REFERENCES

Abul-Fadl, M. A. M. (1948). *Biochem. J.* **42**, xxxvii.
Davidson, J. & Capen, R. G. (1929). *J. Assoc. Off. Agric. Chem.* **12**, 310.
Kirk, P. L., Rosenfels, R. S. & Hanahan, D. J. (1947). *Analyt. Chem.* **19**, 355.
Kun, E. (1947). *J. biol. Chem.* **170**, 509.
Mehlig, J. P. (1939). *Industr. Engng Chem.* (Anal. ed.), **11**, 274.
Mehlig, J. P. (1941). *Industr. Engng Chem.* (Anal. ed.), **13**, 819.
Richards, M. B. (1930). *Analyst*, **55**, 554.
Rowland, G. P. (1939). *Industr. Engng Chem.* (Anal. ed.), **11**, 442.
Strickland, J. D. H. & Spicer, G. (1949). *Anal. Chim. Acta*, **3**, 517.
Szebelledy, L. & Bartfai, I. (1936). *Z. Anal. Chem.* **106**, 408.
Willard, H. H. & Greathouse, L. H. (1917). *J. Amer. chem. Soc.* **39**, 2366.

The Manganese in Bone

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Manganese is generally agreed to be essential for the prevention of perosis, a bone deformity of chicks and turkeys, and it may perhaps also be essential for normal bone formation in mammals. The mode of action of manganese in calcium and phosphorus metabolism has not been established but a role as activator of phosphatase has been suggested because chicks receiving added manganese show a rise in the manganese content of bone and an increase in phosphatase activity.

The present work is concerned not only with the manganese content of bone but also with the distribution of the element among its constituent parts, in the hope of finding new evidence pertinent to the elucidation of function.

EXPERIMENTAL

Analytical methods

Emission spectrography. A Littrow type quartz spectrograph (Hilger E_1) was used in two settings covering the regions 2260–2880 and 2870–5050 A. on 10 × 4 in. (25 × 10 cm.) plates. The light source was a d.c. arc (230 V.) between graphite or graphite-copper electrodes. For most of the work the copper-on-graphite electrodes were used; two 1 cm. lengths of pure copper rod (5 mm. diam.) were pushed into holes bored into the ends of graphite rods (9·5 mm. diam.). When the arc was running the electrodes became much hotter than simple copper electrodes and trace metals present in ash placed on the lower electrode were well volatilized into the flame. The *raies ultimes* of Mn (4030·8, 4033·1, 4034·5 A.) are easily obscured by cyanogen emission bands when graphite electrodes are used and are only shown with the copper-on-graphite arc (5 amp., 3 min. exposure) when the Mn concentration in bone ash is between 1 and 10 p.p.m. The 2800 A. triplet (2794·8, 2798·3 and 2801·1 A.) was more readily recorded than the Mn II *raies ultimes* (2576·1, 2593·7 and 2605·7 A.) and could be seen using graphite or copper on graphite electrodes at 1–10 p.p.m. Mn. Calcium phosphate ($Ca_3(PO_4)_2$) prepared from Analar Na_2HPO_4 and $CaCl_2$ revealed added Mn at 5 and 10 p.p.m. clearly, 2 and 4 p.p.m. with less regularity and certainty, and failed to show up 1 p.p.m.

Periodate method. The method of Skinner & Peterson (1930) was used in a modified form ensuring complete solution of the sample in H_3PO_4.

Materials and procedures

Fresh ox femurs from the abattoir were used.

(i) After removing flesh and marrow, the bone was cut into sections which were then reduced to shavings on a lathe, taking care to avoid contamination. The shavings, consisting of both compact and cancellous bone, were subjected to extraction with glass-distilled water saturated with $CHCl_3$. Three fractions were obtained, an aqueous phase, the residual shavings, and fat removed by means of ether from the extract. The bone marrow was shaken with ether for 4 hr. and on standing aqueous and ethereal phases separated, with gelatinous material at the interface. Much of this material appeared to consist of cell-wall debris from the marrow tissue, and was subsequently separated and washed with glass-distilled water. The two aqueous extracts were separately evaporated to dryness and the residues incinerated. The two ethereal extracts were washed with water, dried over Na_2SO_4, and the ether removed from measured portions and the residues burned off. The shavings and the 'cell-wall debris' were each dried and ashed. The six samples of ash were examined spectrographically on graphite and on copper-on-graphite electrodes.

(ii) (*a*) Fresh bone shavings were decalcified as follows: shavings (20 g.) were shaken with 0·1N-HNO_3 (1 l.) made from redistilled acid diluted with glass-distilled water. The suspension was filtered and the filtrate and residue subjected to emission spectrum analysis.

(*b*) The above treatment was repeated on a fresh sample (20 g.) and the residual shavings were left to stand overnight in a further 0·5 l. of 0·1N-HNO_3. The second extract and the (largely organic) residue were examined spectrographically. The very small amount of ash from the decalcified material was diluted with 'specpure' $(NH_4)_2SO_4$.

(iii) Two types of shavings were obtained from an ox femur, namely compact bone from the shaft and cancellous bone tissue from the head of the femur. Aqueous extracts from each type of material were made and portions were incubated with disodium phenylphosphate at pH 9 with suitable controls. After 16 hr. the solutions were tested for free phenol using 2:6-dichloroquinone chloroimide. The extracts were also tested spectrographically for Mn.

(iv) Another fresh ox femur was cut into sections and the marrow removed as completely as possible. The different portions of bone consisted of (*a*) predominantly epiphyseal bone from the head, (*b*) a mixture of epiphyseal and diaphyseal bone, and (*c*) diaphyseal bone from the shaft.

The separate portions of bone were incinerated and the proportions of ash determined.

(v) A portion of bone shaft was cleaned and turned on the lathe. Three experiments were carried out on the shavings. (*a*) 30 g. were packed in a 'chromatography' tube and covered with 0·1N-citric acid solution (made up in glass-distilled water). A dropping funnel was affixed to the top by means of a rubber bung and 3 l. of citric acid solution were allowed to percolate slowly through the shavings. Finally the contents of the tube were removed, dried and incinerated; the percolate was also dried and reduced to ash. The ash from the partly decalcified shavings was examined spectrographically and the ash from the percolate was analysed for Mn by the periodate method. (*b*) 16·6 g. of shavings were treated similarly and 9·3 g. of ash were obtained from the percolate and 0·31 g. from the residue in the tube. Both fractions were tested for Mn. (*c*) 30 g. of shavings were treated as before and the percolate collected in five fractions which were dried and incinerated separately. The residue in the tube consisted of an upper translucent portion and a lower opaque portion; the two portions were separated and each reduced to ash and examined for Mn.

RESULTS

Exp. (i) showed that the water-extracted shavings contained manganese (< 5 p.p.m.), but the ash from soluble material certainly contained no more. The aqueous extract from the bone marrow did not contain detectable amounts of manganese, but the ash from the cell-wall debris was richer than the total ash; it was enriched in manganese (10 p.p.m.) and in all other metals detected in bone (Al, B, Ba, Cu, Fe, Mg, Na, Pb, Sn, Sr and Zn) except potassium. Manganese was also detectable in the ash from both fatty fractions. The experiment showed that only a minute fraction of the total manganese in bone was present in a water-soluble form, and on the assumption that the bone phosphatase is extractable, little if any manganese can be directly attached to it. The small amount of manganese accompanying the fat may have been attached to the phosphoric acid residues of phospholipins.

Exp. (ii) showed that the organic matter remaining after decalcification with nitric acid retained a small amount of inorganic matter which was richer (on ashing) in manganese, copper, and iron than the original ash from the untreated shavings. The Mn content was about 50 p.p.m.

Exp. (iii) showed that alkaline phosphatase could be extracted by means of water from both epiphyseal and diaphyseal bone. Manganese was detectable only in the ash from the epiphyseal extract. In any case there was no sign of preferential extraction of manganese as a congener or integral part of phosphatase.

Exp. (iv) showed clearly that the percentage of ash varies for the different portions of femur (Table 1).

Table 1. *Ash contents of various parts of an ox femur*

	Wt. of bone (g.)	Wt. of ash (g.)	Percentage ash
Epiphyseal portions (near head)	118	37	31
	97	26	27
	157	51	32
	153	52	34
Diaphyseal (shaft)	23	13	57
	23	13	57
Mixed type	95	37	39
	142	54	38

Exp. (v) (*a*) yielded 14·4 g. of ash from the percolate and 2·8 g. of ash from the residue in the tube (i.e. 57·2 % ash on the 30 g. used). The manganese content of the percolate was estimated, by a colorimetric procedure using periodate oxidation, to be 8 μg. The bone shavings were estimated to contain 15 μg. so that approximately half the manganese was retained. Similarly, in Exp. (v) (*b*), 16·6 g. of shavings yielded 9·3 g. of ash from the percolate and 0·31 g. from the residue, and it is estimated that approximately one-fifth of the total manganese was retained in the latter. In Exp. (v) (*c*) 56 % of ash was obtained altogether, and within experimental error the manganese was extracted with the calcium phosphate, at first slowly and then more quickly. The proportion of the total manganese remaining in the translucent residue was quite small, but expressed in p.p.m. on the ash it was high (100 p.p.m.).

The diaphyseal bone ash contained 1·44 p.p.m. (± 5 %) of manganese and the epiphyseal bone ash 1·41 p.p.m. (± 15 %). Now the fresh diaphyseal bone contains 57 % of ash so that the manganese content is about 0·51 p.p.m. The fresh epiphyseal bone contains 31 % of ash corresponding with about 0·27 p.p.m. of manganese. Thus, although the manganese content of bone varies along its length, this is due entirely to the varying amount of calcium phosphate deposited. It may be concluded that most of the manganese in ox bone is deposited with the inorganic salts, but a small proportion is definitely associated with the organic matrix.

Compared with the total, the amount of manganese (if any) which is attached to water-soluble phosphatases is negligibly small.

SUMMARY

1. Spectrographic studies and determinations based on periodate oxidations show that the manganese content of ox femur is of the order 0·27–0·51 p.p.m. depending on the type of bone, compact or cancellous. Irrespective of the type this corresponds to about 1·4 p.p.m. expressed on the ash.

2. Most of the manganese is associated with the inorganic matrix, but a small proportion is bound to the organic matrix.

3. Little manganese, if any, accompanies the water-soluble alkaline phosphatase.

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REFERENCE

Skinner, J. T. & Peterson, W. H. (1930). *J. biol. Chem.* **88**, 347.

Manganese in Rabbit Tissues

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Although many papers have appeared on the manganese contents of the tissues of various animals, in no case has a survey been recorded of the concentrations in all the tissues of a single animal.

Such a survey might provide a better clue to function than comparisons of the manganese contents of tissues from different individuals or species.

EXPERIMENTAL

Animals and materials

The rabbit was chosen for this investigation because it is of a convenient size for obtaining reasonable amounts of small bodies such as the pituitary and pineal glands. Four animals were used; samples of most tissues were obtained from an adult female chinchilla and of the other tissues from the remaining three. Specimens of incompletely separated adrenal cortex and medulla, thymus and thyroid were obtained from a resting adult female chinchilla; specimens of mammary gland, thyroid and lung were obtained from a lactating female albino, and samples of pancreas, thyroid, testes and spinal cord from an adult male chinchilla. Liver samples from each animal were used as controls. The pineal and pituitary glands from the last two animals were examined. The separation of the duodenal mucosa and muscularis tissue was only approximate. All dissections were made with carefully cleaned stainless-steel instruments.

Using portions of kidney and liver from the first rabbit, homogenates were prepared and dialysed in cellophan bags against changing glass-distilled water for 10 days, toluene being added as preservative. The residue in each bag was divided into two portions and the Mn content of each determined.

Method of analysis

Mn was determined by a modification (Fore & Morton, 1952*a*) of a method described by Kun (1947) based on the catalytic oxidation of diethylaniline by Mn^{++} and periodate to give a coloured product absorbing at 470 mμ. The experimental error is from ± 10 to ± 20 % depending on the size of the sample.

RESULTS

The findings on rabbit tissues are recorded in Table 1. The manganese contents based on fresh tissue weight are listed in decreasing order in Table 2. For comparison, (*a*) the values reported in the literature for each rabbit tissue have been averaged, and (*b*) the values reported for each type of tissue irrespective of species have been averaged. The published figures for the rabbit were obtained from papers by Bertrand & Medigreceanu (1912*a*, *b*; 1913), Ellis, Smith & Gates (1947), Lorenzen & Smith (1947), Lund, Drinker & Shaw (1921), Ray (1938) and Richards (1930). The figures in the right-hand column of Table 2 are averages obtained by attaching the same weight to all the published figures which can be traced. The number of papers consulted is about thirty-five and a full list of references would take up too much space.

The results of the dialyses are given in Table 3, and if the figures 2·0 and 1·2 p.p.m. are accepted for the manganese contents of the liver and kidney homogenates, respectively, it appears that in the liver 74 % of the manganese, 72 % of the dry matter