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On the Mechanisms of Calcification.* (23847)

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Studies of the mechanism of calcification have been dominated by a single approach, that formulated by Robison(1): calcification involves some booster mechanism by which the product of the concentrations of calcium and phosphate is elevated locally so as to induce a precipitation of bone salts from saturated or slightly undersaturated tissue fluids. An important weakness in this approach is the necessary assumption that the tissue fluids are not already supersaturated with respect to bone salt. Actually, the older literature(2,3, 4) and recent results(5,6) have demonstrated rather conclusively that the serum and, most probably, the extracellular fluids are *supersaturated* with respect to the mineral phase of bone. More recently, in recognition of this supersaturated state of body fluids, attention has turned to the possibility that the mechanism of calcification may involve the crystal seeding or a surface-catalyzed induction of crystal formation. Indeed, quite some time ago(7), it was clearly shown that bone mineral would itself induce crystal formation from normal serum ultrafiltrates. These findings were extended and the ability to induce the formation of apatite crystals was shown also to reside in the collagen molecule, although specificity of collagen for such crystal induction was only crudely demonstrated(5, 8). More recently, however it has been claimed, on evidence from electronmicroscopy and X-ray diffraction technics, that only *one* crystallographic form of the collagen molecule is capable of inducing apatite crystal formation from supersaturated solutions of calcium and phosphate(9). Collectively, these studies make the *principle* of catalytic crystal seeding an attractive hypothesis at present. However, several questions arise: Do all collagens catalyze equally well? All connective tissues contain collagen, why don't all connective tissues calcify? Is the same mechanism, crystal seeding, operative in both osteoid *and* cartilage? As stressed recently(10), there are differences between calcification in osteoid and in the provisional zone of calcification in cartilage.

To obtain partial answers to some of these questions, an attempt was made to prepare purified, reconstituted(11) collagen fibers from a number of different tissues. It was hoped that these various collagen preparations could be compared quantitatively for their ability to induce crystal formation from supersaturated solutions of calcium and phosphate. Good yields of relatively pure, reconstituted fibers were obtained from skin and tendon. However, the usual reagents(11) employed in dissolving collagen from other tissues failed to dissolve collagen from bone sections previously demineralized with ethylene diamine tetra acetic acid (EDTA) at pH 7.4. Whether the EDTA-treated bone had been altered by the demineralizing procedure

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or whether the collagen native to bone has properties different from connective tissue collagen is a question not yet fully answered. Similar EDTA-treatment did not adversely affect the yield of reconstituted collagen fibers from skin. In any event, having failed to prepare collagen from bone, the tissue of most interest, a compromise was effected. Sections of skin, tendon and bone were merely demineralized with EDTA and the crude tissue residues tested directly for their ability to induce crystal formation from solutions of varying calcium and phosphate content. While it is not possible to attribute the seeding results obtained to the collagen molecule *per se*, there were no marked differences in the ability of the three tissues to seed crystal formation. Bone matrix seemed to "take up" calcium ions even when crystals did not form.

Methods. Preparation and treatment of tissues. Fresh calf legs and tails were obtained at the time the animals were sacrificed. The epiphyseal portion of the tibia was cleaned free from non-osseous tissue and sawed into blocks of approximately 1 cm^3. The skin and tendons were dissected from the tails and cut into similar small pieces. Samples of 1 g of each tissue were suspended in 300 cc of saturated EDTA (Versene) solutions at pH 7.4 for a period of 12 days, the solutions being changed on the sixth and ninth day to insure complete removal of mineral. After such treatment, bone was found by spectrographic analysis† to contain less than 1 mg of calcium per 100 g of tissue. The tissues were washed free of Versene twice with 500 cc aliquots of normal KCl solution and once with distilled water. All tissues were stored at subzero temperatures until used in the seeding experiments. *Preparation of solutions.* The calcium and phosphate solutions were prepared with CO_2-free distilled water, buffered at pH 7.4 with 0.02 *M* Veronal, and made up to physiologic ionic strength with KCl. The potassium chloride solution was similarly prepared. To inhibit bacterial growth 5 cc of chloroform per liter of solution were added, and the flasks were sealed with paraffin and parafilm(5,12). *Procedure.* Approximately 1 g of "decalcified" tissue was added into 100 cc of solutions of either constant calcium and variable phosphate concentrations or *vice versa*. The flasks were then sealed and equilibrated for 10 days at 37°C with gentle shaking. At the end of this period, the pH of the solutions was recorded and the fluid filtered through molecular filters. The filtrate was then analyzed for calcium by the method of Sobel and Hanok(13) and for phosphorus by the method of Fiske and SubbaRow(14). The calcium and phosphate removed from the original solution by the tissue served as a criterion of its calcification.

† Courtesy Dr. L. Steadman, University of Rochester Atomic Energy Project, Rochester, N. Y.

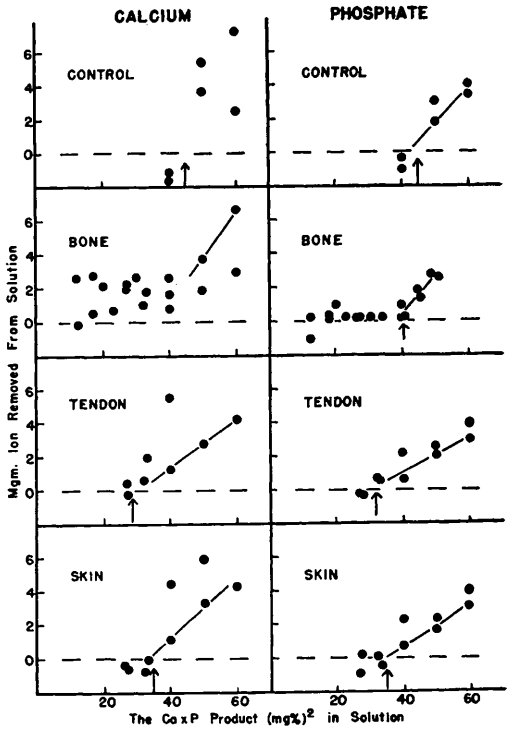

FIG. 1. Induction of crystal formation in the calcium:phosphate:water system by collagenous residues of skin, tendon, and bone. In these graphs, the product Ca × P is plotted *vs* the amount of calcium and phosphate (P) removed from solution by the organic phase. Relative scatter of points is not a true measure of the reproducibility since the solutions varied widely in the Ca/P ratio. Note that, in bone, calcium was removed from solution unaccompanied by phosphate.

Results. The results of these experiments have been summarized in Fig. 1. The degree of saturation of the solutions has been expressed with simple calcium and phosphate product, where calcium and phosphorus val-

ues were expressed as mg %. Based on the ionic strength and pH, this simple ratio can be converted to the thermodynamic product, $a_{Ca^{++}} \cdot a_{HPO_4^{=}}$, by dividing by 18.6(5,12,15). In normal human sera, these products vary from about 18 to 35(16), while the cow normally has a serum product equivalent to about 35(17). In expressing serum products, one must correct for protein-bound calcium; thus a serum containing 10 mg% Ca and 3 mg % P would have an *ion* product of about 18 (6x3(16)).

As previously shown, solutions of calcium and phosphate, in the absence of solid phase, are stable and do not precipitate until the K_{sp} of $CaHPO_4 \cdot 2H_2O$ is exceeded(5). This stability is somewhat surprising since such solutions are frequently very supersaturated with respect to hydroxy apatite, the only stable phase at physiological pH(5). In these experiments, the K_{sp} of $CaHPO_4 \cdot 2H_2O$ corresponds to a Ca x P product of about 45. In confirmation of the earlier results, control experiments (no solid phase) were stable at a Ca x P product of 40 but precipitation occurred when the product was 50 or greater.

Also, in confirmation of earlier studies(4), the addition of collagen induced crystal formation at products below the point of spontaneous precipitation, in the range of 30 to 35. Surprisingly, perhaps, skin and tendon appeared to be at least as efficient in providing nucleation centers as did bone. The one unique property exhibited by bone matrix was its ability to bind calcium. This observation though reproducible and unequivocal may be in part artifactual in origin. Acid mucopolysaccharides have been demonstrated to possess cation-binding properties(18,19). Such substances may have been removed from the skin and tendon preparations by the prolonged EDTA-treatment and frequent washings in KCl buffer. Even so, this represents a major and unique property of bone matrix. It contains cation-binding substances even after prolonged washing and extractions. In these experiments, the binding of calcium by bone matrix renders it difficult to determine precisely the product (Ca x P) at which crystallization first occurred. As plotted in Fig. 1, the value represents the initial product, uncorrected for calcium removed from solution.

We have, however, other results obtained with a slightly modified technic. In these cases, the demineralized bone sections were placed in the solutions as before, but the solutions were decanted and *replaced* several times during the first 24 hours. Under these conditions (where calcium-binding could not materially lower the Ca x P product of the *final* mineralizing solution), the induction of crystal-formation occurred at products in the range of 30 to 35. Similar results were obtained with rat bone (unpublished). Human bone specimens, too, failed to mineralize at products below 30 (unpublished).

These results, in themselves, offer further evidence that the demineralization by EDTA was essentially complete. Had seed crystals remained behind in the tissues, mineralization would have been observed at even the low Ca x P products(5).

These results, then, raise more questions than they answer. How does human bone matrix become mineralized when the normal adult product, Ca x P, in the extracellular fluids is around 18? Presumably, some sort of "booster mechanism" will be required. Though a number of suggested "booster mechanisms" have been made in the literature(1, 20,21,22), none has gained universal acceptance. In the rat and the cow, with normal products of 35, why do not all collagenous tissues calcify? In these species, at least, some inhibitory mechanism must operate to prevent mineralization of extraskeletal collagen fibers. To our knowledge, only one such inhibitory mechanism has been postulated(23).

Clearly, the concept of a simple collagen-induced epitaxy of bone mineral is not adequate to account for the site-specific mineralization seen in the normal animal. Enzymes and cells will, in all probability, be credited with localized functions(24), though their role still remains unclear.

Summary. Specimens of skin, tendon, and cortical bone from the calf were exhaustively demineralized with EDTA at pH 7.4. These tissues were then equilibrated with solutions of varying calcium and phosphorus content. All 3 tissues, presumably because of their collagen content, were able to induce formation of

hydroxy apatite crystals from otherwise stable solutions. In contrast to skin and tendon, bone removed calcium from all solutions, but all three tissues were equally efficient in crystal seeding.

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Effect of Heparin on Serum Lipids Following Intravenous Administration of Fat Emulsion in Dogs.* (23848)

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The evidence that intravenous administration of heparin is capable of clearing alimentary lipemia *in vivo*(1,2,3), and that post-heparin plasma decreases turbidity of lipemic plasma(3,4) and of synthetic fat emulsion(5, 6) *in vitro* is well documented. Bragdon and Havel(7) and French and associates(8) have shown that administration of heparin increased rate of removal of intravenously injected lipoproteins and fatty chyle respectively. Meng and Youmans(9) and Grossman and Strub(10) found that heparin injected simultaneously with synthetic fat emulsion also accelerates the disappearance of the infused fat from the blood circulation. The present work was designed to study: (1) changes in serum lipids following intravenous administration of an olive oil emulsion, and (2) effect of heparin on removal of the infused fat and other serum lipids.

Methods. Healthy adult male dogs weighing from 9 to 15 kg were used. All animals were fasted for 24-36 hours prior to use and experiments were carried out under sodium pentobarbital (Nembutal Sodium) anesthesia. One gram of fat per kilo was infused intravenously as a 10% olive oil emulsion which was stabilized with purified soybean phos-

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