Fibrous Apatite Grown on Modified Collagen

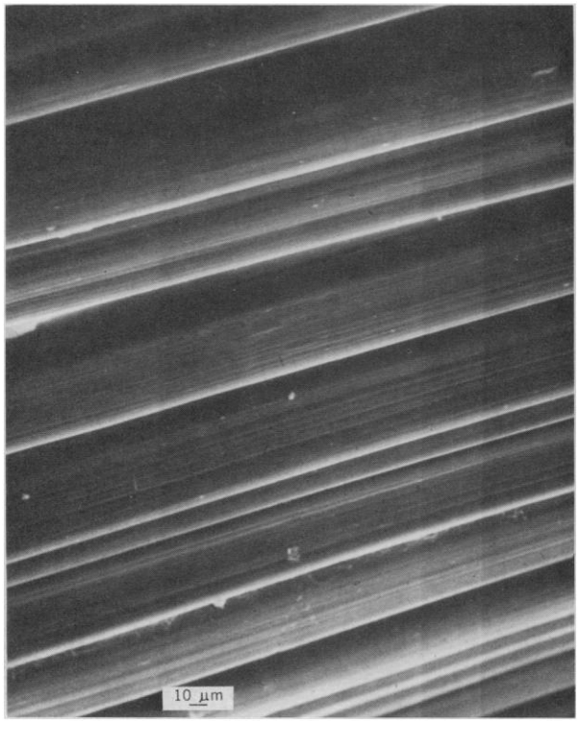
Abstract. *Fibrous apatite has been grown by the enzymatic hydrolysis of calcium β-glycerophosphate on reconstituted calfskin collagen tapes which had been modified by the addition of a phosphoprotein, phosvitin, in the presence of a cross-linking agent, dimethylsuberimidate. The deposits were identified as a carbonate-bearing hydroxyapatite by x-ray diffraction, and scanning electron micrographs confirmed their fibrous character.*

We report here the growth of a fibrous apatite material by hydrolysis of calcium β-glycerophosphate solutions treated with solutions of an alkaline phosphatase enzyme. Highly oriented deposits were formed on oriented tapes of reconstituted calfskin collagen which had been modified by grafting a phosphoprotein, phosvitin, by means of dimethylsuberimidate, a known cross-linking agent for proteins (*1*).

These experiments were part of an attempt to establish an in vitro model of the calcification mechanism proposed in 1923 by Robison (*2*), who observed alkaline phosphatase in ossifying cartilage and suggested that it was an agent in mineralization. Robison's mechanism is no longer accepted, in large part because no concentrated substrate for alkaline phosphatase has been detected in vivo (*3*). Recent studies (*4*) have indicated that calcification is under cellular control. This implies enzymatic action, and it seems plausible that there may be a calcification mechanism in which calcium and organic phosphate are released in response to the release of the phosphatase, or in which the release of calcium and organic phosphate triggers the release of the enzyme. There is certainly no shortage of organic phosphates, such as glucose phosphates, glycerophosphates, and adenosine phosphates, in living cells.

There have been a great many studies of calcification in vitro. Most of them have been performed with inorganic solutions having calcium and phosphate concentrations comparable to those found in blood serum and other biological fluids. The only recent example we could find of such a study in which enzymes were used in vitro is that of McConnell *et al.* (*5*), who used carbonic anhydrase to form deposits of a carbonate apatite on glass plummets dipped into saliva or inorganic solutions having similar concentrations of calcium and phosphate. These polycrystalline deposits were not oriented, although a similar deposit formed on shark connective tissue showed some preferred orientation.

The orientation of apatite crystallites on collagen in various hard tissues is well known, and many recalcification experiments (*6*) on decalcified bone and dentin have seemed to indicate a strong orienting effect of the collagen. However, such experiments may simply show that not all nuclei of crystalline material were removed by the decalcification treatment. For this reason, we sought to develop a "synthetic" substrate as similar to calcifying collagens as possible. Oriented reconstituted calfskin collagen is available, but there is no similar product made from bone collagen. Since a major difference between bone and skin collagens is the presence of phosphoprotein side chains in the former, we studied the effect of phosvitin (*7*), a phosphoprotein, on the orientation of apatite crystallites with respect to the collagen. Preliminary studies (*8*) had indicated that alkaline phosphatase solutions cause precipitation of an apatitic material when added in catalytic quantities to solutions of the calcium salts of various organic phosphates such as adenosine triphosphate, monophenyl phosphate, and β-glycerophosphate. Some orientation effects were found on collagen tapes alternately soaked in enzyme and calcium–organic phosphate solutions; the effects were similar to those described by Glimcher *et al.* (*9*) for such tapes soaked in solutions of calcium phosphate (*10*). We then decided to attempt a systematic study of the effects of various treatments involving the addition of phosvitin and the use of the cross-linking agent. Studies



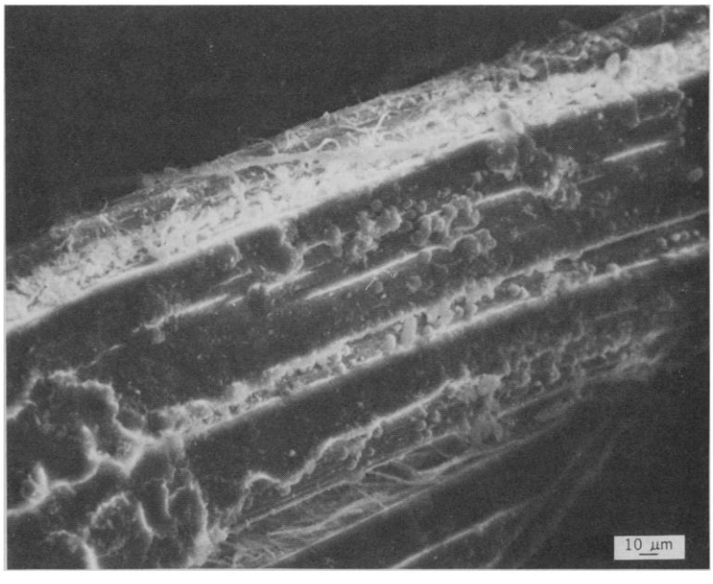


Fig. 1 (left). Scanning electron micrograph of reconstituted collagen tape. Fig. 2 (right). Apatite deposit on collagen tape. Note collagen fibrils not covered by deposit.

were made of the effect of the order of addition of phosvitin, enzyme, and substrate and of the presence or absence of the cross-linking agent. All experiments yielded precipitates of apatitic material, some of which deposited on the tape. The only experiments that yielded continuous, strongly adherent layers on the tapes were those in which the collagen and phosvitin were treated with dimethylsuberimidate.

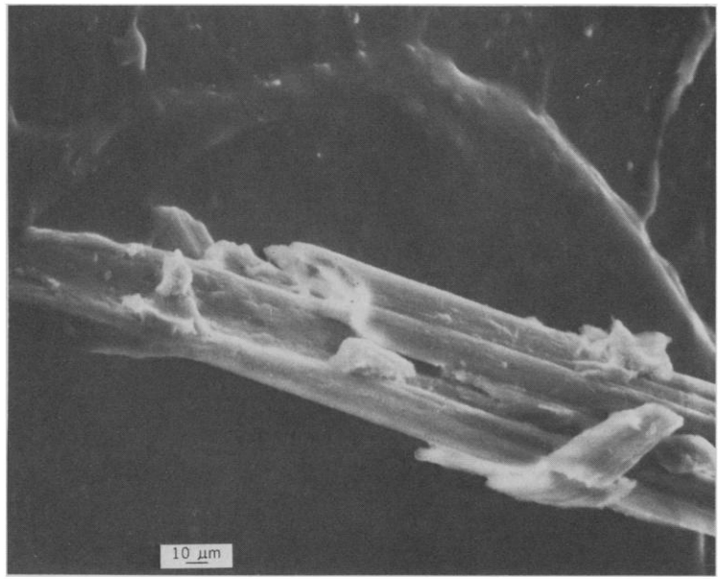
The experimental procedure with which the best deposits were formed was as follows. A frame of stiff copper wire covered with plastic tubing was wound with reconstituted collagen tape (Ethicon). After weighing, the assembly was soaked for 5 days in egg-yolk phosvitin (Sigma) and alkaline phosphatase (Schwartz/Mann). After rinsing in distilled water, the assembly was soaked in an aqueous solution containing 0.2*M* triethanolamine hydrochloride and 0.3 percent dimethylsuberimidate hydrochloride, adjusted to *p*H 8.5 with NaOH. After this treatment, the assembly was washed in several changes of distilled water for 90 minutes to remove unreacted phosvitin and reagent. It was then transferred to a 0.25 percent solution of calcium β-glycerophosphate, which had been prepared by precipitation of the sodium salt by calcium nitrate, recrystallized from hot water, and dissolved in tris (hydroxymethyl)-methylamine buffer at *p*H 9.7. There was no immediate precipitate, although this had happened in earlier runs at slightly higher concentrations. The sample was then alternately soaked in the enzyme-phosvitin solution and the calcium β-glycerophosphate solution for 24 hours each, thoroughly washing with distilled water each time. After 15 changes of solution and a final washing, the calcified tapes were dried, cut from the frame, and refluxed in boiling ethylenediamine to dissolve away the protein. The residue was washed into a Büchner funnel with water and filtered.

The product coming off the tapes appeared to be flat needles up to 2 mm wide and 1 cm long. When the tapes with the deposit were examined in the polarizing microscope before refluxing, they showed birefringence with straight extinction along the axis of the tape, having the appearance of large single crystals. Refractive indices were difficult to measure accurately because of the irregular orientation of the crystals, but values of $n_E = 1.648(3)$ and $n_O = 1.656(3)$ (the extraordinary and ordinary indices of refraction, respectively) yielded a Δn value of -0.008 ± 0.004. Such relatively high birefringence is characteristic of mineral carbonate hydroxyapatites; the relative intensities of the carbonate infrared bands are consistent with such an assumption. It was also noted that the extinction direction followed the curvature of the tape where it had been wound around the frame. This indicated that crystallization had been oriented by the collagen and not simply nucleated at discrete sites.

However, an oscillation x-ray photograph, taken on a Weissenberg camera about the long axis of a needle, resulted in a powder diagram. This suggested that orientation along the fiber direction might be complete, but that at right angles to that direction orientation was random. The unit cell is hexagonal with axial lengths $a = 9.37(5)$ Å and $c = 6.93(5)$ Å. These dimensions are within the range of reported values for carbonate apatites (*11*). In a complete oscillation diagram, the (002) reflection showed intense arcs along the needle axis, indicating a high degree of preferred orientation. A Laue diagram showed a similar effect. Other reflections were rings, indicating random orientation of the *a*-axis.

Infrared spectra of powders made by grinding the deposit (KBr pellet) show the phosphate and OH bands characteristic of hydroxyapatite, with some absorption in the carbonate region and some water absorption. The carbonate bands are not unexpected inasmuch as the material was prepared from solutions at high *p*H exposed to laboratory air for about 20 days.

The fibrous character of the deposit and the orientation along the axis of the tapes are shown in the scanning electron micrographs. Figure 1 is a micrograph of a section of untreated collagen tape. It is composed of parallel bundles of fibers



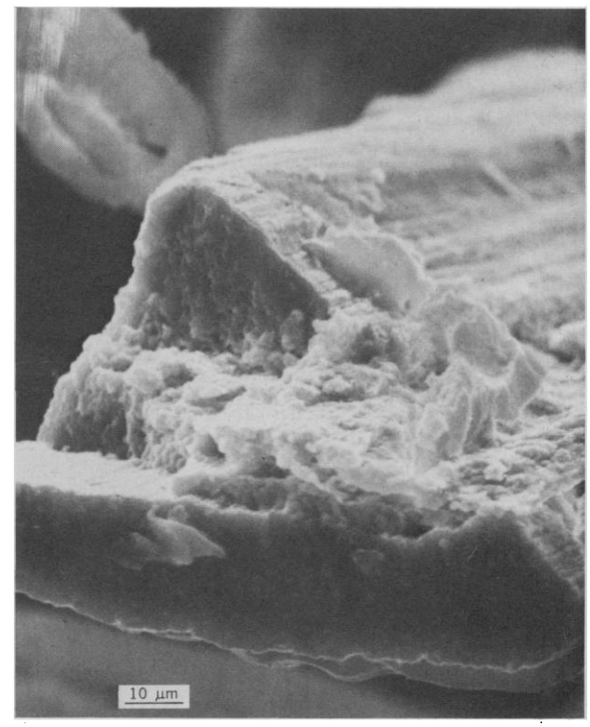


Fig. 3 (left). Fragment of deposit after collagen removal. Note hollow where fiber bundle has been dissolved and parallel arrangement of crystal fibers. Fig. 4 (right). Fractured end of "crystallite." Note parallel striations, fractured ends of microcrystallites.

about 50 μm across, with individual fibers of the order of 2 to 3 μm. Figure 2 shows a section of tape after calcification. The dark areas show regions where coherent mineralization has occurred. Some spherulitic growth is evident on the outer surface of some deposits and especially in the white region where the tape has been torn apart, perhaps by stresses induced during crystallization. One band of collagen is only partially covered, showing the small striations in the underlying fiber.

Figures 3 and 4 are specimens of the apatite material after the removal of collagen by ethylenediamine treatment. Figure 3 shows a bundle of apatite fibers with a hollow in the center, which appears to have grown around one of the bands of collagen about 30 μm in diameter. Note the parallel arrangement of bundles and the curvature of segments which have grown apparently in conformity with the shape of the collagen fiber bundle. Figure 4 shows the fractured end of a piece of fibrous apatite. Beneath the snowlike fine material on the surface, this sample appears to be a mass of parallel crystallites with cross-sectional dimensions of about 1 μm or less; some of these appeared hexagonal in cross section. Parallel striations can be seen on the exposed longitudinal surfaces. The mottled appearance of the transverse surfaces indicates individual crystallite fracture surfaces whose curvature is reminiscent of that of the conchoidal fracture seen in macroscopic apatite mineral specimens. The electron microscopic observations are entirely consistent with the evidence from x-ray diffraction and polarized light microscopy.

The phosvitin-modified collagen tapes used in these experiments can only be a crude approximation to the natural collagen produced under cellular control. In the latter a complex enzyme system catalyzes the formation of precise molecular species, whose conformations lead to structures which must be even more specific in their capacity to control the orientation of crystals of apatite in growing hard tissues. We reemphasize that the only experiments yielding these fibrous deposits were those in which the phosphoprotein was added to the collagen in the presence of a known cross-linking agent.

These experiments do not establish Robison's proposed calcification mechanism. However, they indicate that the failure until now to detect a concentrated substrate for alkaline phosphatase does not, of itself, rule out such a mechanism. If alkaline phosphatase does play a part in biological calcification, it would only require the presence in the tissue of a system that would release a calcium salt of an organic phosphate at a rate appropriate to the rate of release of alkaline phosphatase. Henrichsen (*12*) has added β-glycerophosphate to a tissue culture of chicken heart fibroblasts and showed that calcification occurs only near the sites of dying cells. This could account for abnormal calcification occurring in normally noncalcifying tissues. It would be of considerable interest to carry out a similar study in cultures of osteoblasts, odontoblasts, or similar cells from normally calcifying tissues.

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Localization of the Globin Gene in the Template Active Fraction of Chromatin of Friend Leukemia Cells

Abstract. *Friend leukemia cell chromatin has been fractionated into template active and inactive components. The globin gene sequence is associated with the template active component both prior to and after the cells are induced with dimethyl sulfoxide to synthesize hemoglobin and therefore appears to be in an active configuration in uninduced as well as in induced Friend leukemia cells. In cells which have lost the ability to produce hemoglobin, the globin gene sequence is not associated with the template active fraction of chromatin. These results demonstrate the success of the fractionation procedure.*

Gene regulation in higher organisms is accomplished, at least in part, by the restriction of transcription to selective regions of the genome (*1*). What specific interactions of the proteins, RNA, and DNA of chromatin are responsible for the inactivation of certain regions of the genome and activation of other regions is not known. Early cytological studies suggested that the extended regions of chromatin or euchromatin were active in RNA transcription, whereas the condensed regions or heterochromatin were transcriptionally inactive (*2*). Recent biochemical evidence showing active genes to be more readily digestible by deoxyribonuclease I (E.C. 3.1.4.5) also argues in favor of structural differences between transcriptionally active and inactive regions of the genome (*3*).

The fractionation of template active and inactive components of chromatin has been much investigated (*4–10*). One approach has been to shear chromatin to a size smaller than the average transcriptional unit and subject the material to a physical separation procedure such as differential centrifugation (*4*), sucrose or glycerol gradient centrifugation (*5*), ion exchange chromatography (*6*), selective precipitation (*7*), gel filtration (*8*), thermal chromatography on hydroxyapatite (*9*), or others (*10*). Another approach is based on a brief digestion of chromatin with deoxyribonuclease II (E.C. 3.1.4.6) followed by differential centrifugation and selective precipitation of inactive chromatin with magnesium (*11, 12*).

Although it has proved difficult to obtain a pure fraction of chromatin by any of the reported procedures, highly enriched fractions have been obtained. Indeed, the deoxyribonuclease II procedure has yielded a six- to sevenfold enriched template active fraction of rat liver chromatin (*12, 13*).

We have now used the deoxyribonuclease II procedure to gain insight into