constant for Mn with EDTA ($\log K = 14$) is much greater than that for Mn with ED ($\log K = 2{\cdot}7$) and it may be argued that EDTA removed Mn and ED did not, solely on this account. However, if the affinities of the ligands for the elements were the only factors involved, EDTA should have removed Cu even more efficiently than Mn, the constants being 18 and 14 respectively, but in every test a much smaller proportion of total Cu than of Mn was removed by EDTA. Thus, the extraction by EDTA of most of the Mn, and very little Cu, along with the mineral fraction, provides evidence for a large part of the Mn of normal bone being associated with the mineral fraction.

Bones of widely different Mn content (0·7–4·9 μg/g) were used in these extraction experiments. All but those of the lowest Mn content could be considered normal; when the Mn content was about 1μg/g or greater, neither the appearance of the live chick nor of the bone after dissection revealed evidence of Mn deficiency. Thus, considerably more Mn than that needed for normal bone metabolism was incorporated into the bones of some birds, and this additional Mn, being readily extractable along with bone mineral by EDTA, may be associated with the crystal lattice and be available by exchange, at times of high requirement or dietary shortage. This is being investigated.

The quantity of Mn remaining after EDTA extraction was fairly uniform whether the bone had initially a high or low content. A small amount of mineral matter also remained after extraction, but the quantity of Mn was considerably more than could be accounted for by assuming it to be associated with mineral matter in the same proportion as that removed. It may be concluded that part of the Mn of bone is associated with the organic fraction, a conclusion that was reached by Fore & Morton (1952) from a somewhat different approach. Leach & Muenster (1962) found low concentrations of the components of acid mucopolysaccharides in the bone of Mn-deficient rats, suggesting that Mn was particularly concerned with the formation of the organic fraction, or more specifically the matrix. Whether Mn plays any other part in bone metabolism is not known, but it may be noted from the present study that when bone has a minimum Mn content consistent with normal metabolism, about 1μg/g, this Mn appears to be approximately evenly distributed between the mineral and organic fractions.

As noted earlier, although the stability constant for Cu with EDTA (log K = 18) is greater than that for Mn with EDTA (log K = 14), Cu was extracted less efficiently than Mn, suggesting that Cu is closely bound to a certain fraction of bone. This has to be material resisting the effects of EDTA, and the organic fraction, or the major part of it, fulfills this requirement. Thus, the observation that EDTA fails to remove most of the Cu and the organic matter suggests that the two are closely associated. Positive evidence was provided by the results of the extractions with ED. It removed most of the Cu, notwithstanding the relatively low stability constant for Cu with ED (log K = 10), as well as most of the organic fraction of bone. Further support for the association of Cu with the organic fraction is found in the relatively high Cu content and large proportion of organic matter in the bones of day-old chicks. However, it will be noted that the concentration of Cu of bone organic matter, calculated on the assumption that the element was confined to this fraction, was in the region of 3–4 μg/g for the bones of $8\frac{1}{2}$-week-old (Table 2), $4\frac{1}{2}$-week-old (Table 3) and 3-week-old (Table 1) birds, but in the bones of day-old birds the concentration was about 10μg/g or greater. Thus the high Cu content of whole bone of day-old chicks was not completely explained on the basis of its high percentage of organic matter: the alternatives are that the organic matter of bones of the day-old chick had a higher concentration of Cu than the organic matter of the bones of the 3-week-old chick or else that some of the Cu in the bones of the day-old chick was associated with the crystal lattice.

SUMMARY

1. The bones of day-old and 3-week-old chicks were analysed for manganese and copper contents, either whole or after separation into ends and shafts.

2. The Cu content of bones of day-old chicks was very much higher than the Mn content, but at 3 weeks the Cu content had fallen markedly and was slightly lower than the Mn content, which had changed very little with age.

3. Chick bones were extracted with trisodium ethylene diamine tetraacetate (EDTA) or with ethylene diamine hydrate (ED) and the Mn and Cu contents of extracted bones compared with those of similar unextracted bone.

4. EDTA removed most of the mineral phase including Mn but relatively little Cu, whereas ED removed most of the organic phase including Cu but very little Mn.

5. With bones of different Mn content there was a highly significant positive correlation between the quantity extracted by EDTA and that originally present.

6. The results are discussed in relation to bone metabolism.

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