Effect of Various Metals on Mineralization *in vitro*.* (28126)

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Since the demonstration by Robison(1) that rachitic cartilage matrix can be readily mineralized *in vitro*, this tissue has proven valuable as a means of elucidating factors important to the processes of bone crystal formation and deposition. Certain metals (beryllium, copper, magnesium and strontium) if present in sufficient concentration in the incubating solution are known to inhibit mineralization of cartilage matrix(2). It may be that other metals also inhibit mineralization of matrix and possibly are more potent in this respect than those previously reported.

The present study was designed to examine the effect of a variety of metals on mineralization of cartilage matrix *in vitro*. The data obtained indicate that the elements zinc, cadmium, manganese and cobalt, in addition to those previously reported, do inhibit mineralization of cartilage matrix. Further studies were carried out to observe the effect of these metals on crystal formation *per se*. With the possible exception of cobalt, the inhibitory action of the various metals on mineralization is apparently not due to interference with bone crystal formation.

Methods. The technic of inducing mineralization *in vitro* used in this study has been described(3). The composition of the basal salt solution used in mM/l was: Na 127, K 5.7, Mg 1.0, Cl 108, HCO_3 2.6, SO_4 1.0, Ca 1.75 (7 mg/100 ml), and P 1.60 (5 mg/100 ml). Incubation of rachitic cartilage in this solution results in prompt mineralization of the cartilage matrix. The effect of various metals on mineralization *in vitro* of rachitic cartilage slices was assessed by addition to the basal solution of each of the following substances at concentrations ranging from 0.0005 mM to 0.5 mM: zinc, cadmium, cobalt and chromium as chloride salts and manganese as an acetate salt. Five to 40 tibial sections were incubated at all concentrations of each metal tested with the larger number of sections being used at those concentrations which caused partial inhibition of mineralization. A maximum of 5 sections was incubated in each flask which contained at least 20 ml of incubation solution per section. Additional studies were conducted with the selected elements using the basal salt solution modified by the omission of magnesium. Observations were also made as to the degree of mineralization occurring on addition of various metals to a basal salt solution containing both magnesium (1.0 mM) and zinc (0.001 mM). As a positive control for each of the foregoing series of observations, one cartilage slice from each animal used was incubated in the standard mineralizing solution unmodified by addition of the various metals.

In the second portion of this experiment the potential of the ions studied to inhibit apatite crystal formation *per se* was determined by a modification of the method of Solomons and Neuman(4). Crystals were observed to develop in the basal incubating solution containing no cartilage slices when maintained at 37.5°C for at least 8 days. To accelerate this crystal formation the basal solution was altered by an increase in concentration of calcium to 9.6 mg/100 ml. All observations were conducted in duplicate using 50 ml aliquots of the above solution modified by addition of various concentrations of those substances which inhibited mineralization in the preceding portion of this experiment. Also tested for a possible effect on crystal formation were several compounds (cyanide, fluoride and iodoacetate), which had been reported previously to inhibit mineralization of rachitic matrix *in vitro*(5). All solutions were incubated in 125 ml unsiliconized flasks at 37.5°C with gentle agitation for 8 days, or for shorter periods if crystal development was evident. Before and after incubation aliquots of the incubating solution were filtered through millipore filters

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TABLE I. Inhibitory Effect of Metallic Ions on Mineralization.*

Metal tested	Other additions to basal solution (mM)	Concentration (mM) of metal tested							
		0	.0005	.001	.005	.01	.05	.1	.5
Zn	0	4	4	4	3	3	0	—	—
	1.0 Mg	4	4	3	1	0	0	—	—
Mn	0	4	—	4	—	4	1	0	0
	1.0 Mg	4	4	3	3	2	0	0	—
	.001 Zn + 1.0 Mg	4	4	2	—	1	0	—	—
Co	0	4	4	4	4	4	2	1	—
	1.0 Mg	4	4	—	3	3	1	0	—
	.001 Zn + 1.0 Mg	4	—	—	3	1	0	—	—
Cd	1.0 Mg	4	—	4	2	2	1	0	0
Cr	1.0 Mg	4	4	3	—	3	—	1	0

* Degrees of mineralization are graded 0 to 4 with 4 indicating complete mineralization of cartilage matrix. Therefore, as control sections were fully mineralized, less extensive mineralization indicates inhibition.

and the filtrates were analyzed for calcium (6) and phosphorus(7). A reduction in concentration of calcium and phosphorus in the filtrates from incubated solutions served as chemical confirmation of crystal formation. Two control solutions containing no additives were incubated concurrently with each series of test solutions.

Certain of the metals under study frequently exist as minor contaminants of the salts used to prepare the incubating solution. However, with the exception of small amounts of manganese, the solutions employed in this experiment were found to contain very little of these metals which inhibited mineralization. The maximal existing concentration of the metal impurities was as follows: zinc <0.1 μM(8), cadmium <0.04 μM(9), chromium <0.2 μM(10), cobalt <0.4 μM(11), and manganese <0.8 μM(12).

Results. Effect on mineralization. Extensive mineralization of cartilage matrix regularly occurred on incubation of rachitic cartilage slices in the basal solution. However, incorporation of a number of metals individually in the basal incubating medium resulted in partial or complete inhibition of matrix mineralization. The results obtained by such procedures are recorded in Table I. When the metals studied were added to an incubating solution that did not contain magnesium, a greater concentration of the respective element was required to inhibit mineralization than would be required for inhibition in the presence of magnesium. Furthermore, and as might have been predicted, the presence of small amounts of zinc (0.001 mM) in the incubating medium (containing magnesium) enhanced the inhibitory effect of other metallic ions on mineralization. Of the elements tested zinc was the most potent inhibitor of mineralization. A 0.01 mM concentration of this ion in the presence of magnesium prevented detectable mineralization, whereas lesser concentrations were only partially inhibitory. The respective order of inhibition observed was as follows: zinc, manganese, cadmium, cobalt and chromium. Manganese was only slightly less effective than zinc in preventing mineralization, a 0.05 mM concentration of the former element being sufficient for complete inhibition. Preliminary observations as to the effect of copper, strontium and molybdate (not recorded in Table I) indicate that a much higher concentration of these substances (>0.1 mM) than of the above elements is required to inhibit mineralization.

No analyses were conducted to determine the concentration of the added metals remaining in solution after incubation. Conceivably, significant amounts of these substances might have been bound by portions of the tibial slices with resulting reduction in the amounts remaining for action on the mineralization process. Contrariwise, concentrations of certain of the elements in question (*e.g.*, magnesium and zinc) may actually have increased in the incubation media as a result of leaching of these ions from cut surfaces

of the cartilage sections. Therefore, the actual concentrations cited above may have been slightly altered by such phenomena.

Crystal formation. In the study of crystal formation, myriads of white needlelike crystals regularly appeared after 4 to 8 days of incubation of the slightly modified (calcium present at 9.6 mg/100 ml) basal solution containing no tibial slices. Cobalt was the only metal that prevented detectable crystal formation during the 8 days of incubation when this ion was present at concentrations greater than 0.001 mM. Zinc, cadmium and manganese failed to inhibit crystal formation at concentrations up to 0.1 mM, and fluoride, cyanide and iodoacetate also failed to block crystallization at concentrations preventing mineralization of cartilage matrix(5). However, it is noteworthy that appearance of crystals was delayed in the presence of cadmium, fluoride or cyanide as compared with the rate of crystal formation in control solutions.

Discussion. Several possibilities may be considered in delineating the factor, or factors, which may account for the observed inhibition of cartilage matrix mineralization *in vitro* by the elements tested. Lack of mineralization may result from blockage of crystal nucleation sites in the matrix, interference with apatite crystal formation *per se,* inhibition of enzyme activity necessary for the mineralization process, or a combination of such effects.

It has long been known that the development of crystals from a solution may be unaffected, impaired or even accelerated by the presence of various "impurities"(13). In the present experiment it was demonstrated that cobalt at concentrations required to inhibit mineralization of matrix prevented crystal formation from a solution containing calcium and phosphorus at 9.6 and 5.0 mg/100 ml, respectively. Thus, the effect of cobalt on apatite crystal formation may, though not necessarily does, account for the inhibition of mineralization observed when this ion was present in the incubating solution.

The nature of the binding sites for apatite crystals in bone matrix has not been definitely established(14), although there are data to suggest that reactive epsilon amino groups of lysine and hydroxylysine in matrix may be the critically spaced groupings necessary for crystal nucleation in bone(15). However, regardless of the structure of crystal formation sites, successful competition with nucleating ions for such sites affords a theoretical means of preventing mineralization. In the present experiment the divalent cations zinc, cadmium and manganese blocked mineralization when present in the incubating medium at concentrations one thirty-fifth that of calcium. At such disparate concentrations interference with binding of calcium would be dependent on groupings possessing a particular affinity for these inhibitory cations. Small amounts of histidine and sulfur containing amino acids do exist in matrix(16), and the affinity of such compounds for zinc or cadmium is well known(17). However, evidence is lacking to implicate such groups as being of importance to crystal nucleation sites.

The role of enzymes in the process of mineralization of bone and cartilage is poorly understood. Manganese and magnesium are known to be essential cofactors for enzymes involved in glycolysis, but these ions, if present in concentrations greater than necessary for enzyme activation, may actually block activity of the respective enzyme(18). It appears likely that some of the elements observed to inhibit mineralization in the present experiment may have done so by an effect on matrix enzyme activity. Zinc, cadmium and manganese are most suspect in this regard. That magnesium potentiated the inhibitory effect of the elements assayed is consistent with enzyme inactivation, since magnesium alone in sufficient concentrations inhibits matrix mineralization(19).

The presence, as contaminants in the basal salt solution, of trace amounts of those metals which affected mineralization may have modified slightly the results obtained. Manganese was the only metal detected in any appreciable concentration (0.0008 mM) and here the amount was less than one-tenth of that required to inhibit mineralization of matrix in the presence of magnesium.

That the observations reported herein are pertinent to *in vivo* states remains to be determined. Most of the elements tested are

present in plasma at various concentrations ranging from 0.001 to 0.01 mM where apparently they are largely protein bound(20), but the concentrations at sites of biological mineralization are unknown. Zinc was the only element used that completely inhibited mineralization at concentrations detected in plasma, *i.e.*, 0.015 mM(8), but the data reported indicate that the inhibiting metallic ions may act additively in preventing mineralization. Therefore, total concentration of the various inhibiting ions may be more pertinent than that of a single inhibitory element to problems of bone mineral deposition. Whether aberrations in transport or concentration of these elements in any way contribute to clinical disorders of mineralization requires further study.

Conclusion. The inhibitory effect of various metals on mineralization *in vitro* and on apatite crystal formation *per se* have been determined. Zinc, cadmium, manganese and cobalt inhibited mineralization of rachitic cartilage matrix when present at concentrations of 0.10 mM or less. Cobalt was unique in preventing apatite crystal formation at concentrations inhibiting mineralization of matrix. These observations indicate that the possible inhibitory effect of certain metals must be considered in any study of mineralization involving use of biological fluids.

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Experimental Infection of Tissue Cultures with Certain Mycoplasma (PPLO).* (28127)

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For some time virologists have been aware of complications which might arise as a consequence of tissue culture contamination with pleuropneumonia-like organisms (PPLO). The problem has become increasingly important since so many host-cell systems can now be grown in continuous culture as well as by primary explant technics(1,2). Soon after

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