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The Distribution of Trace Metal Ions in Bone and Tendon

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The natural trace metal compositions of human bone mineral and demineralized bone were measured by emission spectroscopy and compared with those of the original whole bone and human tendon. Cu, Fe and Zn remained in the collagenous matrix of bone and were found in similar quantities in tendon. It is suggested, therefore, that these ions are chemically bound to the collagen matrix in these tissues *in vivo*, and that the binding has a part in their activity. Most of the Pb, Si, Sr, and V in bone remained with the mineral portion, probably as substituted or interstitial ions. Zn is divided between the organic and mineral phases.

Key words: Collagen—Bone—Tendon—Trace Metals—Spectrographic Analysis.

La composition en éléments métalliques, trouvés à l'état de traces, dans le minéral de l'os humain et dans l'os déminéralisé est mesurée par spectroscopie d'émission et comparée à celle de l'os original entier et le tendon humain. Le Cu, Fe et Zn restent dans la matrice collagène de l'os et se retrouvent en quantités équivalentes dans le tendon. Il semble donc que ces ions soient liés chimiquement à la matrice collagène de ces tissus *in vivo* et que cette liaison joue un rôle dans leur activité. La majorité du Pb, Si, Sr, et V de l'os reste dans la fraction minérale, probablement sous la forme d'ions substitués ou interstitiels. Le Zn se répartit entre les phases organiques et minérales.

Die natürliche Zusammensetzung der Spurenelemente im menschlichen Knochenmineral und im demineralisierten Knochen wurde mittels Emissionsspektroskopie gemessen und mit jener im ursprünglichen, intakten Knochen und in der menschlichen Sehne verglichen. Cu, Fe und Zn blieben in der Kollagenmatrix des Knochens zurück und wurden in ähnlichen Mengen in der Sehne gefunden. Deshalb wird angenommen, daß diese Ionen in vivo chemisch an die Kollagenmatrix dieser Gewebe gebunden sind und daß diese Bindung einen Anteil an ihrer Aktivität hat. Der größte Teil des Pb, Si, Sr und V des Knochens blieb in der Mineralfraktion, vermutlich als substituierte oder interstitielle Ionen. Zn ist gleichmäßig auf die organische und anorganische Phase verteilt.

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A. Introduction

Trace metals are thought to play several roles in the synthesis, cross-linking, calcification and diseases of connective tissues (Sobel and Hanok, 1952; O'Dell *et al.*, 1966; Schiffmann *et al.*, 1966; Chvapil and Hurych, 1968). In previous investigations (Becker *et al.*, 1968; Ellis *et al.*, 1969) of the trace element composition of whole human cortical bone and beef tendon, we have observed a regularity in the appearance of certain metals, namely Cu, Fe and Zn. These, and possibly other, ions may be structurally incorporated in the collagenous matrices of these tissues. Recent experiments have suggested that the collagens are not merely passive cellular products, but are themselves active substrates for initiating the growth and repair of connective tissues (Grobstein and Cohen, 1965; Friedman *et al.*, 1968; Urist *et al.*, 1968). If this is so, then the incorporation of specific metal ions in collagen matrices is important.

Only two reports have been located dealing with the natural distribution of trace elements among the components of bone. Fore and Morton (1952) in their excellent investigation of Mn in bone, found that although most of the 1.4 ppm of Mn in ox femur was located in the mineral portion, a small amount was associated with the organic matrix, and no detectable Mn was found in the water-soluble fractions. Ellis (1964) found, in qualitative studies, that Cu and Fe were more consistently associated with the organic fraction of human bone, and Pb and B with the mineral.

We present below data on the concentrations of several naturally-occurring trace metals in the organic and mineral fractions of normal human cortical bone. In addition, we report the results of an analysis of several samples of normal human tendon, which is mostly collagen. To our knowledge, no trace analyses of human tendon have been previously reported.

B. Methods

1. Sample Preparation

Sections of cortical bone were isolated from the midshafts of either tibiae or femora of five individual amputee patients, the operations being necessitated by mechanical orthopedic conditions. Only the medial portions, exclusive of highly vasculated and trabecular bone, were used. Human tendon was obtained during similar amputations from Achilles, and foot extensor, tendons of four individuals. After dissection and removal of unwanted tissue, the tendon sections were air dried and stored in plastic containers until analysis.

A set of representative bone samples was cut into small chips and refluxed with ethylenediamine in acid-cleaned glassware by the technique of Williams and Irvine (1954). This procedure was extended for at least 60 distillation cycles (3 days). The mineral was then washed for 24 h in distilled, de-ionized water. Another set of bone samples was treated for five days with a 5% solution of formic acid to dissolve the mineral. The resulting samples of bone organic matrix were washed for 24 h in distilled, de-ionized water before analysis.

These procedures limited the interpretation of analytical results. Ethylenediamine is a well known metal chelating agent and may dissolve some of the mineral, or sequester loosely-bound ions during refluxing. During demineralization, the formic acid solution probably dissolves some of the collagen and other proteins; it may also remove loosely-bound ions, and expose the protein to the dissolving minerals.

Nevertheless, an analysis of the organic and mineral phase of bone prepared as above could be expected to localize the more tenacious metallic ions and identify the more weakly-bound ions. Complete recovery would be unlikely. The residual Ca in the demineralized bone

samples was less than 1 ppm of dry weight in this experiment, and ignition to 450° of the extracted bone mineral produced weight losses of only a few percent from the same mineral dried at 110°. Thus, the separations, however inefficient, were virtually complete.

II. Spectrographic Analysis

All samples were dried at 110° for at least 18 h and weighed. Demineralized bone sections were dry-ashed in covered crucibles by slowly raising the oven temperature to 350° over a 2 day period. The remaining black ash, resembling graphite, weighed about 35% of the original dry material and was powdered. The dried tendon sections were treated similarly, raising the temperature to 400° over a four-day period, producing a 12% ash/dry-weight ratio. These organic ashes were mixed in the ratio of one part ash, one part Li_2CO_3, and two parts pure graphite (including 0.05% $(NH_4)_2PdCl_4$ providing Pd as a general internal standard). The mixtures, along with similarly-prepared, multi-element graphite standards, were ignited in an arc and the spectra recorded as described previously (Ellis *et al.*, 1969). Fortran programs designed in this laboratory for use on the IBM 360–50 were employed to perform emulsion calibrations and estimate concentrations from log-intensity vs. log-concentration working curves (Spadaro, 1969b).

The samples of extracted bone mineral were ground to a fine powder in a dental shaker, mixed 1:1 with palladiumized graphite and ignited in an arc with specially-designed external standards previously reported to be effective for the analysis of bone ash and mineral (Spadaro, 1969a). Both the precision and accuracy of this method have been shown to be about 8%, averaged over 18 common elements (Spadaro, 1969b). The average precision of the demineralized bone and tendon analyses was estimated to be from 10% to 20% and generally below sample variations. In all cases, spectrographic grade reagents (Johnson-Matthey) and graphites (National) were used; all vessels were washed in dilute sulfuric acid and rinsed thoroughly in distilled, de-ionized water.

C. Results

I. Human Bone Mineral and Bone Collagen

The average concentrations of detected elements in samples of bone mineral and demineralized bone are shown in Fig. 1, adjacent to the results for the original 5 whole bone samples for comparison. The concentrations in all three materials are given in equivalent ppm of 110° dried whole bone to make the comparison easier[1]. As can be seen in Fig. 1, Pb, Si, Sr and V, while found at reduced concentrations in bone mineral, seemed to be absent from demineralized bone to relatively low limits. Therefore, part of the content of these metals in bone is probably tenaciously bound to the mineral, either as substituted, or interstitial, ions. This has been previously shown for Pb and Sr using X-ray diffraction (MacDonald *et al.*, 1951). Results for Mn and Sn are inconclusive, but they appear to be only weakly bound, if at all, to the organic matrix.

In contrast, part of the Cu and most of the Fe remained with the decalcified organic matrix, with little or none remaining with the bone mineral. This is an important observation, because it indicates that these ions are relatively strongly-bound to the bone collagen and may therefore be functionally important to it. The actual average concentrations of Cu and Fe in the organic matrix in terms of dry demineralized bone were 2.7 ± 0.4 ppm and 8.4 ± 3.7 ppm respectively[2]. Although the results for Zn were only approximate, due to technical difficulties

1 Here it was assumed for the purpose of calculation that the mineral was 68% and decalcified matrix about 30% of whole dried bone, and that processing weight losses were small.

2 The errors expressed are average deviations in five samples.

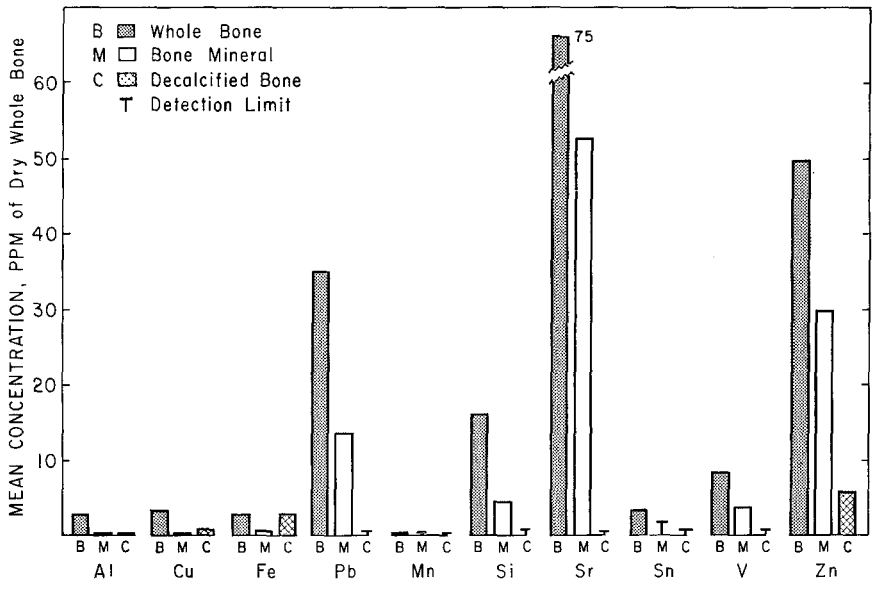

Fig. 1. Trace analysis of bone mineral and demineralized bone compared to whole bone. Concentrations in ppm per equivalent dry weight of whole bone in all three cases

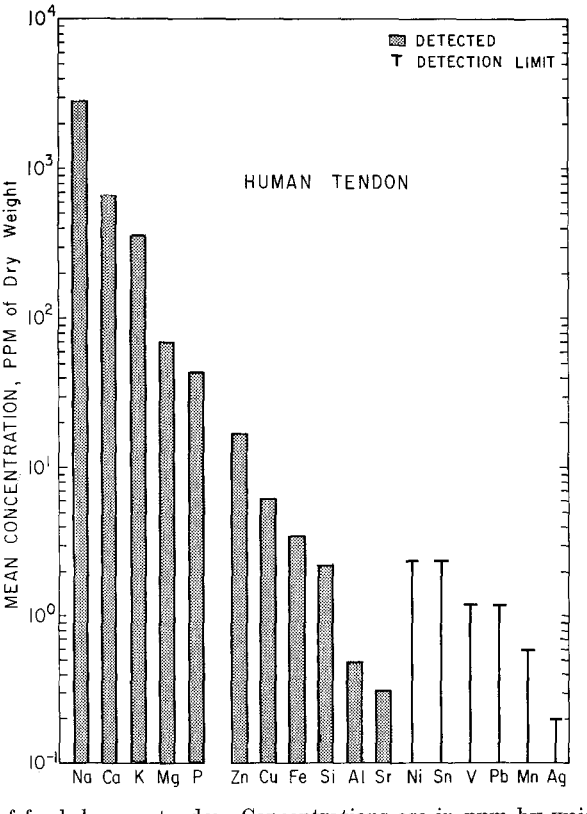

Fig. 2. Analysis of fresh human tendon. Concentrations are in ppm by weight of dry tissue on a log scale. Detection limits for some elements not detected are shown with solid lines. The results of three Achilles, and one foot extensor tendon, all from different individuals, were averaged. P was detected in only one sample, however, and is not averaged

in using the Zn line 2138, it appeared as if part of the Zn in bone is firmly bound to the mineral, and part (about 6 ppm) to the collagen matrix. Zn was detected in all whole bone samples and in their extracted components.

II. Human Tendon

The average concentrations of metals in human tendon, expressed in ppm of dry tissue (110°), are shown in the graph in Fig. 2. The detection limits for the last six elements are shown because they had been found previously in either human bone or beef tendon (see references cited above). The large concentrations of Na, Ca, K, Mg and P are included for completeness (P was detected in only one sample and had a detection limit of 12 ppm in this analysis). The results in Fig. 2 are similar to those for whole and purified beef tendon (Ellis *et al.*, 1969) and demineralized bone (Fig. 1). Zn, Cu and Fe were detected in human tendon in concentrations between 3 and 20 ppm of dry tissue. Metal concentrations in the current analysis showed average sample-to-sample deviations of from 10–30 %

The small amounts of Si, Al and Sr found in tendon may also have been present in the demineralized bone, since detection limits in the analysis of the latter were not as low as for the tendon analysis.

D. Discussion

The most important observation made during the present experiments was that Cu, Fe and Zn, in small but relatively constant amounts, are found both in the insoluble organic fraction of human bone (despite extensive demineralization) and in human tendon. This result, and the similar concentrations previously found in whole bone (Becker *et al.*, 1968) and in beef tendon (Ellis *et al.*, 1969) lead us to assert with confidence that these ions are in some way bound to the collagen matrix and not merely present in the interstitial fluids. Attachment to the non-collagen proteins or to protein-mucopolysaccharides, while not ruled out, is unlikely, due to the fact that Cu, Fe and Zn have been found in similar concentrations in acid-demineralized bone collagen and in salt-extracted tendon collagen (Ellis *et al.*, 1969), as well as in all untreated collagenous tissues examined so far. Furthermore, it has been shown that collagen, especially in matrix form, binds these ions rather strongly *in vitro* (Spadaro *et al.*, 1970)[3]. The question is, what role, if any, do these ions play in the growth and/or function of collagenous tissues ?

The observed concentrations imply, if the distribution is homogeneous, the presence of only one atom of metal for about 10 or more molecules of tropocollagen. The roles would then involve (1) direct interactions on the supramolecular level of organization, or (2) indirect interactions, for example through the control of enzymatic activity.

These interactions are not, in fact, mutually exclusive. It is possible that the metal ion *in situ* is responsible for enzyme activation; that is, the collagen matrix-metal ion complex may confer activity on an enzyme, mostly as a co-factor. Alternatively, the collagen matrix, through its binding specificity, could concentrate necessary ions which would be released to the appropriate enzyme by ion

3 In most cases, the binding constants of Cu^{2+}, Zn^{2+}, Fe^{3+} were found to be about 100 times that of calcium under identical conditions.

exchange. An active role of the collagen matrix has, in fact, been suggested by recent bone induction experiments (Friedman *et al.*, 1968; Urist *et al.*, 1968). Cu, Fe and Zn are excellent candidates for such interactions. They have been enzymatically linked to collagen synthesis and cross-linking and to bone calcification; they are strongly bound by collagen matrices *in vitro*; and they are present consistently in bone and tendon collagen in appropriate concentrations.

With regard to the human tendon analysis, it is worth noting that the Mg/Ca weight ratio in tendon is approximately 0.1, or about 10 times that of bone, The Sr/Ca ratio, however, is found to be 0.45×10^{-3} in dry human tendon, exactly as found by Thurber *et al.* (1958) in their exhaustive study of Sr in human bone. Since this latter figure is 1/15 of that found in average rock and soil and even for human muscle (Report of Committee II on Permissable Dose for Internal Radiation, 1960), it is clear that tendon discriminates against Sr (in relation to Ca) to the same extent as does bone, although the latter is over $^2/_3$ mineral. Further investigations of these relationships seem to be required.

References

Becker, R. O., Spadaro, J. A., Berg, E. W.: The trace elements in human bone. J. Bone Jt Surg. **50**-A, 326–334 (1968).

Chvapil, M., Hurych, J.: Control of collagen synthesis. In: International review of connective tissue research (ed. by Hall, D. A.), v. 4. New York: Academic Press 1968.

Ellis, E. H.: The fluorescence of bone. Thesis, Syracuse University, 1964.

— Spadaro, J. A., Becker, R. O.: Trace elements in tendon collagen. Clin. Ortho. Rel. Res. **65**, 195–198 (1969).

Fore, H., Morton, R. A.: The manganese in bone. Biochem. J. **51**, 598–600 (1952).

Friedman, B., Heiple, K. G., Vessely, J. C., Hanaoka, H.: Ultrastructural investigation of bone induction by osteosarcoma using diffusion chambers. Clin. Ortho. Rel. Res. **59**, 39–57 (1968).

Grobstein, C., Cohen, J.: Collagenase: effect on the morphogenesis of embryonic salivary epithelium in vitro. Science **150**, 626–628 (1965).

MacDonald, N. S., Ezmirlian, F., Sapin, P., McArthur, C.: The ultimate site of skeletal deposition of strontium and lead. J. biol. Chem. **188**, 387–399 (1951).

O'Dell, B. L., Elsden, D. F., Thomas, T., Partridge, S. M., Smith, R. H., Palmer, R.: Inhibition of the biosynthesis of cross-links in elastin by a lathyrogen. Nature (Lond.) **209**, 401—402 (1966).

Report of Committee II on permissable dose for internal radiation. Hlth Phys. **3**, 1–151 (1960).

Schiffmann, E., Corcoran, B. A., Martin, G. R.: The role of complexed heavy metals in initiating the mineralization of "elastin" and the precipitation of mineral from solution. Arch. Biochem. **115**, 87–94 (1966).

Sobel, A. E., Hanok, A.: Calcification VII; reversible inactivation of calcification *in vitro*. J. biol. Chem. **197**, 669–685 (1952).

Spadaro, J. A.: Use of the direct current arc voltage in determining the carbonate content of bone. App. Spectry. **23**, 125–128 (1969).

— Trace metal ions in bone and collagen. Thesis, Syracuse University (1969b).

— Becker, R. O., Bachman, C. H.: Size-specific metal complesing sites in native collagen. Nature **225**, 1134—1136 (1970).

Thurber, D. L., Kulp, J. L., Hodges, E., Gast, P. W., Wampler, S. M.: Common strontium content of the human skeleton. Science **128** (256–257, 1958).

Urist, M. R., Dowell, T. A., Hay, P. H., Strates, B. S.: Inductive substrates for bone formation. Clin. Ortho. Rel. Res. **59**, 59–96 (1968).

Williams, J. B., Irvine, J. W.: Preparation of the inorganic matrix of bone. Science **119**, 771–772 (1954).