REVIEWS OF MODERN PHYSICS VOLUME 31, NUMBER 2 APRIL, 1959

42

Molecular Biology of Mineralized Tissues with Particular Reference to Bone*

MELVIN J. GLIMCHER†

Department of Biology, Massachusetts Institute of Technology, Cambridge 39, Massachusetts

INTRODUCTION

THERE are several reasons for presenting the topic of biological mineralization. In the first place, the organization of these tissues, from the macromolecular level to the level of the macroscopic tissue elements, offers superb examples both of structural order in biological systems and of the relation between tissue architecture and tissue function. Secondly, the *process* of mineralization illustrates the way in which the physiological function of a tissue or organ may be interpreted on the basis of the molecular structure and macromolecular organization of its components, and in terms of fairly well-characterized physicochemical phenomena. Thirdly, many of the unsolved phenomena in mineralization provide challenging problems, particularly for those with training in the physical sciences.

Certain tissues of the organism must perform a variety of mechanical functions, such as the maintenance of the form and shape of the organism against the forces of gravity, and the protection of certain delicate organs (or the organisms themselves) by enclosing them in rigid vaults. As sites of attachments for muscles and by virtue of articulations, they also provide the organism with a system of movable but structurally rigid levers. Thus, certain organisms have not only definite form and shape, but also flexibility and a means for locomotion and prehension.

Nature has devised a number of different ways of differentiating these specialized tissues. In the case of certain insects, the polymeric chitinous-protein procuticle becomes highly crosslinked providing the structural properties required for the exoskeleton. In the case of certain *Elasmobranchii*, such as the shark, a fibrous, gel-like structure, cartilage, provides both resiliency and structural rigidity. A third method, the subject of this paper, is the deposition of a substantial amount of inorganic crystals within an organic matrix: *tissue mineralization*. This process is widespread in biology (Table I), both in the plant and in the animal kingdoms, ranging from the most primitive to the most highly ordered species. Classical examples are the exoskeletons of certain marine mollusks (the shells of clams, oysters, etc.) and the endoskeletons of the vertebrates (calcified cartilage, and bone). Also, a combination of methods is used, such as the crosslinking of the chitinous-protein shell of the lobster and the collagen matrix of bone, both of which are also mineralized.

The inorganic crystals not only serve to confer new structural properties on the tissues, but also provide a storehouse of inorganic ions which may be used to help maintain the constancy of the ionic environment of the organism.

Space does not permit the discussion of the molecular biology of all of the mineralized tissues. Illustrated here are some of the major points and problems in one representative tissue, bone.

COMPOSITION AND MOLECULAR STRUCTURE OF THE MAJOR COMPONENTS OF BONE

Analytically, on a dry-weight basis, bone consists roughly of 65 to 70% of the inorganic crystals of the calcium-phosphate salt, apatite, and 30 to 35% of organic matrix of which collagen makes up the major fraction (95 to 99%).[1,2] The collagen in bone appears to be *structurally* similar to that in other tissues, although it is probably highly crosslinked.[3] The other components in the organic matrix include a number of ill-defined proteins, as well as the acid mucopolysaccharides, and constitute part of the ground substance. Some of the mucopolysaccharides probably are present as mucoproteins (noncollagenous protein complexes). The exact anatomical location of these components at a macromolecular level is not certain, and their state of aggregation and polymerization is not well known.

Although it had been known for nearly two hundred years that bone contains calcium and phosphate, it was not until 1926 that DeJong[4] demonstrated by x-ray diffraction that the crystal structure was similar to that of the apatites, more specifically hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$], the structure of which is illustrated in Fig. 1. The exact nature of the apatite in bone, however, is still being debated.[5–11] This is partly because of the very broad x-ray diffraction reflections resulting from the extremely small crystal size, preventing crystallographic differentiation between a number of very similar proposed structures.

Further difficulties arise because the stoichiometry

* These studies were aided by research grants E-1469 (C1) from the National Institute of Allergy and Infectious Diseases, and A-2317 from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare, and by research grant 12A from the Orthopedic Research and Education Foundation. Many of the major concepts involved have already been dealt with in an earlier paper [M. J. Glimcher, A. J. Hodge, and F. O. Schmitt, Proc. Natl. Acad. Sci. U. S. 43, 860 (1957)]. The detailed evidence and documentation for these concepts are currently in the press.

† Special Postdoctoral Research Fellow of the National Heart Institute, National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

TABLE I. Examples of biologically mineralized tissues.

Species	Tissue mineralized	Crystal chemistry	Mineral form	Major organic matrix components
Plants	Cell wall	$CaCO_3$	Calcite	Cellulose, Pectins, lignin
Radiolariens	Exoskeleton	$SrSO_4$	Celestite	(?)
Diatoms	Exoskeleton	Silica	(?)	Pectins
Mollusks	Exoskeleton	$CaCO_3$	Calcite, aragonite	Chitin, protein
Arthropods	Exoskeleton	$CaCO_3$	Calcite, aragonite	Chitin, protein
Vertebrates	Endoskeleton			
	Bone	$Ca_{10}(PO_4)_6(OH)_2$	Hydroxyapatite	Collagen
	Cartilage	$Ca_{10}(PO_4)_6(OH)_2$	Hydroxyapatite	Collagen, acid mucopolysaccharides
	Tooth			
	Dentin	$Ca_{10}(PO_4)_6(OH)_2$	Hydroxyapatite	Collagen
	Cementum	$Ca_{10}(PO_4)_6(OH)_2$	Hydroxyapatite	Collagen
	Enamel	$Ca_{10}(PO_4)_6(OH)_2$	Hydroxyapatite	Eukeratin

of the bone mineral (and many synthetically prepared apatite crystals) departs from the theoretical value of hydroxyapatite (lower Ca/P ratio). Because of their extremely small size and, therefore, large surface area, attempts have been made to explain this nonstoichiometry on the basis of the surface adsorption of excess phosphate on stoichiometric hydroxyapatite.[12,13] Aside from a number of theoretical objections to this explanation, direct experimental evidence has not verified this hypothesis.[11,14]

Other investigators have felt that lattice defects account for the aberrant stoichiometry.[6,11] In most instances, these investigators have proposed that calcium atoms were missing from the atomic structure (either internally or on the surface) and were replaced either by other cations, water, or by protons (as hydrogen bonds between the oxygens of orthophosphate groups and protons). The latter have been referred to as defect apatites.[11] However, defect crystals in which there are vacancies in certain lattice positions are ones in which the order of magnitude of these "holes" or "defects" is possibly one per thousand or ten-thousand

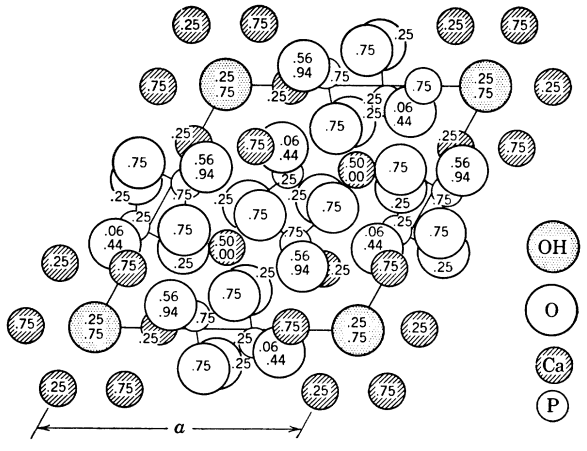


FIG. 1. Atomic arrangement of the constituents of hydroxyapatite projected on the 001 plane. The numbers refer to the fractional height in the unit cell of the atoms in the plane perpendicular to the paper (c-axis), as reported by Carlström[6] [from A. S. Posner, Clin. orthoped. **9**, 5 (1957)].

atoms. To satisfy the requirements in this particular case, however, one or even two calcium atoms out of every ten would have to be absent from the lattice structure. It is very doubtful that so many calcium atoms could be absent or substituted for without some structural change in the lattice.

Such structural changes have been postulated in the case where water or hydronium ions replace Ca atoms in the lattice structure. Experiments have shown (1) that a considerable amount of water is lost both from bone apatite crystals and *in vitro* prepared apatite crystals when subjected to progressively increasing temperatures after initial dehydration,[8–10,15] and (2) that apatite crystals precipitated from aqueous solutions have associated with them an amount of water many times greater than that adsorbed by initially dry crystals from a vapor phase of water.[16] This "excess" water cannot be separated from the crystals by mechanical centrifugation at 80 000 g.[16]

Since the structure of hydroxyapatite allows for no water of crystallization, several explanations have been offered to account for this "excess" water.

Thus, the substitution of water (or hydronium ions) for Ca atoms, would not only explain the low Ca/P ratios, but would also be consistent with the data on water discussed above.

The objection that such a relatively large replacement of Ca atoms would lead to structural changes in the lattice (*vide supra*) is acknowledged by one group which believes that there *are* structural differences between hydroxyapatite and the bone apatite crystals, and refers to the latter as α-tricalcium phosphate or hydrated tricalcium phosphate without specifying either the exact differences in the structural parameters or the position of the water in the new structure.[8–10,17]

Considering the water as specifically replacing columnar calcium atoms in the apatite lattice, it has been proposed also that certain low-ratio apatites are layered structures consisting of "sheets" of hydroxyapatite held together by hydrogen bonds between the phosphate groups and water.[18]

This layered apatite structure referred to as octocalcium phosphate (OCP) gave an almost identical

x-ray diffraction pattern as that of "pure" hydroxyapatite except for several additional reflections,[5,17] presumably thought to be owing to the superlattice effect of the layered water.

On the other hand, the "excess" water (particularly in the case of *in vitro* precipitated apatite crystals in aqueous solutions) has also been postulated to be the result of a large hydration shell composed of about 60 molecular layers of water "bound" to the crystals because of an electrical-field asymmetry of the crystal surfaces.[16] This amount of water is many times greater than that adsorbed by initially dry crystals from a vapor phase of water and consists of many more molecular layers of water than usually considered possible for surfaces to bind as a result of electrical-field effects.

It appears possible from the data on the replacement of calcium atoms by water (or hydronium ions) that, when apatite crystals are precipitated in aqueous solution, part of the "excess" water may be accounted for by its incorporation *into the crystal lattice*, resulting in a phase change of the solid.

Another factor in determining the amount of water associated with apatite crystals, particularly those precipitated in aqueous solutions, is the size, shape, and interaction properties of the crystals which are discussed later in this paper (page 386). Recently, however, single crystals of OCP have been prepared, and it has been shown to be a distinct compound whose structure, while closely related, is not identical to hydroxyapatite.[19] These investigators also felt, however, that OCP was also a layered structure with the layers separated by water molecules.

Dehydration of OCP caused a characteristic x-ray reflection (18.4 A) to shift to progressively lower spacings and to disappear completely upon elimination of approximately two-thirds of the water. The crystals then showed a typical apatite pattern, indicating that, with loss of a certain amount of water, the OCP structure is unstable and a true phase change occurs from OCP to apatite.

Octocalcium-phosphate (OCP) crystals prepared in the laboratories of A. S. Posner, American Dental Association,[20] Research Division, National Bureau of Standards, have been well characterized by x-ray diffraction and have shown minor differences both in the lattice spacings and intensities of the reflections as compared to those of hydroxyapatite. These OCP crystals have been examined in this laboratory by electron-microscopy and electron diffraction. The electron-diffraction patterns were indistinguishable from those of hydroxyapatite indicating that, under the conditions of vacuum and temperature in the electron microscope, a phase change had occurred from an OCP lattice to an apatite lattice by the dehydration process, confirming the report[19] referred to earlier.

Another problem is the determination of the position and state of aggregation of carbonate, which has been postulated to exist in the crystal lattice *per se* (as a carbonate apatite) as a separate phase (calcium and magnesium carbonate) or adsorbed on the crystal surfaces as carbonate ions.[7–10,21,22]

These examples illustrate that, although the evidence is quite substantial that the lattice structure of the inorganic crystals in bone is either that of hydroxyapatite or of something similar, the intimate details such as the relationship between OCP, other hydrated calcium phosphate compounds, defect apatites, hydroxyapatite, and the structure and nonstoichiometry of the bone crystals and synthetically prepared "apatite" crystals is obviously not yet clear.

ANATOMICAL STRUCTURE

Figure 2(a) demonstrates the gross appearance of the upper end of a femur. The honeycomb-like appearance of the head and metaphysis is referred to as the *spongiosa* and is composed of delicate spicules of bone called *trabeculae*. The bone in the cortex of the shaft is much more densely packed and is referred to as *compact bone*. In both cases, the structure of the adult bone consists of a series of layers or lamellae. In the cortex of many long bones, the lamellae are further arranged concentrically around a central canal forming hollow "cylinders" containing small blood vessels. These small cylinders of bone are the basic units of such compact bone and are called *osteones* or *Haversian Systems* (Fig. 3). They are longitudinally oriented in the general direction of the long axes of the long bones (Fig. 4).

The collagen fibers in a lamella are arranged in small bundles which encircle the canal in continuous spirals crossing one another and resulting in a trellis-like arrangement.[23,24] Although the general direction of the bundles in the same lamella is similar, it varies from one lamella to the next, giving a characteristic appearance when viewed in polarized light.[25] This arrangement of the fibers within any one lamella and in consecutive lamellae imparts maximum structural properties to the tissue.

The inorganic crystals of apatite are deposited in this highly organized and ordered matrix of collagen and ground substance. Their distribution can be visualized by the use of microradiography (Fig. 5). Note the inhomogeneity, not only with respect to the entire section, but even within any one Haversian system.

The trabeculae, or "bone girders" of the spongiosa are oriented functionally in that they closely parallel the trajectories of maximum stress [compare Figs. 2(a) and 2(b)]. This allows the structure to resist mechanical stress and strain in the most efficient manner and with the greatest economy of material in accordance with sound engineering principles.

The *size*, *number*, *disposition*, and *orientation* of trabeculae of the spongiosa also can *change* in response to altered mechanical demands. This functional adaptation of bone to mechanical stress was emphasized first by Wolff in 1892 (Wolff's law),[26] yet, to date, no adequate explanation of the mechanism has been offered.

The structural order evident in the arrangement of the macroscopic trabeculae and in the microscopic Haversian systems of bone is also evident at a lower order of magnitude. In Fig. 6, an electron micrograph of a longitudinal section of cortical bone, the collagen fibrils are well oriented and the 640-A axial repeat of the fibril is accentuated in many areas by the specific location of the dense inorganic crystals. The crystals are well oriented with their long axes parallel to the collagen fibrils and intimately related to them. Electron diffraction (Fig. 7) confirms that it is the crystallographic *c*-axis of the inorganic crystals which is parallel to the fibril axis of the collagen.

Most workers have felt that the inorganic crystals of adult bone were in the ground substance between the collagen fibrils,[27,28] although more recent work indicates that some of the crystals might be within the fibrils.[29] Interpretation of electron micrographs has been difficult because of the dense packing of the collagen fibrils (Fig. 5), making it impossible to say with certainty whether the crystals were within or on the surface of the collagen fibrils, or just generally associated and oriented with them by virtue of the over-all organization, orientation, and dense packing of the fibrils in the tissue. Our laboratory has recently resolved this issue by studying bone in which the collagen fibrils are widely separated, and it is possible to see the relationship between individual fibrils and individual crystals by high-resolution electron microscopy.

Figure 8, an electron micrograph at low magnification, indicates the regular arrangement of the collagen fibrils in alternate layers and the relative looseness in the packing of the individual fibrils. It is quite obvious that the crystals are *within the fibrils* and not in the intervening ground substance. Figures 9–12 are longitudinal and cross sections at higher magnifications, and more clearly emphasize the position of the inorganic crystals *within the collagen fibrils*. That this is not an initially random crystal precipitation between the fibrils, later reorganized within the fibrils by recrystallization, is evident from an examination of electron micrographs of the earliest stages of calcification in

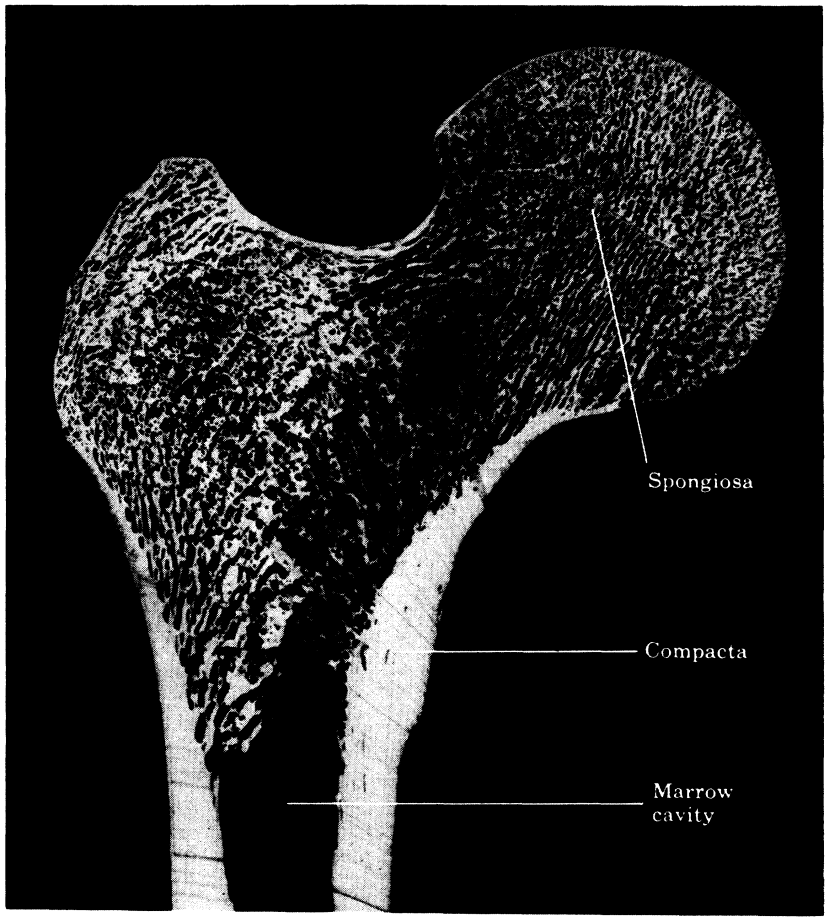


FIG. 2(a). Coronal section of the upper end of a human femur [from D. W. Fawcett, in *Histology*, R. Greep, editor (The Blackiston Company, New York, 1954), p. 133; reproduced from original kindly supplied by the author].

embryonic bone.[30,31] Figures 13 and 14 are electron micrographs of the initial stage of calcification in embryonic bone. These clearly show that the earliest crystals are regularly spaced and deposited *within* the collagen fibrils.

The location, distribution, and orientation of the crystals, by virtue of their position within the fibrils, provide the most efficient arrangement for effectively resisting mechanical stresses.

The extremely small size of the crystals (200 to 400 A $\times$15 to 30 A) and the fact that one and probably two of their dimensions consist of only a few unit cells, result not only in a tremendous surface area, but also in a large percentage of the atoms being in surface or near-surface positions. This easy accessibility to the crystal interior allows for a rapid exchange of ions between the crystals and the interstitial fluids.

In summary, starting with the major components themselves (collagen and apatite) which are ordered materials, the organization of the tissue, from a macromolecular level to the arrangement of the macroscopic trabeculae, constitutes a highly ordered, well-organized structure, superbly adapted both mechanically and chemically to perform its biological functions.

MECHANISM OF CALCIFICATION

Introduction

The many theories of calcification may be classified, in general, into two groups. The first, referred to as the "booster" theory,[16] proposed that a specific enzyme (or enzymes) exists in the areas undergoing calcification and that it splits off inorganic phosphate from an organic substrate.[32] This local "boosting" of the concentration of phosphate then exceeds the level of spontaneous precipitation and results in crystallization.

A second concept, first proposed in 1921[33] and recently revived, suggests that the *organic matrix* of calcifiable tissues initiates the formation of crystals. Recent biochemical evidence[34] and the inadequacy of the "booster" theory in explaining the precise localization of the

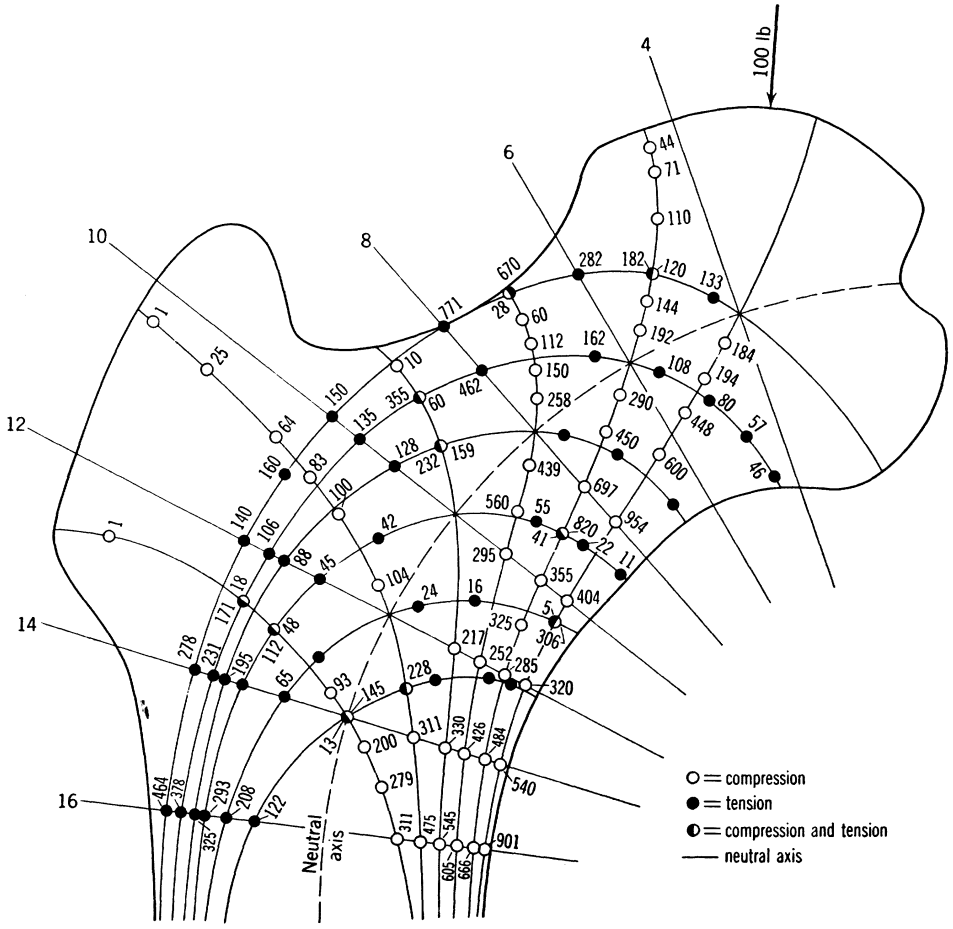


FIG. 2(b). Diagram of the lines of stress in the upper femur (after Koch) [from *Gray's Anatomy*, W. H. Lewis, editor (Lea and Febiger, Philadelphia, 1942), twenty-fourth edition, Fig. 245].

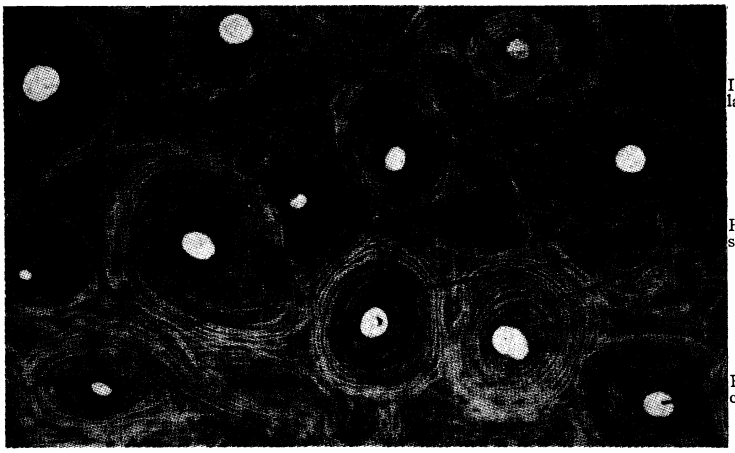


FIG. 3. Cross section of decalcified compact bone ×80. Note the alternating dark and light layers in the Haversian systems and interstitial lamellae attributable to consecutive changes in collagen-fiber orientation [from D. W. Fawcett in *Histology*, R. Greep, editor (The Blackiston Company, New York, 1954), p. 134; reproduced from original kindly supplied by the author].

crystals at an ultrastructural level strongly support the role of the organic matrix in calcification. Opinion as to what substance (or substances) in the organic matrix is responsible for the induction of crystallization and the nature of the *mechanism* of induction has varied.[35–42]

Because of the complexities in biological mineralization, it is important that the physicochemical nature of the mineralization phenomenon be clearly defined before proceeding to the experimental evidence and hypothesis which follow. Crystallization, or the formation of inorganic crystals from solutions where none previously existed, represents a *phase change*. This physical change in state can be divided arbitrarily into *crystal nucleation*, the process of forming the initial fragments of the new phase, and *crystal growth*, the subsequent growth of these fragments into clearly defined crystals. In addition, the phenomena of recrystallization (the growth of large crystals at the expense of smaller ones), may also play a role even after the solid state has been achieved. Failure to distinguish these interrelated but separate phenomena and failure to differentiate between the many *regulatory* processes controlling them and their underlying *mechanisms*, can be confusing. A brief review of some of the thermodynamic and kinetic principles related to *phase transition* is given in the Appendix at the end of this paper.

FIG. 4. Longitudinal section of compact bone illustrating Haversian systems (original magnification ×120) [from J. P. Weinmann and H. Sicher, *Bone and Bones, Fundamentals of Bone Biology* (C. V. Mosby Company, St. Louis, 1947); reproduced from original kindly supplied by the authors].

Certain misconceptions concerning the formulation of apatite crystals from solution are clarified by the phenomenological description in the Appendix regarding the manner in which phase changes occur.

It has been assumed[6,16,43,44] that, in the precipitation of apatite crystals, the formation of brushite ($CaHPO_4 \cdot 2H_2O$) must occur first, later to be hydrolyzed or otherwise converted to apatite [$Ca_{10}(PO_4 \cdot 6(OH_2)$]. This assumption has been based on the incorrect premise that an 18-body collision is required for hydroxyapatite formation whereas only a two-body collision is required for brushite formation.

Several conceptual errors need clarification. In the first place, when calcium and phosphate ions in solution aggregate to form a crystal, the change in state which occurs is a *physical change in state*—i.e., a change in the state of aggregation of the ions from a solution phase to a *solid* phase. The empirical formulas of the solids in such cases [$CaHPO_4 \cdot 2H_2O$ *or* $Ca_{10}(PO_4) \cdot 6(OH_2)$] do not refer to a molecule or molecules of brushite (containing 2 ions and 2 water molecules) or of apatite (containing 18 ions), but merely represent the *ratios* of all of the constituent ions of the *solid phase* in terms of smallest whole numbers. The ions are part of a crystal-

line solid containing *thousands* or usually *millions* of ions arranged in a definite spatial configuration characteristic for the particular crystal.

Secondly, even in chemical reactions where a physical change of state does not occur, neither the reaction *order* nor the reaction *mechanism* can be deduced simply on the basis of the chemical formulas of the reactants or the reaction products. In the nucleation of new phases, nuclei probably arise by the stepwise addition of single molecules, atoms, or ions[45,46], and there is no obvious association between the empirical formula of the solid and the size and composition of the nucleus or the order of the reaction.

Inspection of the empirical formulas of various possible solids in no way permits one to predict the *probability* that the formation of *one* particular solid is *more likely* than another. This, of course, will depend on the relative amount of work which is necessary for the formation of a cluster of a particular composition, size, shape, and configuration under the specific conditions of the experiment, i.e., *p*H, temperature, etc.

Some simple examples illustrate the foregoing points. The formation of ice from water may be written H_2O(liquid)→H_2O(solid). It is obvious that one is not dealing with single "molecules" of ice, but with a *solid* containing many water molecules in a specific steric configuration. It is also apparent that neither the *size* of the critical cluster nor the *reaction kinetics* can be deduced, either from the chemical formula or from the equation which describes the change in state. Physicochemical evidence[46] suggests that the nucleus which initiates this phase change is composed of about 80 to 100 water molecules. These nuclei do not form by the simultaneous collision of 80 to 100 water molecules, but are believed to arise as the result of the stepwise addition of single water molecules. The reaction is, therefore, bimolecular in *mechanism*, and of the 80th to 100th *order*.

As a second example, the formation of sodium chloride crystals from solution may be written as Na+ (ion aqueous) +Cl− (ion aqueous)→NaCl (solid). This is a physical change in state—i.e., a change in the state of aggregation of the sodium and chloride ions from ions in solution to ion constituents of a crystal. In this solid, there are no NaCl "molecules," but an array of Na+ and Cl− ions, with each sodium ion equally shared by six chloride ions and each chloride ion by six sodium

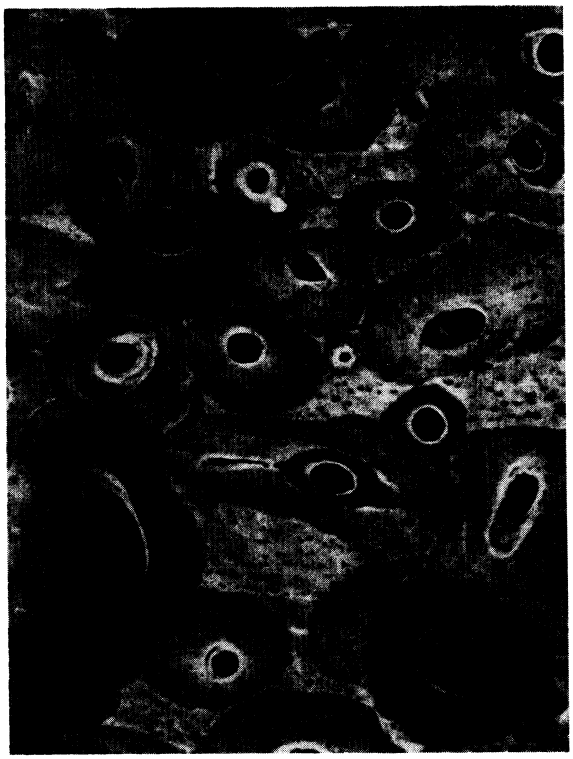


FIG. 5. A microradiogram of a thin cross section of normal cortical bone. Note the variation in density (indicating inorganic-crystal concentration) within any one Haversian system, as well as within the section as a whole. Reproduced from original kindly supplied by A. Engström, Karolinska Institutet, Stockholm, Sweden.

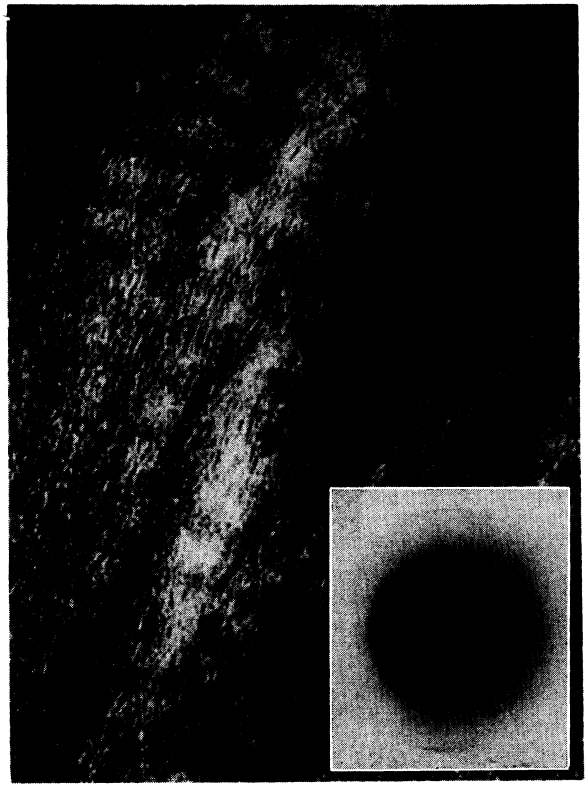


FIG. 6. Electron micrograph of a longitudinal section of compact bone. Note the accentuation of the characteristic axial repeat of the collagen fibrils in some areas by the small inorganic crystals, whose long axes approximately parallel the collagen-fiber axis. ×100 000.

FIG. 7 (lower right-hand corner). Selected-area electron diffraction of the specimen in Fig. 6, showing characteristic pattern of apatite. Arcing of the 002 and 004 reflections indicates that the crystallographic *c*-axes of the crystals are oriented parallel to the collagen-fiber axis, and, therefore, correspond to the long axes of the crystals.

Fig. 8. Electron micrograph of a cross section of fish bone. Note the alternating direction of the collagen fibers in consecutive layers and the packing of the fibrils. Even at this low magnification it is obvious that the dense inorganic crystals are *within* the fibrils and not in the intervening ground substance. ×58 000.

ions. The chemical formula of the solid (NaCl) obviously does not indicate that this change occurs as a result of a 2-body collision.

There appears to be no doubt that, under *certain* physicochemical conditions (low *p*H, 6.0–6.2), one *can* precipitate the calcium phosphate solid, brushite, and that this can in turn be hydrolyzed or otherwise converted to apatite under the proper physicochemical conditions (raising the *p*H, for example). However, one cannot infer that, when a calcium-phosphate salt is precipitated, the formation of brushite is more probable or more likely than apatite, simply because its *empirical* formula contains fewer atoms and ions.

MECHANISM OF CRYSTAL INDUCTION

The paper by F. O. Schmitt (p. 349) notes that not only is the collagen macromolecule a "crystalline" protein, but also the collagen *fibril*—by virtue of its *ordered aggregation* of such macromolecules, and high degree of structural regularity, as seen both by electron microscopy and by low-angle x-ray diffraction—may be considered "crystalline."

These facts and the obvious association between the apatite crystals and the collagen fibrils in normally calcified tissues recommended investigation of the possibility that this intimate anatomical relationship was evidence that either the macromolecular *structure* of collagen or the macromolecular *aggregation state* of the collagen fibrils was responsible for the *induction* of calcification,[40] by acting as a catalytic heterogeneity for the nucleation of apatite crystals. Other studies had also suggested a role for collagen[38,39] or collagen chondroitin sulphate complexes in calcification.[35,36]

For such a mechanism to be operative, however, the solutions from which the crystals are formed must be in metastable equilibrium. It is, therefore, important to establish the state of equilibrium of the fluids immediately surrounding the fibrils in relation to the formation of this new phase (apatite crystals) *in vivo*. Although it has been demonstrated that the bulk of the extra-

FIG. 9. Electron micrograph of fish bone in a region of loosely packed fibrils such as in Fig. 8. Despite variations in individual fibril direction, long axes of inorganic crystals are parallel to the individual collagen fibrils with which they are associated by virtue of their position within the fibrils. ×75 000.

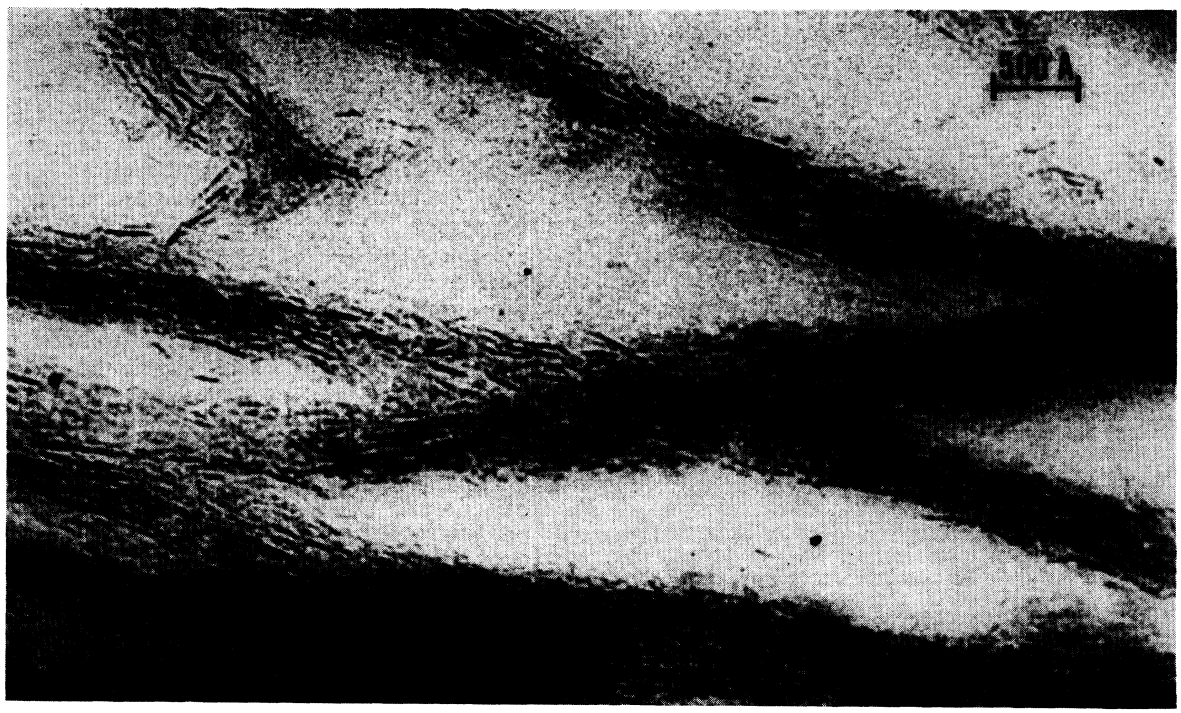


FIG. 10. Higher magnification of an area in Fig. 9. The crystals appear to be rod-shaped, approximately 200 to 400 A long and 15 to 40 A wide. ×225 000.

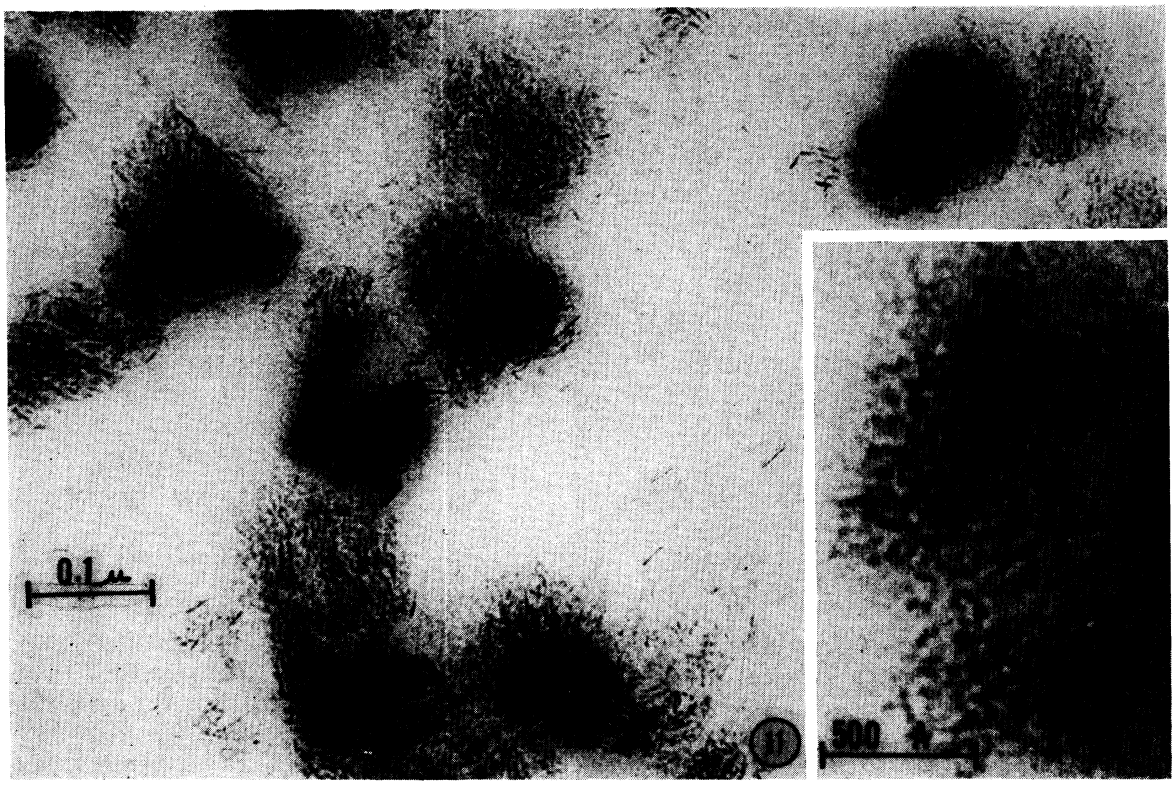


FIG. 11. Higher magnification of cross sections of fibrils in fish bone. The apatite crystals are obviously within the collagen fibrils. ×165 000.

FIG. 12. Cross section of two fibrils in an area similar to Fig. 11. The crystals have appearance of rods viewed on end and appear to be hexagonally packed. ×430 000.

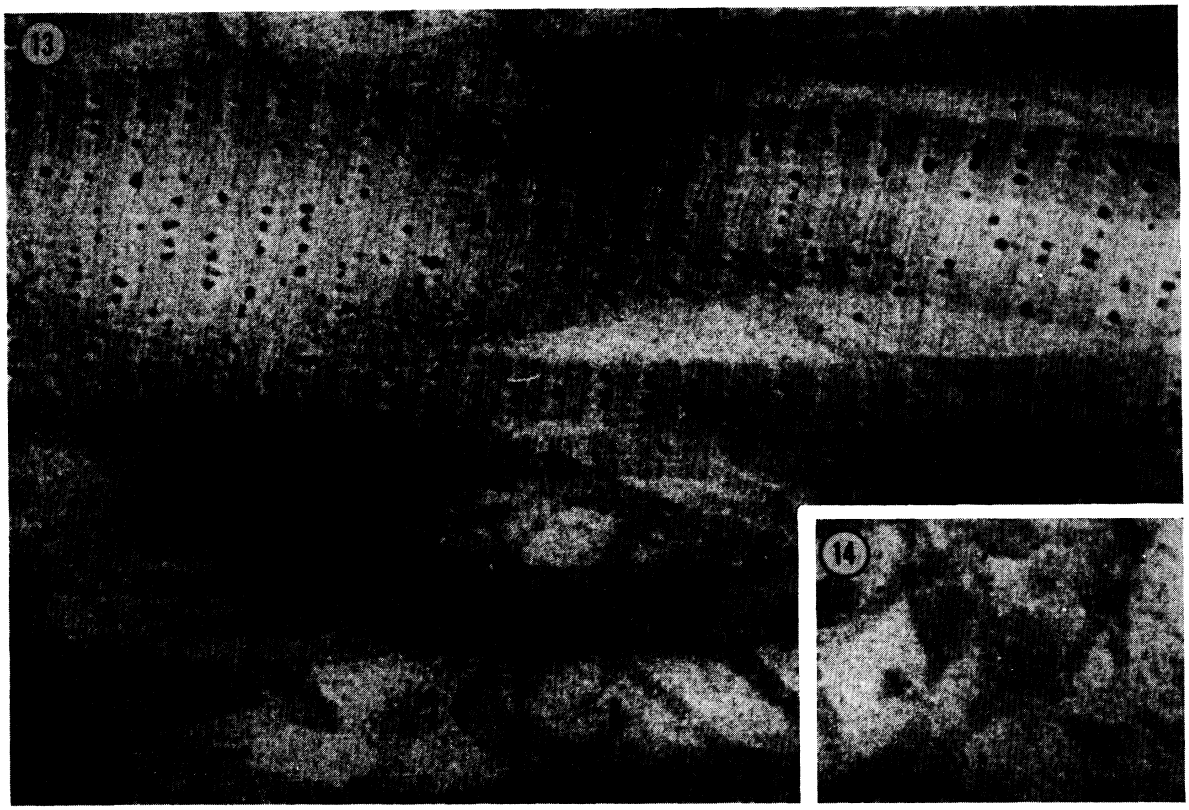
cellular interstitial fluid is metastable with respect to the formation of apatite crystals,‡ the *local* values immediately surrounding the collagen fibrils in any particular tissue might be markedly different because of active cellular-controlled compositional changes, or active or passive transport and diffusion phenomena.

Although it is informative to examine the data with regard to the state of equilibrium of the unaltered extracelluar fluids, not only with reference to the mechanism of induction but also particularly with regard to the *regulation* and *control* of this process in other non-mineralized tissues, the *immediate objective*, however, was to establish whether or not collagen *could* induce the formation of apatite crystals from solutions which *were* metastable, and to determine the nature of this specificity (if any) and its mechanisms. The experiments discussed in the following were conducted at the Massachusetts Institute of Technology in collaboration with A. J. Hodge and F. O. Schmitt, and were designed to answer these questions.§

The collagen of many connective tissues can be dissolved in a number of weak acids and neutral buffers yielding viscous solutions of the macromolecules (see F. O. Schmitt, p. 349). These can subsequently be reaggregated and reconstituted into fibrils with the typical and characteristic axial repeat and intraperiod fine structure of native fibrils. By appropriate treatment of the connective tissue prior to dissolving the collagen and by several recrystallizations, the collagen fibrils can be prepared relatively pure, with only minute traces of ground-substance constituents.

‡ The ion product ($a_{Ca}++\times a_{HPO_4}-$) in the serum of many vertebrates is considerably higher than the same product in solutions of equivalent ionic strength, *p*H, temperature, etc., where equilibrium has been approached either through precipitation or through dissolution of apatite crystals.[38,39] Since this product appeared to be critical in determining whether or not precipitation of a solid phase occurred, it was concluded that serum and interstitial fluid were supersaturated with respect to the bone mineral. While these thermodynamic data are not conclusive, direct experimental evidence supports this supposition. Inorganic solutions with ion products ($a_{Ca}++\times a_{HPO_4}-$) similar to serum were stable for indefinitely long periods of time, but showed rapid separation of *more* solid when exposed to bone mineral[47] or to synthetic apatite crystals.[48] It would appear, therefore, that the unaltered extracellular interstitial fluid is metastable with respect to the formation of apatite crystals.

§ Although we are fully aware of the limitations of a model system which is admittedly a great deal simpler than the process as it occurs *in vivo*, it has enabled us to characterize and distinguish what appears to be the *mechanism* of crystal induction as well as several closely allied phenomena in the process of calcification and to gain an insight into their mechanisms. Many of the minute details and a quantitative description of these phenomena and the manner in which they are biologically *regulated* will, of course, have to be gathered from the intact organism. However, one must remember that it is difficult even to know *which* details to search for if the basic underlying mechanisms are not at least clearly defined conceptually.



FIGS. 13 and 14. Electron micrographs of avian embryonic bone in the earliest stages of calcification. Note the regular and periodic arrangement of the dense apatite crystals within the collagen fibrils [from S. Fitton-Jackson, Proc. Roy. Soc. (London) **B146**, 270 (1957); reproduced from originals kindly supplied by the author]. ×100 000.

The first experiments were carried out by exposing such reconstituted 640-A axial-repeat collagen fibrils to calcium-phosphate (Ca-P) solutions shown previously to be metastable with respect to the formation of apatite crystals. Because of the technical difficulties involved, and because the thermodynamic properties of calcium-phosphate solutions and their relation to the formation of solid phases are not well-enough characterized, it has not yet been possible to procure quantitative data on nucleation rates, crystal growth rates, free energy of nucleation, etc. The more qualitative methods used were designed to detect the ability of the test materials to *induce* the formation of apatite crystals, and depended mainly on the identification of the formed crystals. They did not distinguish between nucleation rate, crystal growth, recrystallization, etc.

The results of these experiments demonstrated that native-type, 640-A axial-repeat, reconstituted collagen fibrils, prepared from normally uncalcified tissues such as rat-tail tendon, calf skin, guinea-pig skin, fish swim bladder, etc., were able to nucleate apatite crystals from metastable calcium-phosphate solutions. Figure 15 is an x-ray diffraction pattern of such a calcified collagen preparation showing the typical diffraction rings of apatite.

Ironically, we were not able to prepare reconstituted collagens from bone, presumably because of its highly crosslinked nature. However, bone could be decalcified under a variety of conditions, which maintained the 640-A structure of many of the collagen fibrils. This

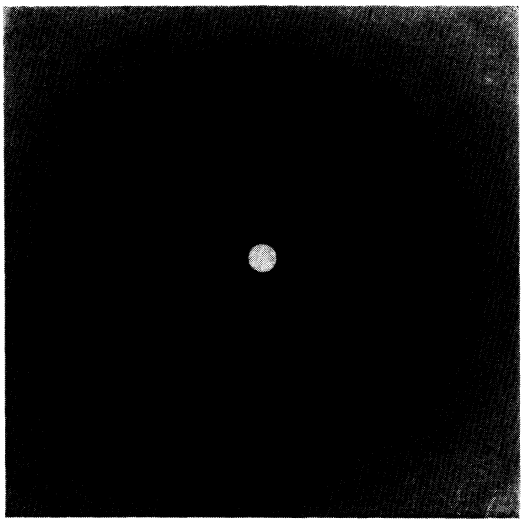


FIG. 15. X-ray diffraction pattern of *in vitro* calcified collagen. Note the lack of crystal orientation (evidenced by complete rings) and the broadness of the diffraction lines attributable primarily to small crystal size.

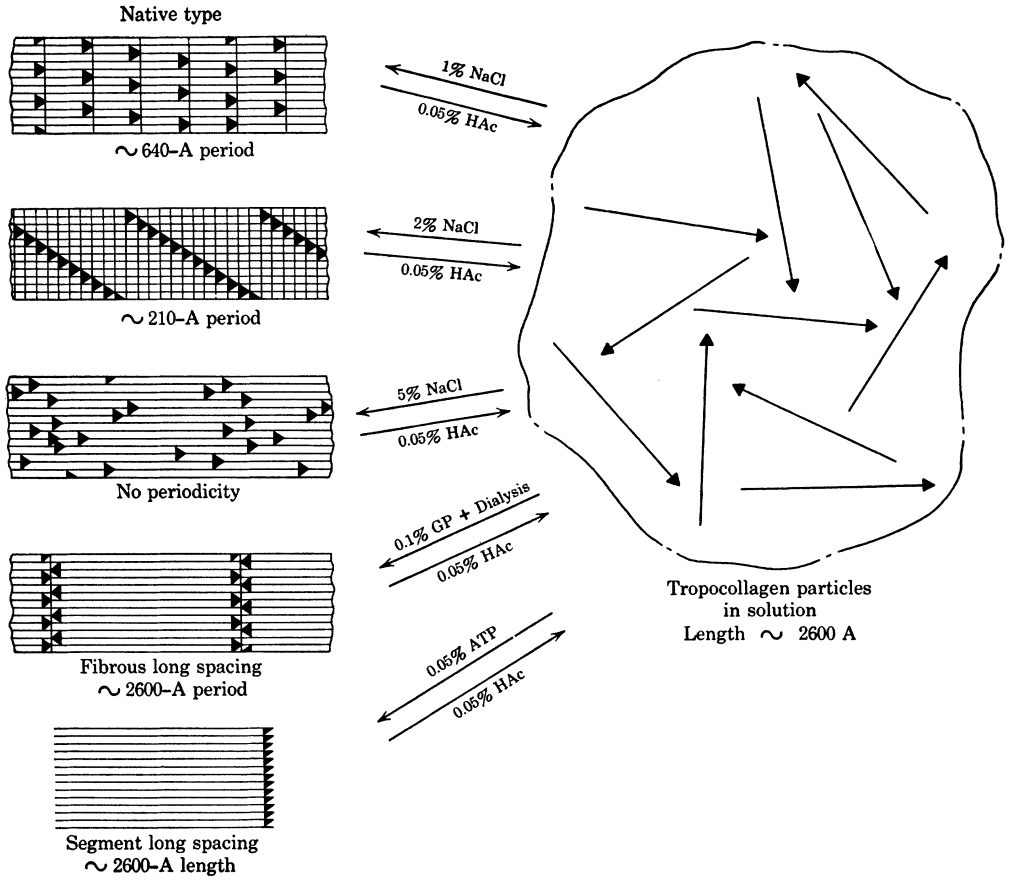


FIG. 16. Modes of aggregation of collagen macromolecules *in vitro* [from F. O. Schmitt, Proc. Am. Phil. Soc. **100**, 476 (1956)].

preparation was also able to induce crystallization of apatite. Although it is very difficult to make quantitative comparisons, it appeared that many of the decalcified bones were not grossly as potent in this respect as the reconstituted collagens, presumably because of changes in the structure of many fibrils and to alteration of certain important functional groups during decalcification.[49–51]

Since, in nucleation phenomena, a number of discontinuities are able to induce phase changes, the next problem was to determine whether or not this property was specific for collagen and, if so, where the specificity resided. Paramyosin fibrils, another well-organized and ordered fibrous protein structure, showing a regular periodic pattern by electron microscopy and low-angle x-ray diffraction, were obtained from the adductor muscles of clams, and used in similar experiments under identical physiochemical conditions. They were not able to induce this phase change.

A number of other fibrillar forms can be reconstituted *in vitro* from the same solution of collagen macromolecules (see F. O. Schmitt, p. 349). These include fibrils with 220-A axial periods, structureless fibrils with no discernible band pattern, fibrous long-spacing (FLS), and segment long-spacing (SLS) (Fig. 16). These are *different* ordered-aggregation states of the *same* macromolecules and reflect differences in the packing and intermolecular geometry of the fibrils. These differences are, of course, also reflected in differences in the *kinds* of amino-acid side chains which interact, and in differences in their *stereochemical relations* to one another. In addition, the isolation of the collagen macromolecules and linear polymers of collagen macromolecules (protofibrils), both in solution and in the solid state, provided a unique system with which to determine the steric nature of the specificity.

Figure 17 is a schematic representation of the basis for these experiments. Using SLS as a typical example with which to compare the native-type fibril, it is apparent that within any region of the fibril, although the *same* macromolecules and, therefore, the *same* amino acids are present, the *steric* relations between amino-acid side-chain groups are different.

The macromolecules, protofibrils, and the various aggregation states were exposed to metastable Ca-P solutions. Neither the macromolecules, linear aggregation of macromolecules, nor any of the fibril forms, *other than those with the native-type 640-A axial repeat,*

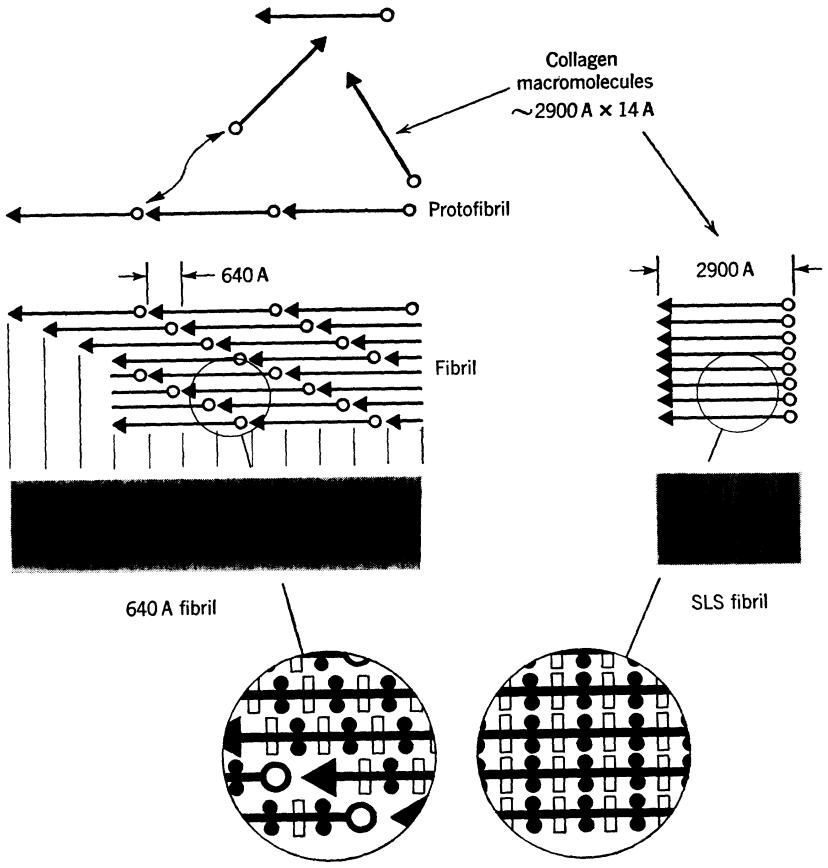


FIG. 17. The steric differences between the reactive side-chain groups of adjacent macromolecules in the native and SLS forms of collagen diagrammatically portrayed.

were able to initiate this phase change under identical physicochemical conditions. Since it was possible to pass reversibly from one fibril form to another and to demonstrate nucleating ability *only* while in the native 640-A form, it was evident that failure to initiate crystal formation was the result of the unfavorable configurations of the other types of reconstituted collagen fibrils and not the result of denaturation or other changes in the macromolecules produced in aggregating them from solution, or of the nature of the metastable solutions.

These experimental findings indicated that the process of *induction of crystallization* was a heterogeneous nucleation of apatite crystals from metastable Ca-P solutions by the collagen fibrils, and was *not* dependent on the macromolecules *per se* or on single linear adlineations of macromolecules, *but on groups of macromolecules and protofibrils polymerized laterally and longitudinally in a highly specific fashion characteristic of native collagen.* Thus, it would appear that the particular type of aggregation and packing of macromolecules characteristic of native-type fibrils creates highly specific steric relationships between reactive amino-acid side-chain groups from adjacent macromolecules within the fibril which serve as centers for nucleation.

Additional confirmation of the necessity of a *specific juxtaposition* of *certain* reactive *groups* was obtained by subjecting aliquots of reconstituted native-type collagen fibrils and demineralized bone to a number of physical and chemical agents, without altering the reactivity of the amino-acid side chains, and demonstrating that the ability to induce crystallization had been lost.

These included the effect of heat, acid, and alkali. In the case of heat, no change was noted *until* the thermal-shrinkage temperature was reached. Thermal shrinkage of collagen results in a phase change as regards its state of aggregation similar to gelatinization.[52,53] This disrupts both the molecular structure and the macromolecular-aggregation state of collagen.

In the case of acid-treated demineralized bone, or alkaline-treated reconstituted fibrils, although the collagen fibrils undergo a good deal of swelling and distortion, leading to loss of the typical 640-A repeat by low-angle diffraction and electron microscopy, the *macromolecular structure* remains intact. In both cases, the amino-acid side chains are not *chemically* altered, but only their *steric* interrelations are changed.

With reference to the phenomenon of nucleation, three distinctly different aspects of the relation between collagen and apatite must be considered. The first is the stereochemical relationship between the reactive side-chain groups which constitute a nucleation center. The second is the demonstration that there are *preferred*

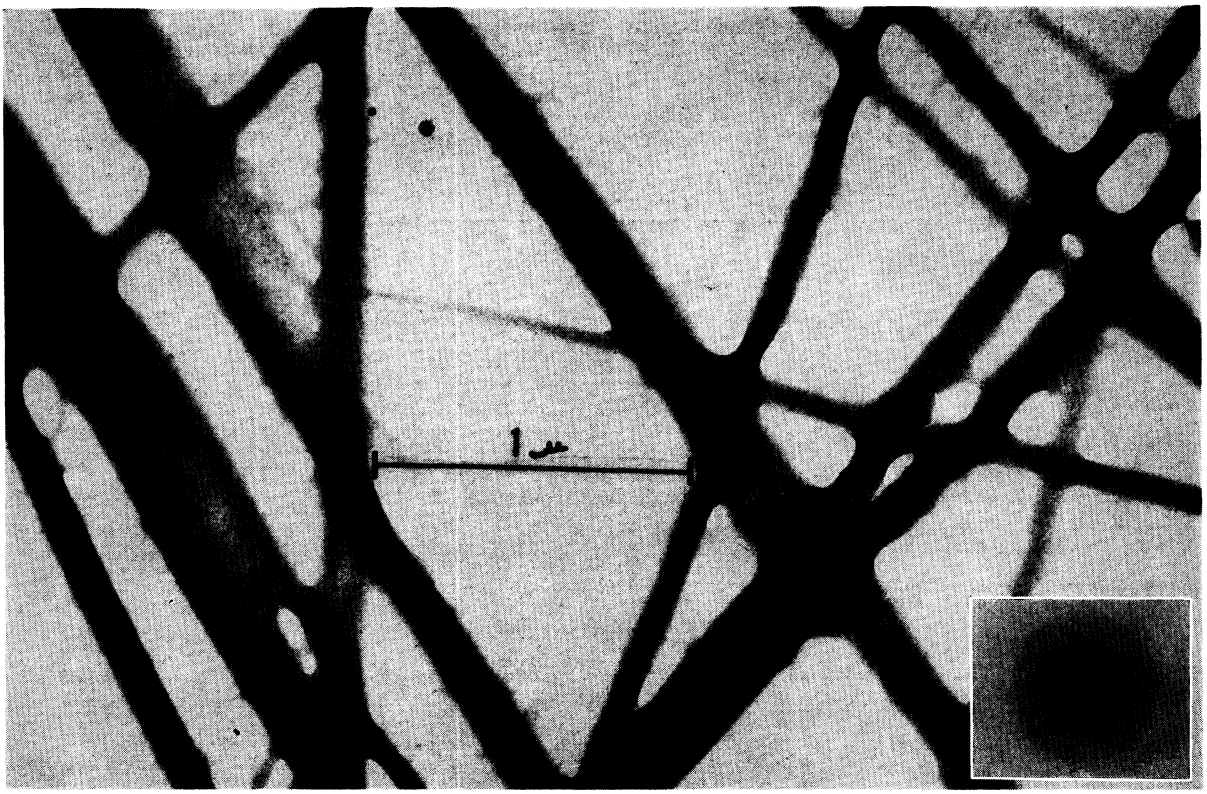


FIG. 18. Unstained, unshadowed preparation of an early stage of *in vitro* calcification of collagen fibrils. Note the regular and periodic distribution of the crystals along the collagen-fibril axis corresponding to the intraperiod fine structure of the fibrils and occurring primarily once per axial period.

FIG. 19 (lower right-hand corner). Selected-area electron-diffraction pattern of preparation similar to Fig. 18, showing the characteristic apatite reflections. There is no evidence of preferred crystal orientation, even though the diffraction pattern was obtained from an area where the fibrils were well oriented. ×42 000.

nucleation centers and the determination of their location with respect to the known electron-microscopic intraperiod fine structure of the collagen fibrils. The third is the nature of the chemical groups in the nucleation centers, and the nature of the intermolecular forces between these groups and the mineral ions.

An undue emphasis seems to have been placed on the *absolute* value of *640 A*, characteristic of native-type fibrils, and its relation to nucleation and other calcification phenomena. It should be apparent, however, that there is nothing specific about this *absolute* value, since it is merely a visible manifestation of the particular aggregation state of the collagen macromolecules and acts as a "fingerprint" for the recognition of the native-type fibrils. Its importance lies in the fact that when the macromolecules are arranged in a specific three-dimensional array so that this characteristic electron-density distribution occurs along the fibril, *certain regions* are created within the fibril whose reactivity and steric relations permit them to act as sites of heterogeneous nucleation. It is this stereochemistry *between reactive side-chain groups within the nucleating regions* which is directly related to the mechanism of nucleation, and this has no direct relation to the *absolute* value of 640 A.

In attempting to correlate the molecular structure of collagen and the property of nucleation, therefore, one must distinguish clearly between the structural characteristics of the *macromolecules* and the higher-ordered configurations which are the result of the specific *arrangement* of the *macromolecules* in the *fibril*.

Unfortunately, unlike the case of nucleation of inorganic crystals by other inorganic crystals, a direct correlation cannot be made between the wide-angle diffraction spacings of collagen and the known lattice spacings of apatite, as has been attempted by one group of workers.[16] The wide-angle diffraction pattern of collagen cannot be treated as arising from simple Bragg planes as in inorganic crystals, but must be interpreted on the basis of the theory of helical diffractors.[54,55] Except for the 10- to 12-A equatorial spacings, indicating the distance between the centers of adjacent macromolecules, the wide-angle reflections are characteristic of the triple-chain helical-coiled structure of the *macromolecule* and give *no* information as to the interatomic distances and configurations of the side-chain groups in the *fibril*, *which* information *is* directly related to the nucleation phenomena. It should be clearly understood that not only do the 640-A-type

FIG. 20. Higher magnification of an area in Fig. 18. ×80 000.

fibrils give the characteristic wide-angle x-ray diffraction pattern, but also the macromolecules themselves and all other fibrillar forms, as well as cold gelatin films.

To date, neither the exact sequence nor stereochemistry of the amino acids in the collagen macromolecule are well enough known to permit determination of this specific configuration, although some general statements may be surmised from other data presented below.

As regards the second point—the demonstration of specific nucleation centers and their location—we have attempted to elucidate this aspect by several different methods. Direct visualization by electron microscopy and correlation of the changes in electron density, either in electron micrographs or from low-angle x-ray diffraction patterns, have been tried.[56] Interpretations based on electron-micrograph density changes and low-angle x-ray diffraction density changes are fraught with technical and theoretical difficulties, and no definite statement is as yet possible from these data.

Electron micrographs taken during the course of time-sequence studies of *in vitro* calcification (Figs. 18–20) and during the earliest stages of *in vivo* calcification of embryonic bone (Figs. 13, 14) provide direct evidence that there are specific regions in the fibrils which act as nucleation centers. These show that, during the earliest stages of calcification *in vitro* and *in vivo*, small dense particles, varying in size from 20 to 150 A, are deposited *regularly spaced* along the fibrils. Selected-area electron-diffraction of these particles in both cases revealed that they are crystals of apatite (Fig. 20).

Since the smallest crystals visualized in electron micrographs represent the *summation* of nucleation and crystal growth, interpretations based on such visual observations must be viewed with some caution. It is entirely possible that regions most favorable for crystal growth may be quite different from those where nucleation occurs. Also, there may be different areas capable of nucleation but differing in their degree of catalytic potency.

In addition, it should be pointed out that the nucleating center is not represented by an entire "band" or "interband" in the collagen fibrils, but only by *regions* within such transverse sections of the fibril. This can be deduced easily if one considers that it takes a certain number of repeating groups from adjacent macromolecules in just the right configuration to make a nucleation center, and that statistically there will only be a finite number of such groups with the proper steric configurations within any one transverse section.

Although it has not been possible to identify the exact location of the nucleation centers with respect to the intraperiod fine structure of the collagen fibrils, observations thus far indicate that these centers do correspond to certain of the so-called band (electron dense) regions. This would correlate well with related chemical and low-angle diffraction studies[57,58] which have shown that the band regions are the most reactive sites for many electron stains, tanning agents, etc., and that these areas probably contain many of the amino acids with long, reactive, polar side-chain groups.

In this respect, it is interesting to note the recent demonstration of the direct correlation between the degree of mineralization and the reactivity of the ϵ-amino groups of lysine in bone and tooth during demineralization,[50] since lysine is considered to be a major constituent of the band regions.[57–60]

Whereas these experiments have indicated the *steric*

specificity in the heterogeneous nucleation of apatite crystals by native-type collagen fibrils, they do not provide information concerning the manner in which these steric factors operate or concerning the nature and importance of the chemical interaction between collagen and the mineral ions, and the specific amino acids and mineral ions involved.

In this regard, certain general principles may be stated. Thus, while it is obvious that some sort of chemical interaction must occur between certain reactive groups in the collagen fibrils and the appropriate ions for nucleation to occur, it is imperative that the nature of the forces involved be such that the ions are still capable of interacting with the other constituent ions of the crystal lattice. For, if either calcium or phosphate ions were very *strongly bound* by the collagen fibrils, collagen would act as a *demineralizer* similar to chelating agents, unless a second mechanism were invoked which *released* the ions after they were bound.

There are several distinct ways in which the steric factors could operate to induce crystallization. The simplest case would be by facilitating the local concentrations of calcium and/or phosphate ions without requiring that the ions themselves be arranged in any particular steric fashion. This local increase in ion concentration would lead to crystallization by exceeding the metastable limit.

Another possibility is that the precise array of groups necessary for nucleation imparts a specificity for the *selective adsorption* or binding of the calcium or phosphate ions, either randomly or in specific steriochemical fashion, and that they then interact with the other constituent ions of the lattice to produce the first fragments of the new phase.

A third method would be the most specific of all, and would statistically require the fewest number of atoms. In this case, the precise array of reactive groups would sterically closely approximate certain low-index planes of apatite in a fashion similar to that proposed by Turnbull *et al.*,[61–63] and discussed in the Appendix. Thus, calcium, phosphate, and hydroxyl ions, either singly or in combination, depending upon which atomic plane of apatite is involved, would be "lightly" bound in such a *geometric configuration* that they would constitute a reactive nucleus.

Of course, one must consider the possibility that certain *clusters* of ions are bound by the reactive groups in the collagen structure. This is really a semantic issue, however, since the binding of such clusters, which presumably have the configuration of the bulk-solid phase, would require similar steric considerations.

Experiments in which nucleation failed to occur, after native-type reconstituted fibrils and demineralized bone were alternately exposed many times to solutions containing *either* calcium or phosphate ions in concentrations equal to or several times greater than those of metastable solutions from which nucleation readily occurred, confirmed the supposition that the intermolecular forces between collagen and the inorganic ions must be just strong enough to affect their interaction energies without *firmly* binding them to the collagen structure.

As to the specific organic groups and mineral ions involved in calcification, most investigators have felt that the initial step in calcification involves the presence in the organic matrix of an anionic group which combines with calcium ions. The anionic site has been variously assigned to chondroitin sulfate,[37] to chondroitin sulfate-collagen complexes,[35,36] or to a phosphorylated polysaccharide.[64] In addition to the fact that the experimental evidence indicates that the nucleating centers consist of reactive amino-acid side-chain groups in the collagen fibril, other considerations cast some doubt on the hypothesis of the *primary* role of calcium-binding in crystal induction.

In the first place, the apatites are *phosphate* salts, and the structural characteristics of the apatite lattice are primarily attributable to the phosphate groups, not to the calcium atoms which can be replaced by a number of other cations (Sr, Pb, etc.) without changing the major features of the crystal structure and symmetry. Since the phosphate groups are the "backbone" of the lattice, their role in the formation of the initial crystal structure would appear to be equally as important, if not more important, than that of the calcium ions.

The findings of Solomons *et al.*,[50] referred to earlier, which showed a direct correlation of the available ϵ-amino groups of lysine (and hydroxylysine) and the degree of mineralization in bone and tooth also is very suggestive, and may indicate that the primary collagen-mineral-ion interaction is between the ϵ-amino groups of lysine and the phosphate ions. Phosphorylation of NH_2 groups has been proposed also by Polonovski and Cartier[65] as the initial step in calcification. Experiments, now under way in this laboratory, in which specific amino-acid groups are being blocked singly and in combination, should provide the necessary data for the interpretation and elucidation of the actual molecular mechanism of the nucleation process.

LOCALIZATION, REGULATION, AND INHIBITION OF CRYSTALLIZATION

The phenomena of *localization*, *regulation*, and *inhibition* of the physicochemical mechanism which initiates crystallization both in normally mineralized and unmineralized tissues are different but closely related. On the basis of the hypotheses and experimental data presented, it would appear that all collagenous matrices are *inherently capable* of nucleating apatite crystals from metastable solutions. Since collagen is the major fibrous protein of all of the connective tissues (skin, tendon, ligaments, etc.), the questions arise: Normally, why do all of these tissues *not* calcify? Why *do* they calcify in certain pathological conditions? Also, even under normal circumstances, apatite crystals are deposited in tissues which do *not* contain collagen fibrils (enamel),

and in abnormal states in other noncollagenous tissues as well.

It is impossible to answer all these questions definitively at the present time, but the controlling factors and the circumstances under which they may be operative can be developed within the framework of the physicochemical concepts of solution metastability and heterogeneous nucleation sites.

Localization of the Crystals to Specialized Tissues

It is not suggested that collagen is the *only* organic compound capable of nucleating apatite crystals. This is quite unlikely, as, in all nucleation processes, many materials can act as nucleation catalysts with varying degrees of potency. In the case of tooth enamel, the apatite crystals are closely associated with another structural and "crystalline" protein, a eukeratin,[66–68] and it is likely that a similar mechanism for crystal induction exists here.

Regulation and Inhibition

Since the native collagen fibrils of most connective tissues do *not* calcify under normal conditions, one of several general situations or a combination of them must exist. Either the collagen in those tissues *not* normally mineralized is different from that of bone or calcified cartilage; or the degree of metastability of the extracellular fluids immediately surrounding and within the collagen fibrils in the various tissues is different; or other local phenomena increase the catalytic potency of the collagen fibrils in the normally mineralized tissues.

Since *reconstituted* native-type collagen fibrils from a wide variety of tissues normally *not* calcified *were* able to initiate crystal formation *in vitro* in our experiments, *failure* to mineralize, if attributed to the collagenous component, would involve subtle *structural* differences between native and reconstituted fibrils, as yet unresolved by the physical and chemical methods employed to date. However, it is possible that, during the extraction and reconstitution of the fibrils, parts are lost of the collagen macromolecules which normally inhibit calcification *in vivo*. There are no data available with which to evaluate the feasibility of this suggestion.

With respect to the second possibility, that differences in the degree of solution metastability account for the specific localization and regulation of calcification, two different points of view are possible. If one assumes that the degree of metastability of the unaltered interstitial fluid is sufficiently great so that collagen *can* induce perceptible rates of nucleation, some mechanism must be operative in the normally unmineralized tissues which *prevents* crystallization. If the degree of metastability of the unaltered interstitial fluids is *not* sufficiently high, either an *increase* in the degree of metastability or an increase in the catalytic potency of the collagen fibril is necessary for crystallization even in the normally mineralized areas, and a *minimal* protective mechanism is required in the normally uncalcified regions.

These variations in the degree of metastability could result either (a) from cellular-controlled compositional changes in the interstitial fluids, or (b) from the presence of other substances in the tissue which actively or passively controlled diffusion and specific ion transport and transfer, or competed with the mineral ions for active sites in the collagen fibril.

Cellular-controlled compositional changes of the interstitial fluids include variations in the calcium and/or phosphate concentrations, calcium to phosphate ratios, *p*H, ionic strength, ion complexes, etc. These could be mediated directly by the cells or by substances secreted by the cells, such as enzymes. For example, the demonstration that phosphorylative glycogenolysis can produce a local increase in phosphate concentration in epiphyseal cartilage[34] illustrates the role of such a device in the *regulation* of calcification in *normally mineralized tissues*, either by merely making more phosphate available, or by participating in another enzymatic cycle[69] which actively transfers phosphate ions to specific groups in the collagen fibril.

As to another method of *regulating* the degree of metastability (particularly in *normally unmineralized tissues*) by controlling diffusion, ion transport etc., the possible role of the ground substance and certain of its components have also been investigated.

Although much research has been done on the ground substance of connective tissues, its exact composition, state of aggregation, and anatomical distribution are not clear. Prominent among its components are the various acid mucopolysaccharides. These are thought to exist in the tissues as complexes with noncollagenous proteins.[70,71] In the past, most investigators have postulated that, of the ground-substance components, the chondroitin sulfates specifically, either alone or in combination with collagen, have played a role in the initiation of calcification.[35–37]

That it is not solely the *amount* of the acid mucopolysaccharides which determines whether calcification is initiated is obvious from the fact that hyaline cartilage —one of the richest sources of this material—is normally uncalcified, whereas bone, which does calcify, contains extremely small amounts of these substances.

Differences in the *kinds* of mucopolysaccharides present is not a plausible explanation, since the various acid mucopolysaccharides found in adult bone or in the growing ends of bone (bone, epiphyseal cartilage, etc.) are present in other tissues.[72]

It is instructive to review the findings as to the nature of the ground substance in cartilage, since a transition occurs from the normally uncalcified hyaline portion to the normally calcified epiphyseal region over a relatively short anatomical distance.

When tissue sections of hyaline cartilage, epiphyseal cartilage, and bone are stained with metachromatic dyes or by the Hotchkiss procedure, definite differences

are noted.[43] The staining characteristics of hyaline cartilage gradually change as the epiphyseal cartilage is approached. This change is also evident in bone which is being actively deposited or resorbed. Since metachromatic dyes react with negatively charged, high molecular-weight compounds, these staining characteristics have been linked primarily to the sulfated mucopolysaccharides in such connective tissues. The change in metachromasia and in the staining properties of these tissues by the Hotchkiss procedure has been interpreted as evidence for the depolymerization of the anionic mucopolysaccharides, as the zone of calcification is approached and reached.[41–43]

Although there is obviously a number of other possible reasons for this change in staining properties, the important point is that *there is some alteration*, either in the amount, state of aggregation, or reactivity of charged groups, etc., in the ground substance, and that this change accompanies calcification. Analytical data[73] which demonstrate a very marked loss of organic sulfate during cartilage calcification and during the formation of bone matrix indicate that it is the *depolymerization* and subsequent *removal* of these compounds which are related to calcification.

In attempting to assign a specific role to the ground substance in calcification, two properties stand out as important. One is that its state of aggregation is probably that of a gel in which the mucopolysaccharides exist complexed with a protein moiety as a mucoprotein.[70,71] Such a physical state of aggregation might well limit the diffusion of interstitial fluids and ions to the collagen fibrils.

The anionic groups of this mucoprotein are free and reactive,[74] which accounts for the second property of importance—the large cation-binding capacity of the ground substance. Under normal circumstances, therefore, the mucoproteins may help to inhibit calcification by limiting the available mineral ions, both by diffusion and by selective cation (calcium) binding. It is also possible, but less likely (since collagen and the chondroitin sulfates, for example,[75] do not react with collagen above *p*H 4.0), that the reactive groups of the mucopolysaccharide portion of the mucoprotein compete with the mineral ions for positions in the collagen fibrils, but it is possible that the noncollagenous protein portion of the mucoprotein may interfere with the process by just such a mechanism.

Depolymerization and removal of these compounds by eliminating the diffusion barrier, decreasing the cation-binding capacity of the ground substance, and possibly "freeing" some of the reactive groups of the collagen from their interaction with the protein moiety of the mucoprotein, would allow the mineral ions to react with the collagen. The cation-binding capacity of the remaining depolymerized mucoprotein components would also most likely be decreased, since it has been shown that metallic cations are more strongly bound to high molecular-weight acids and bases than to their monomeric compounds.[76]

In vivo, the rapid depolymerization of the mucopolysaccharide-protein complexes and the subsequent decrease in their cation-binding properties might well lead to a local release of free cations including calcium, so that the resultant *increase* in calcium-ion concentration might then actually *aid* the initiation of crystallization by increasing the degree of metastability and making additional calcium ions available.

This hypothesis as to the role of the ground substance is supported by a series of *in vitro* experiments, conducted in our laboratory, in which native collagen-rich tissues, such as rat-tail tendon, calf skin, guinea-pig skin, etc., *failed* to mineralize under physicochemical conditions identical with those used in testing *reconstituted* native-type fibrils *from these same tissues*. In addition, when these tissues were treated so as to *extract* many of the components of the ground substance, including the chondroitin sulfates, either directly or by enzymatic depolymerization, these same native tissues readily mineralized.

Further support for this hypothesis comes from the experimental observation that hyaline cartilage, rich in the acid mucopolysaccharide-protein complexes *fails* to mineralize *in vitro* but selectively *removes* Ca^{++} from the calcium-phosphate mineralizing solutions. This property has been shown to be related to the sulfate groups of the chondroitin sulfates.[77]

This "shielding" of reactive sites in the collagen fibrils by ground substance components in intact tissues has also been noted in the tanning and dying of skins, a process which depends on the interaction of certain dyes, complex metallic ions, etc., with specific groups in the collagen. It was found that such interactions were markedly facilitated when the tissues were first tested by procedures designed to extract the ground substance components, and even more so by the prior depolymerization of the chondroitin sulfates by testicular hyaluronidase.[78] The latter method, in addition to being more specific, was also carried out under milder conditions so that the collagen fibrils were presumably less distorted and less swollen.

Figure 21 is a schematic representation of many of the experiments described. It illustrates both the specificity of the collagen macromolecular-aggregation state in *initiating* calcification, and the *possible* role of the ground substance as *a factor* in inhibiting it.

Since nucleation rate is so markedly dependent on the degree of metastability (p. 390), the organism has the dual problem of keeping the metastability of the extracellular fluids at a sufficiently high level for collagen to induce a perceptible rate of nucleation in normally mineralized tissues, and at the same time not so high that aberrant calcification cannot be safely controlled. It seems likely that a compromise is obtained by maintaining the degree of metastability in unaltered extracellular fluid *just* below the point where collagen is very effective. The rate of nucleation in bone and cartilage could then be increased and controlled by very small increases in the local concentration and/or active

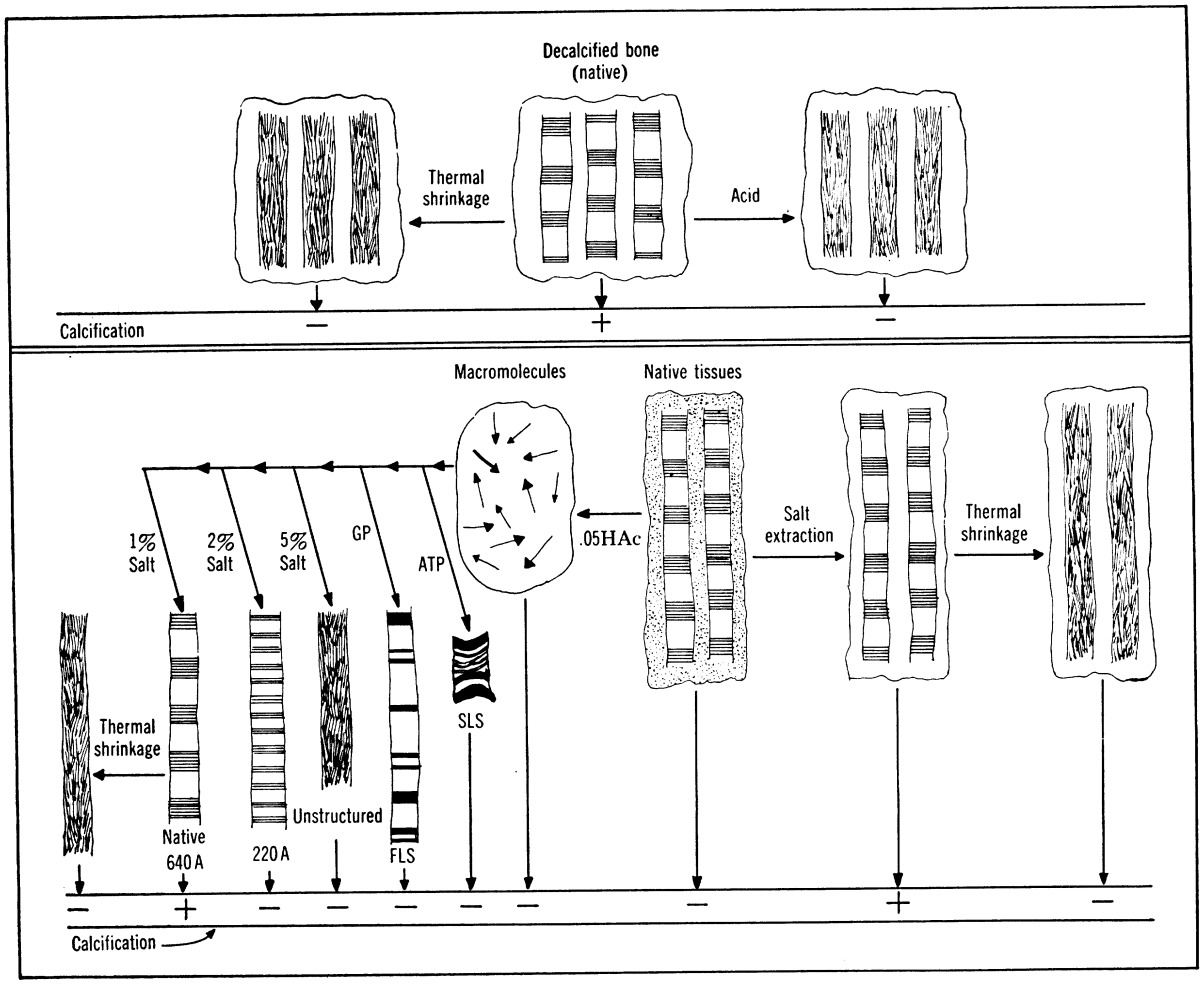


FIG. 21. Composite and diagrammatic illustration of the experiment demonstrating the specificity of the macromolecular aggregation state of native-type collagen fibrils in calcification, and the possible role of the ground substance (heavily stippled regions surrounding fibrils in native tissues) in inhibiting and controlling it. Enzymatic treatment of the native tissue is not shown, since it is not yet certain whether depolymerization is itself effective, or whether depolymerization and subsequent removal from the tissue is necessary.

transfer of mineral ions by enzyme mechanisms, such as the one mentioned earlier for phosphate ion, and the prevention of pathological calcification assured by decreasing the degree of metastability by utilizing the properties of the ground substance, as enumerated in the foregoing.

There is every reason to expect that, like other functions of such vital importance to the organism, mineralization is under the control of many factors, delicately balanced to provide *biological* and *cellular regulation* of the physiochemical *mechanism* which initiates crystallization.

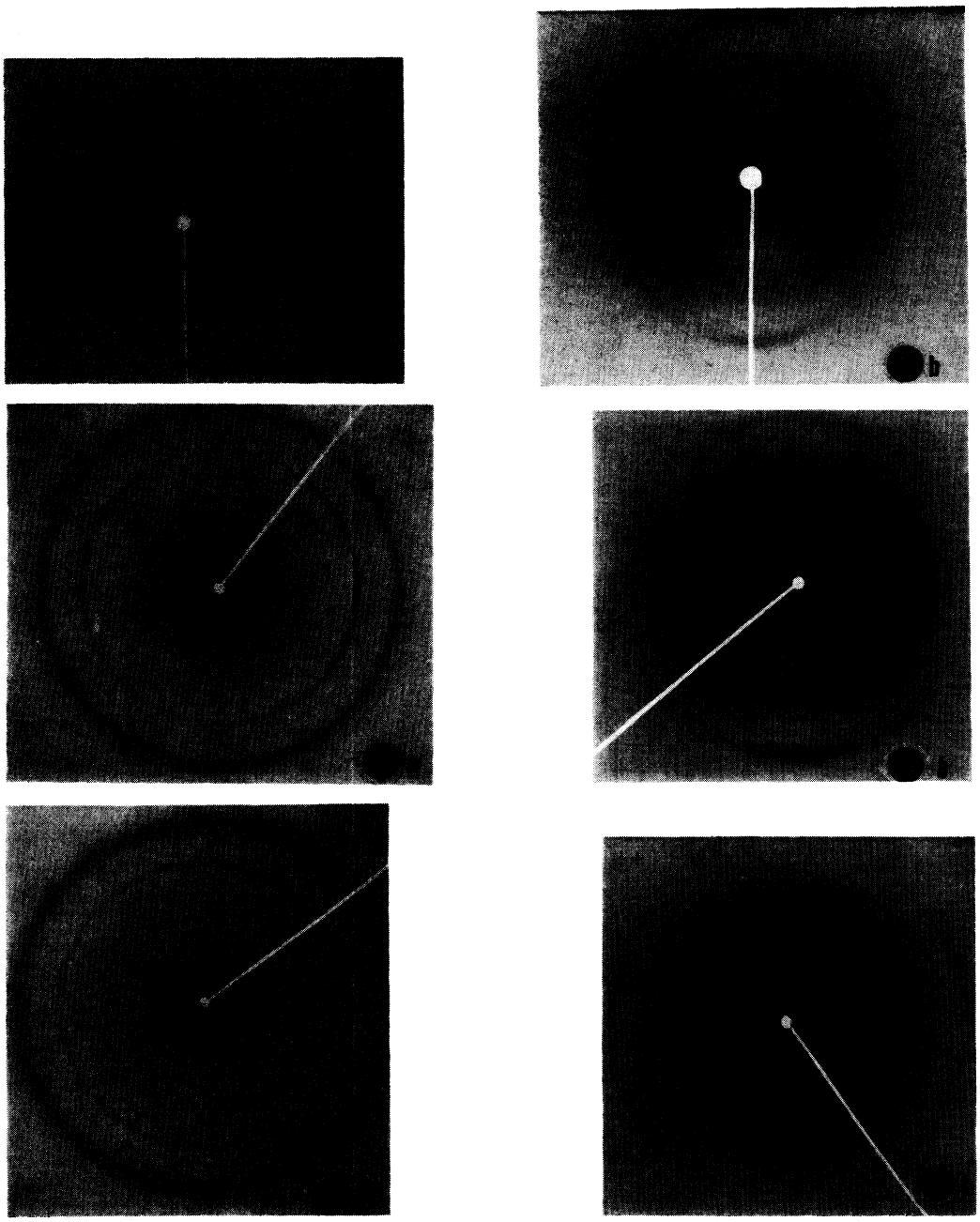
COLLAGEN-INORGANIC CRYSTAL RELATIONSHIPS

Location of the Inorganic Crystals with Respect to the Collagen Fibrils

The location of the crystals within the collagen fibrils supports the theory of heterogeneous nucleation as presented, since the probability of arranging a number of side-chain groups in the proper steric configuration is statistically higher *within* the fibrils, and where the density, intermolecular forces, and interaction energies are highest.

The longitudinal position of the crystals tends in many instances to be localized to certain regions accentuating the 640- to 700-A axial repeat of the collagen fibrils. It is difficult to say exactly where these regions are in relation to the intraperiod fine structure of the fibrils, although density considerations and shadowed preparations indicate that they are most likely in those regions where the density of the bands is greatest. This is especially true in the less heavily calcified fibrils. In many other fibrils, however, especially where calcification is quite high, no such localization is apparent. It would appear, therefore, that—although *initially* there are preferred regions in the fibril for nucleation and for crystal growth—as the fibril continues to calcify, crystals are deposited throughout the fibril.

X-ray diffraction evidence that the average size of the crystals is approximately 220 A led some workers to



FIGS. 22, 23 and 24. X-ray diffraction patterns of longitudinally oriented native, calcified fish bone [Fig. 22(a)], rat femur [Fig. 23(a)], and embryonic metatarsal rudiment [Fig. 24(a)], showing apatite orientation; same specimens [Figs. 22(b)–24(b)] decalcified, showing collagen orientation.

speculate that three such crystals were aligned longitudinally per axial period of the collagen fibril.[79] Such an arrangement would result in the structure having an axial repeat of 220 A. This has not been borne out by direct visualization of the fibrils and the inorganic apatite crystals by electron microscopy.

Inorganic Crystal-Collagen Fibril Coorientation

Earlier workers have demonstrated by electron microscopy that the long dimension of the inorganic crystals, while closely parallel to the fibril axis in bone, is for the most part randomly oriented in calcifying cartilage.[80] Since the process of orientation was felt to be a true-oriented overgrowth directed by certain crystallographic planes in the collagen fibrils (epitaxy)[5,16] and directly related to the process of mineral phase induction, it was suggested that *possibly* different mechanisms were involved in the initiation of calcification in these two closely related tissues.[16]

This was based also on electron-microscopic evidence

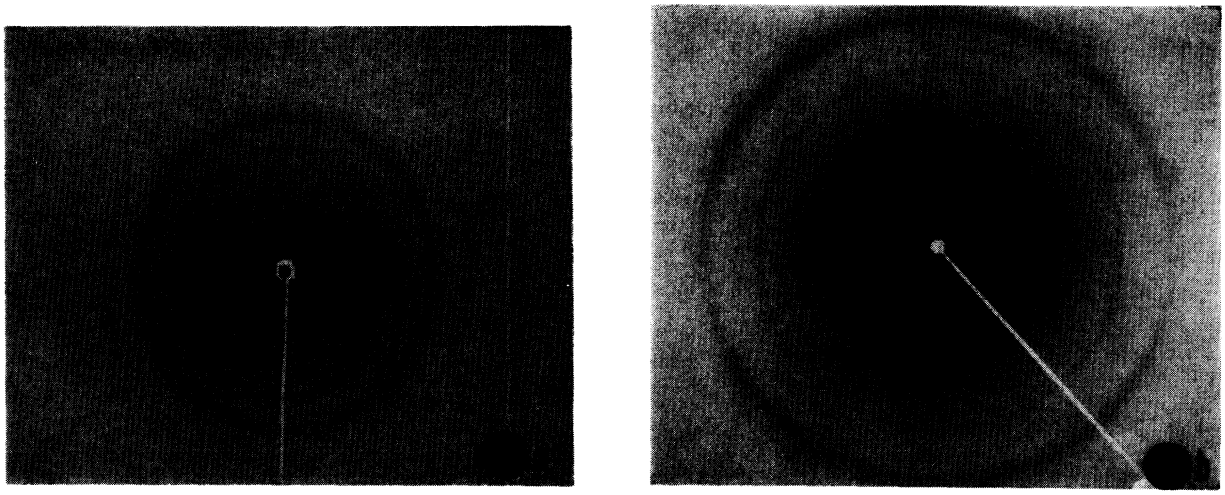


FIG. 25 X-ray diffraction patterns of *in vitro* (a) recalcified fish bone and (b) rat-femur bone. Note that, although there is *some* orientation of the apatite crystals as evidenced by the arcing of the 00*L* reflections, it is *not* as prominent as in the original native, calcified bone [compare Fig. 22(a) with Fig. 25(a), and Fig. 23(a) with Fig. 25(b)].

that, in the zone of provisional calcification in cartilage, the size, appearance, and organization of the collagen fibrils were markedly different from those in bone.[80] More specifically, the collagen fibrils in this calcifying zone of cartilage are quite thin (50 to 250 A) and do not show any visible interperiod fine structure. The fibrils are, in addition, widely separated and randomly oriented. Correlations of this type, however, depend both on an intimate knowledge of the *mechanism* of crystal orientation and its relation to the *induction* of crystallization, and on information concerning the macromolecular organization of the *apparently* "structureless" collagen fibrils. Furthermore, several recent studies also have demonstrated that the embryonic *bone* of some species (and even early postfetal bone in others) does not show any preferred orientation of the inorganic crystals[81–83] (as deduced by x-ray diffraction, based primarily on the arcing of the 00*L* reflections of apatite), and that crystal orientation varies not only from bone to bone, but also in different areas of the same bone.[82,83]

As in other problems in calcification, the term "orientation" must be defined carefully. Orientation of the inorganic crystals has been related to the long axis of the entire bone and to the various hierarchies of collagen structure. These definitions obviously are not identical, and the orientation of the crystals with respect to the *collagen fibrils with which they are associated* is the relationship of interest.

The evaluation and interpretation of such coorientation by x-ray diffraction evidence alone is limited and may be quite misleading. The difficulties stem both from the basic nature of the method and from the failure to interpret the results in the light of the gross and microscopic arrangements of the collagen fibers in the tissue.

An x-ray beam, regardless of how small a collimator is used, integrates the orientation of the collagen and crystals over an enormous volume as compared with the order of magnitude discussed here. Since the x-ray diffraction data are summations from many crystals and fibers, it is not possible to discern *individual inorganic crystal-collagen fibril* relationships, particularly if the collagen fibrils are themselves randomly dispersed or if account is not taken of the higher-ordered organization of the structures in the tissue.

Electron microscopy, on the other hand, gives a direct result if the crystals can be visualized with respect to individual fibrils. In cases where small local areas are in question, selected-area electron diffraction—since it integrates over a much smaller volume than x-ray diffraction—also can be of great help.

In order to evaluate whether the marked variation in crystal orientation as deduced by x-ray diffraction is indicative of varying degrees of collagen fibril-inorganic crystal orientation, or whether it is the result of the geometric factors in tissue organization and of x-ray technique, a number of specimens showing the complete spectra of orientation was examined by electron microscopy, electron diffraction, and x-ray diffraction. Figures 22–24 are x-ray diffraction patterns of fish bone, rat bone, and 16-day-old embryonic chick bone, calcified and decalcified. In the case of fish bone, there is marked orientation of both the apatite crystals and the collagen fibrils. The rat bone shows less orientation of both the apatite crystals and the collagen fibrils, and in the embryonic bone, no orientation of the apatite is present and there is very little evidence of collagen orientation.

Electron micrographs and selected-area electron diffraction of the two extreme cases (fish bone and embryonic chick bone) clarified the issue. Figure 6 demonstrates that the fish bone consists of extremely well-oriented collagen fibrils with the inorganic crystals lying with their long axes (crystallographic *c*-axis) parallel to the collagen-fibril axis. Figure 26 is an electron micrograph of embryonic bone which reveals the general randomness of the collagen fibrils. Within very

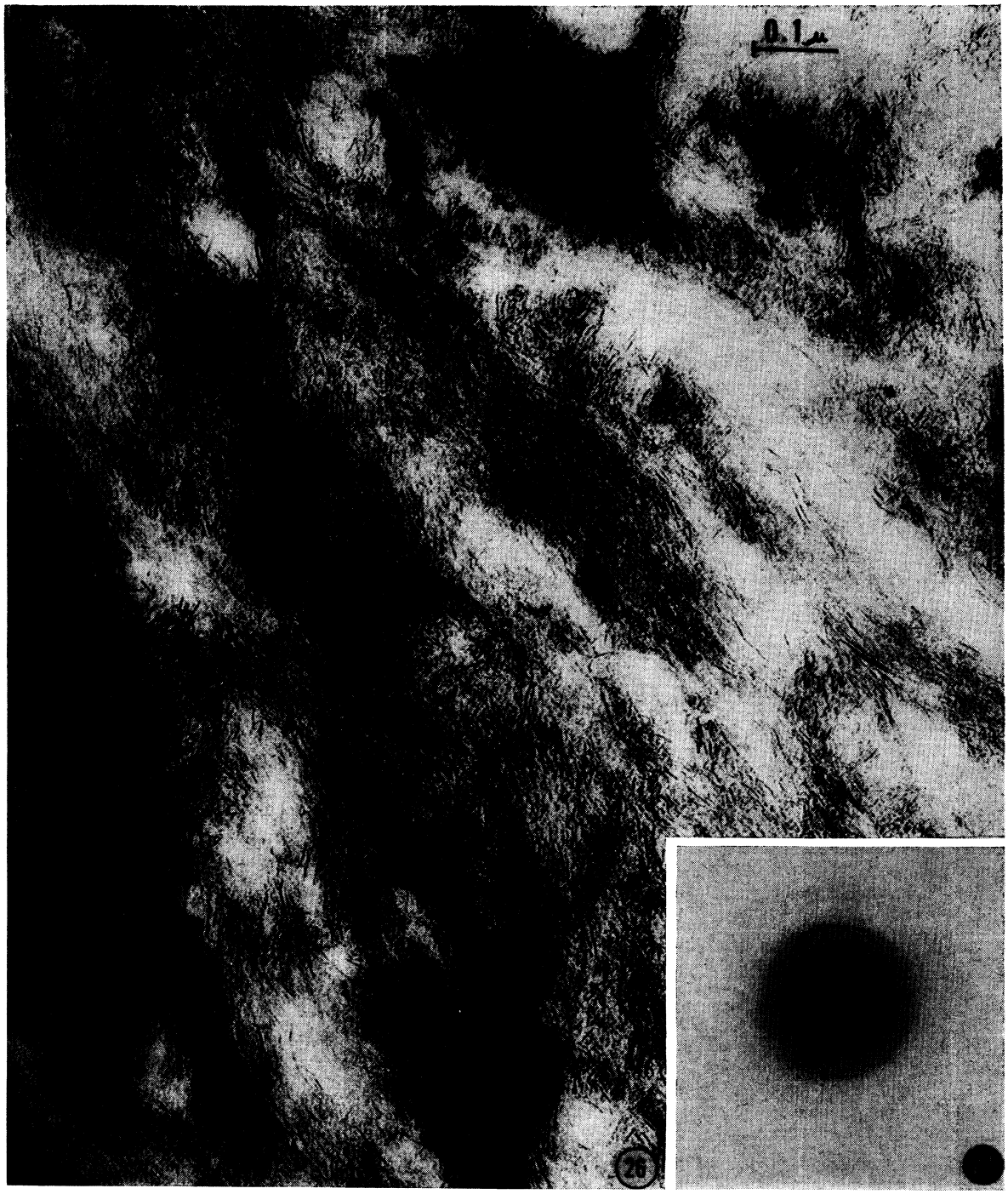


FIG. 26. Electron micrograph of a section of the metatarsal rudiment of embryonic chick bone (16 days). Despite the general randomness of the collagen fibrils, the crystals are oriented in local regions corresponding to the individual collagen-fibril directions. The size and shape of the crystals are similar to those in adult bone. ×40 000.

FIG. 27. Selected-area electron diffraction of specimen shown in Fig. 26. The preferred orientation of the crystals is evident by the arcing of the 002 and 004 reflections.

local areas, however, *the same general parallel alignment of crystals and the collagen fibrils is present*, confirmed by selected-area electron diffraction (Fig. 27). The orientation of individual crystals with *the fibrils with which they are associated* is also apparent in certain adult bones where the collagen fibrils are clearly sepa-

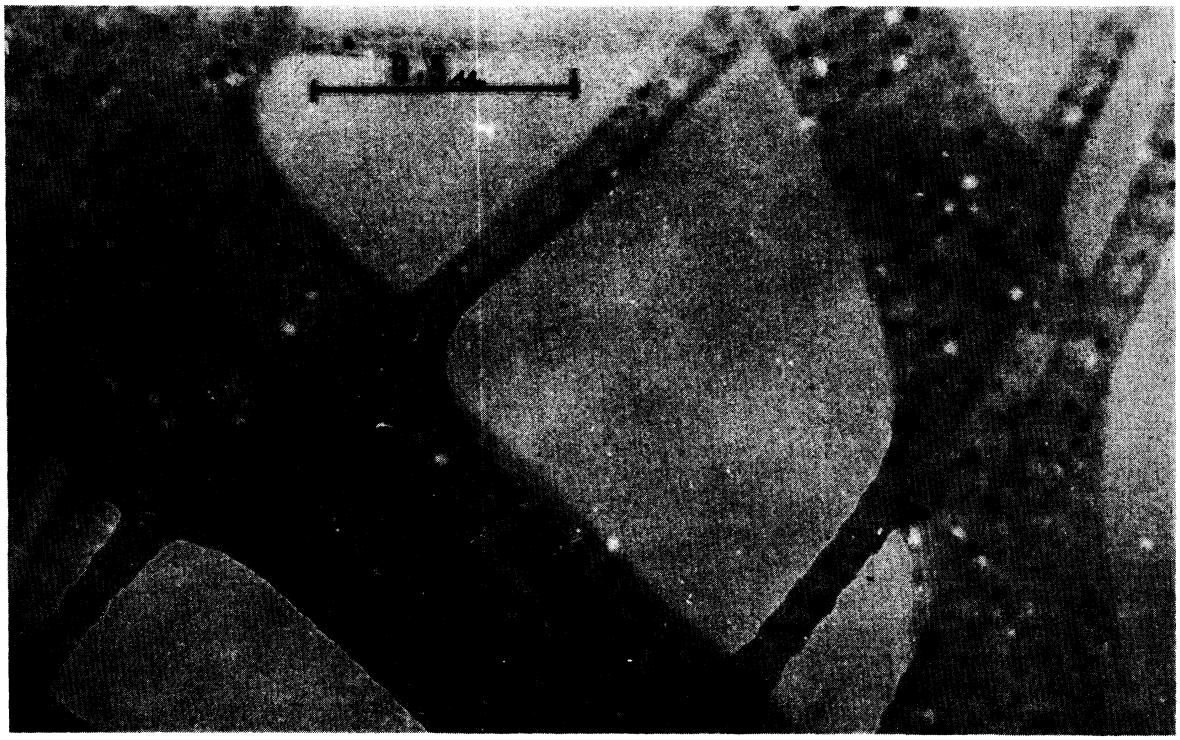


FIG. 28. Unstained, unshadowed preparations of *in vitro* calcified collagen at an early stage. Although the crystals are situated regularly spaced in certain regions of the fibrils, the haphazard arrangement of the crystal reflections (white spots) indicates lack of preferred *orientation* of individual crystals with the fibrils with which they are associated. ×70 000.

rated, as shown in Figs. 8 and 9. It is clearly evident, therefore, that x-ray diffraction evidence *alone* cannot be used as a criterion in assessing or interpreting crystal orientation, it may, as in this case, be quite misleading. Thus, the complete lack of preferred crystal orientation as evidenced by x-ray diffraction data in the embryonic bone is simply the result of the randomness of the collagen fibrils themselves, and, as far as the crystals within any one fibril with which they are associated are concerned, they are well oriented similar to adult bone.

Mechanism of Crystal Orientation

As further experimental results unfolded, it became apparent that the reasons for crystal orientation were twofold: *the size and habit of the inorganic crystals, and the location of the crystals within the fibrils.*

Electron micrographs taken during time-sequence studies of *in vitro* calcified reconstituted collagen have shown a striking similarity in the appearance of the inorganic crystals to those of embryonic bone during the initial stages of calcification (Figs. 13, 14, 18, and 20). In both cases, the crystals appeared dot-like with none of the axes elongated in any direction, and in both cases selected-area electron diffraction (Fig. 19) showed *no* preferred crystal orientation from areas where the collagen fibrils were relatively well oriented. On the other hand, in later stages of embryonic bone, once crystal growth (and/or recrystallization) has occurred, leading to *small* asymmetric crystals *within* the fibrils, the crystals do become oriented with their long axes approximately parallel to the collagen fibrils *with which they are associated* (Figs. 26 and 27).

Although it was impossible to obtain single-crystal patterns from the *in vitro* calcified specimens, individual diffraction spots, rather than complete rings, were obtained in many instances from small local areas with relatively few crystals and in regions where there were several well-oriented fibrils.

In order to obtain more information about the relationship of individual crystals to the collagen fibrils, recourse was had to another experimental device. The objective aperture of the electron microscope was removed and the specimen photographed slightly out of focus. The resultant crystal reflections, seen as white dots in Fig. 28, are from a definite set of crystal planes, confirmed by measuring the distance from the crystals to the reflections. One easily can see the haphazard patterns of these reflections, which clearly indicate that the individual crystals *are not oriented with their crystal axes parallel to the fibril axes, but are randomly oriented.* These data indicate that the *initial* crystals *in vivo* and *in vitro* have not grown in an epitatic fashion and that their *subsequent* orientation must be attributable to other factors.

A detailed electron-microscopic examination of calcification in cartilage has been made by Robinson *et al.*[80]

FIG. 29. Electron micrograph of *in vitro* apatite crystals precipitated under conditions similar to those of the *in vitro* collagen-nucleation studies. Note large hexagonal-type crystals. ×50 000.

They observed that, in the initial stages of cartilage calcification, the crystals *are* directly related to the collagen fibrils and are propagated in the intervening interfibrillar areas. The crystals are not preferentially oriented, except for a few which lie directly on, or possibly in, the fibrils.

The other point concerning calcifying cartilage—that is, the lack of the usual interperiod fine structure of the collagen fibrils—appears to be attributable to the very small size of the fibrils and possibly to the large amount of ground substance surrounding them. The question of collagen-fibril size is also important from the standpoint of the number of collagen macromolecules that must be polymerized laterally in order to constitute a nucleus.

With these factors in mind, reconstituted collagen fibrils were prepared, approximately 50 to 100 A in diameter and not showing cross-striations when stained with phosphotungstic acid (PTA). Well-oriented fibers of this material, however, demonstrated the characteristic 640-A low-angle x-ray diffraction pattern, indicating the *macromolecular organization* to be similar to the larger fibrils. Preparations of these fibrils were able to initiate crystallization of apatite *in vitro* from metastable solutions in experiments similar to those described.

The data may be summarized as follows. In calcifying cartilage, embryonic bone, and *in vitro* calcified collagens, crystallization is initiated in *direct relation to the collagen fibrils*. During the *first stages* of mineralization in embryonic bone, reconstituted collagens, and calcifying cartilage, the inorganic crystals are *not* oriented with respect to the collagen-*fibril axis*. In both embryonic bone and reconstituted collagens, the crystals are strikingly similar: dot-like with *none of the crystallographic axes elongated.*

Further crystal growth results in assymetric crystals with one of their axes elongated. In bone, this occurs primarily *within the fibrils* and is *associated with orientation of the crystals*. That the orientation of the crystals is not a function of the over-all compactness or organization of the collagen fibrils in the tissue, but is dependent upon the *location* of the crystals within the fibrils, is shown by the observations on embryonic bone and on certain adult bone. In the former (Fig. 26), despite the general randomness of the fibrils, and in the latter (Figs. 8 and 9), although the collagen fibrils are clearly separated and somewhat randomly directed, the inorganic crystals are well oriented with their long axes parallel *to the collagen fibril axis with which they are associated.* In calcifying cartilage, however, because of the very small diameter of the fibrils, crystal growth is propagated primarily *between* the widely spaced fibrils, and these crystals, therefore, *incapable* of being oriented by the fibrils, are randomly oriented.

The conclusions are reached that: (1) the basis for the orientations of the inorganic crystals is the asymmetric growth of small crystals *within the collagen fibrils, between tightly packed, longitudinally oriented chains of macromolecules* (*protofibrils*), which necessarily results in the crystals being similarly oriented; (2) the process of crystal orientation, therefore, is unrelated to the *mechanism of induction* of crystallization; (3) since crystal orientation is a function of the *size* and *shape* of the *crystals* and their position *within the fibrils* and not part of the crystal induction mechanism, the *lack* of preferred orientation in cartilage in no way suggests that the *mechanism* of crystal formation (heterogeneous nucleation by the collagen fibrils) is different in cartilage from that in bone.

In fact, the theory of heterogeneous nucleation of apatite crystals by collagen fibrils may be more convincing in cartilage than in bone, since the collagen fibrils are so closely packed (particularly in compact bone) that there is very little space for crystal formation *except* within the fibrils, whereas in cartilage, the fibrils are relatively far apart and randomly oriented with large amounts of intervening interfibrillar ground substance available for crystallization. Despite this, calcification does *not* begin randomly in the tissue, but is initiated in *direct relation* to the very thin fibrils and is then propagated throughout the intervening ground substance.

CRYSTAL HABIT AND SIZE

A good deal of disagreement exists as to the exact habit and size of the apatite crystals in bone. Electron micrographs obtained in our laboratory, in agreement with those of Speckman and Norris,[84] and Fernández-Morán and Engström,[85] show what *appear* to be rod-shaped particles, the majority varying in thickness from 15 to 30 A and in length from 200 to 400 A. It is possible, however, that these represent extremely thin, lathe-like crystals, several of which are stacked together. Perfect cross sections of well-oriented fibrils are obtained with great difficulty, but a number of fibrils in many fields appear to show a dot-like appearance in cross section consistent with a rod-like habit of the crystals (Fig. 12).

Because the size of the crystals approaches the limit of resolution of the electron microscope, particularly in a tissue technically difficult for sectioning such as bone, and because of other factors such as overlap, etc., it is not possible to be absolutely certain of the exact size and shape of the inorganic crystals. In any event, the issue is somewhat semantic: when does a rod become a plate? There may be variations in the habit of the crystals from true rods to a *somewhat* lathe-like habit where one of the faces is *slightly* larger than the other. But from many cross sections, longitudinal sections and oblique sections, one can say with certainty that the crystals are *not* the large hexagonal plates (500×250×100 A) reported by some workers on the basis of the examination of blended or autoclaved bone.[27,28]

On the other hand, the apatite crystals formed by simple precipitation in the test tube,[86] or crystals nucleated and *grown in the presence of collagen*, are usually hexagonal plates quite unlike those occurring in bone.[87] Occasionally, lathe-like crystals can be formed in the test tube, but again these are several orders of magnitude larger than the bone crystals.[86] Thus, although the early crystals nucleated by collagen *in vitro* are similar to those nucleated *in vivo*, further crystal growth appears to be quite different.

This difference in crystal habit and size between bone crystals and precipitated calcium phosphate crystals might be explained on the basis of the mechanical factors resulting from crystal growth within the fibrils, or by an active process controlled by the structure of collagen such as has been suggested by others.[79] It is also possible that it is entirely unrelated to the structure of collagen.

The position of the bone crystals within the collagen fibrils suggests that the collagen fibrils play a primary role in determining crystal size and shape. The finding, however, that collagen fibrils were *not* able to alter the size or the habit of the crystals under the physicochemical conditions of the *in vitro* experiments casts some doubt on the validity of this hypothesis. This is also confirmed by an examination of electron micrographs of calcifying cartilage which reveals that the crystals lying in the interfibrillar space and *anatomically unrelated to the collagen fibrils* are the same size and shape as those within the collagen fibrils of bone.

Since it is a well-established fact that the size and shape of crystals can be altered by changes in the physicochemical environment—including the addition of a number of substances bound on certain crystal faces and limiting their further growth—studies were carried out on the precipitation and growth of apatite crystals from solution under a wide variety of experimental conditions.

Figure 29 is an electron micrograph of a calcium phosphate precipitate in which the physicochemical conditions were similar to the *in vitro* collagen-nucleation experiments. These large, hexagonal plates are somewhat larger than those usually seen under these circumstances, but serve as a control to illustrate the progressive change in size and habit that can be produced *in vitro* in the absence of collagen. X-ray diffraction of this preparation revealed a typical apatite pattern without any evidence of the presence of octocalcium phosphate or brushite which also crystallize in large plate-like form.

Figure 30 is another preparation in which an attempt was made to alter crystal size and shape. One can easily see the marked difference in the size of the crystals, although they are still in the form of hexagonal plates. Note that where the crystals overlap there is a suggestion of a dense "rod," particularly if viewed without reference to the neighboring crystals.

Figures 31–33 are electron micrographs of apatite preparations where the crystal size was further reduced, and it appears that the habit has also been altered so that a number of rod-like crystals have formed. It is impossible, however, to tell whether these are truly rods or represent extremely small, thin plates stacked together or supporting each other and viewed along their edges. The "rods" are now approximately the size of the bone crystals.

Figure 34 is an electron micrograph of another preparation in which the crystals are even thinner (many ~10 A or less) and very similar in size and appearance to the bone crystals, and in which there is no apparent evidence of hexagonal plates. If one examines the elec-

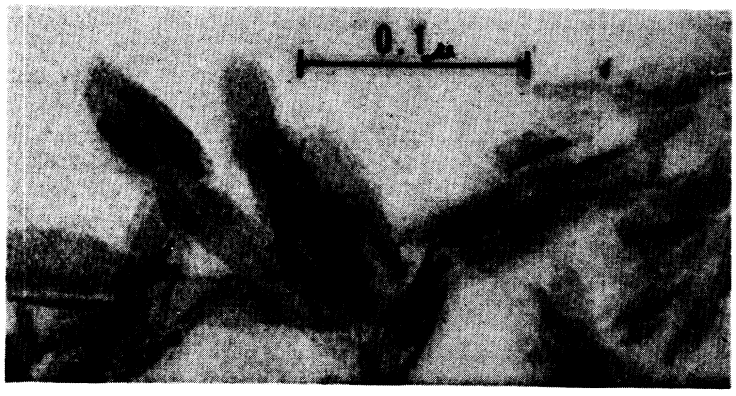


FIG. 30. Under altered physicochemical conditions, crystal size decreases markedly, but crystal habit remains the same. ×300 000.

FIG. 31. Further alteration in the physicochemical environment produces not only a further decrease in crystal size but also, for some, a change in the crystal habit to a rod-like appearance. ×180 000.

tron micrographs carefully, however, it is obvious that there is a minimal but definite background electron density which may indicate that these crystals are extremely thin plates, some of which are stacked together or are upright and supporting each other, and, when viewed along their edges, give the appearance of rods. The fact that many of the rods appear to be bent in several directions may indicate that this is the correct interpretation. This same appearance has been seen in very lightly calcified collagen fibrils of bone.

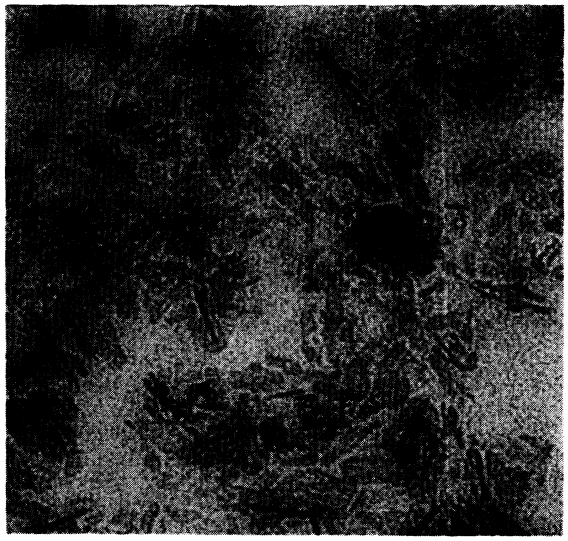


FIG. 32. Higher magnification of an area in a sample similar to Fig. 31, where the "plates" predominate. ×260 000.

Although it is still impossible to state dogmatically the exact size and shape not only of the bone crystals but also of many *in vitro* precipitated crystals, these experiments show that *it is possible to grow from solution under controlled physicochemical conditions apatite crystals whose size, shape, and appearance in electron micrographs and whose x-ray diffraction characteristics are similar to those of bone crystals.*

The facts (1) that *in vitro* collagen *per se* is not able to modify the crystal habit or size, (2) that *in vivo* (calcified cartilage) crystals similar in size and shape to the bone crystals lie in the interfibrillar spaces *between* the collagen fibrils and anatomically unrelated to them, and (3) that crystals *can* be grown *in vitro* similar to the size and habit of bone crystals in the absence of collagen, indicate that the *size* and *shape* of the *apatite crystals are primarily controlled by other physicochemical factors in vivo* and *not by the collagen fibrils.*

Obviously, the conditions under which crystal growth occurs within longitudinally oriented and closely packed fibrils may exert some secondary influence on the crystal size and habit, both mechanically and by selectively facilitating and inhibiting the diffusion of ions; but

when the other physicochemical conditions are not met, crystal growth is *not altered* by the collagen fibrils.

Under such conditions, the crystals ordinarily tend to grow to a relatively large size as compared with normal bone crystals, and since such crystal growth cannot take place within the fibrils, further seeding, recrystallization, etc., occurs *outside* the fibrils. The results of experiments in which crystals initially nucleated within the collagen fibrils *in vitro* proceeded to recrystallize and grow outside of the fibrils, substantiates this conclusion, and clarifies the observations related to crystal orientation in *in vitro* calcified collagens including bone.

Thus, *in vitro* calcified, reconstituted collagens showed *no* preferred inorganic crystal orientation by x-ray diffraction, even though the collagen was moderately oriented. On the other hand, specimens of bone recalcified *in vitro* did show some preferred inorganic crystal orientation, but not as much as similar samples of native bone, as shown in Figs. 22(a), 25(a) and 23(a),25(b). In the case of the recalcified bone, the degree of orientation of the crystals was related to the degree of orientation of the collagen matrix [Figs. 22(b) and 23(b)].

Electron micrographs of heavily calcified preparations of *in vitro* calcified collagens demonstrated that the majority of the crystals were in the interfibrillar space and *not* within the fibrils. In addition, the crystal habit and size were unlike that of native bone and resembled the large *in vitro* precipitated hexagonal plate-like crystals. Because of size and shape, crystal growth was more favorable in the interfibrillar regions rather than within

FIG. 33. Higher magnification of an area in a sample similar to Fig. 31, where the rod-like crystals predominate. ×260 000.

FIG. 34. *In vitro* precipitated apatite crystals. Note the rod-like appearance without evidence of plate formation, except for the minimal background electron density. Crystals vary from 8 to 20 A in width, and many appear to be "bent" similar to areas of lightly calcified bone. ×260 000.

the fibril. These crystals were, therefore, in no way capable of being oriented by *the individual fibrils.*

In samples of reconstituted collagens, the interfibrillar spaces were large, and the over-all organization of the fibrils was also not able to influence the orientation of the crystals (similar to cartilage). In the case of demineralized bone, however, the collagen fibrils are more closely packed and well oriented. Under these circumstances, the elongated plate-like crystals mechanically orient with their flat, thin faces between adjacent fibrils and their long dimensions approximately parallel to the collagen fiber axis. This arrangement gives a co-oriented x-ray diffraction pattern of apatite and collagen but not as good a one as that in native bone, where the majority of the crystals are within the fibrils.

Experiments are now underway in which the physicochemical conditions during crystal *growth* as well as crystal *induction* are being carefully controlled in an attempt to grow the crystals primarily within the collagen fibrils similar to that of native bone.

Crystal Nonstoichiometry

As mentioned in the beginning of this paper, one of the perplexing problems in the study of the nature of the bone crystals or of *in vitro* precipitated apatites has been their nonstoichiometry. Recall that, in bone and in some *in vitro* precipitated crystals, the average width of the rods or of the stacked plates (or both) varied from 10 to 30 A. This dimension corresponds to the *a*- or *b*-axis of the unit cell which is approximately 9.43 A. Thus, the crystals are composed of 1 to 3 unit cells in this dimension. Although the unit cell of hydroxyapatite may be represented as in Fig. 1, it must be remembered that it depicts only *conceptually* the *spatial relations* of the constituent atoms and molecules, and that many of the individual atoms and molecules are shared by adjoining unit cells in an actual crystal.

Therefore, the stoichiometry of the solid phase may be represented by the structure of the unit cell *only* when there is a very large number of unit cells comprising the crystal, so that the unit cells or the portions of them making up the surface are not statistically significant. When the entire crystal is composed (in any dimension or dimensions) of only a few unit cells, however, and if the planes on which crystal growth ceases are relatively uniform along the length of the crystal, *it is not possible to obtain the theoretical stoichiometry.* The *actual* stoichiometry in these cases will be determined by the atomic planes in which crystal growth ceases. If the crystal surface planes on which crystal growth ceases vary a good deal, it is possible that a fortuitous combination might occur which would result in a theoretical stoichiometry, but this is highly unlikely. In *in vitro* precipitated crystals, however, there is ordinarily a very wide range of crystal size and shape (and probably hydration), and the statistical summation of the calcium and phosphorus content of such an heterogeneous sample, whether stoichiometric or not, would not be very meaningful. Although there is obviously a number of other previously mentioned factors such as internal lattice substitution, surface adsorption, vacant lattice sites, etc., which may influence the stoichiometry, the point is that, *if none of these existed, it still would not be possible to obtain stoichiometric hydroxyapatite* with crystals the size of those obtained in bone or prepared *in vitro.*

The larger "defect" apatite crystals reported by Posner and Perloff[11] of course poses another problem, but it is possible that such large crystals represent a somewhat different structure than hydroxyapatite and are similar to OCP.

The small size of the crystals also may be an important factor in determining the amount of water associated with apatite crystals precipitated from aqueous solutions as discussed on p. 361. This "excess" water is many times greater than that adsorbed by initially dry crystals from a vapor phase of water and consists of many more molecular layers of water than that usually considered possible for a surface to bind because of electrical-field effects.

In addition to the fact that some of the water may enter the lattice structure itself, since the minute apatite crystals in aqueous suspension tend to stick together and form stacks and bundles, the very large surface tension and capillarity effects between such crystal surfaces could well "trap" a large amount of water (in addition to a bound monolayer) and still resist separation from the crystals by mechanical centrifugation.

Closing Comments

As regards other mineralized biological tissues, one can see from Table I that the various crystals are anatomically related to specific organic matrices. Note the wide variety both of inorganic crystals and of organic matrices in which mineralization occurs. In fact, in some marine mollusks, calcium carbonate exists in two of its three possible crystallographic forms (calcite and aragonite) *in the same shell*, in well-demarcated regions which *border* on each other.

Another example of such an intimate chemical and ultrastructural relationship between the inorganic and organic phases is that which occurs in oyster shells. Electron micrographs have shown a close and ordered relationship between the calcium carbonate crystals and the organic matrix of conchiolin.[88–90] Here too, calcification is initiated by small seed-like crystals which are initially deposited in a regular fashion in the organic matrix and appear similar to synthetically grown crystals from *supersaturated* solutions.[90]

Although the mechanisms by which mineralization is initiated and regulated in the organism are undoubtedly far more complex than those in the relatively simple model system described, the process of the heterogeneous nucleation of inorganic crystals by highly specific regions in the organic matrix as the result of a characteristic stereochemical array of certain reactive groups is

probably a fundamental one, not only for the calcification of bone and cartilage but also for biological mineralization in general.

APPENDIX A. SOME THERMODYNAMIC AND KINETIC CONSIDERATIONS IN PHASE TRANSFORMATIONS‖

Crystallization is a specific case of a more general phenomenon in which a change in state involves a phase transformation. A complete consideration of phase transformations would encompass (1) the conditions under which a system can or cannot persist in a distinct, physically homogeneous state (a single phase); (2) a *mechanism* for the formation of new phases from such systems; and (3) a quantitative description of the time-dependence of the process.

Since the primary purpose of this exposition, however, is to provide a background of some basic concepts in phase transformations in order to understand better the general phenomenon of crystallization and of biological mineralization, attention is directed primarily to some general considerations of the first two aspects.

Equilibrium and the Stability of Phases¶

In most general terms, Gibbs[91] has defined equilibrium as a state independent of time, that is, a state in which *all* of the sensible properties which describe the system do not vary with time. Thus, in formulating both the necessary and sufficient conditions of equilibrium in terms of thermodynamic functions (e.g., energy, entropy, free energy, etc.), Gibbs considered equilibrium with respect to *all possible variations* of the state of the system, and *not* with respect to *certain* variations only. In practice, however, it is necessary to idealize most systems by imposing certain conditions of restraint and then by studying them with respect to *certain* possible variations only. In the case at hand, the possible variation of interest is the formation of entirely new phases from an initially homogeneous phase.

The usual criteria of equilibrium [for example, that for all possible variations in the state of an isolated system $(\delta E)_S \geqq 0, (\delta S)_E \leqq 0, (\delta F)_{p,T} \geqq 0$, etc.] do not give the information necessary for the interpretation of phase transformations, since many systems which meet these general criteria of equilibrium may vary widely in their *relative* tendency or ability to form new phases. On the basis of this *relative stability*, Gibbs[91] distinguished four kinds of equilibrium: (1) stable, (2) neutral, (3) unstable, and (4) metastable equilibria.

‖ The reader is referred to the following article for a more comprehensive review of the subject: D. Turnbull, Advances in Solid-State Phys. 3, 225 (1956).

¶ The material presented here has been compiled from a series of lecture notes from a graduate course in Chemical Thermodynamics, based on the writings of J. Willard Gibbs, given at the Massachusetts Institute of Technology, Department of Physical Chemistry, by Professor James A. Beattie. I would like to thank Professor Beattie for permission to use some of this material, including Figs. 35 and 36, and for many helpful suggestions and criticisms in the preparation of the Appendix.

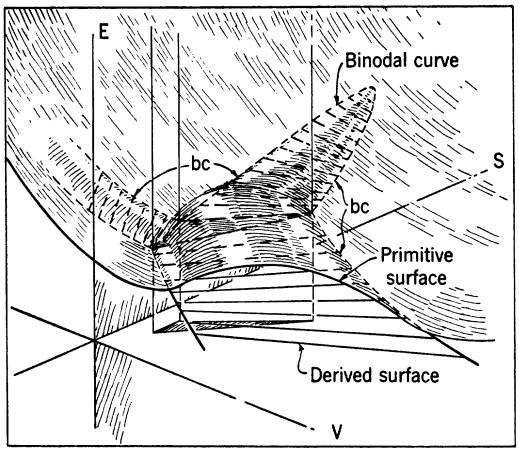


FIG. 35. Artist's (P. Lund) conception of the Gibbs energy-entropy-volume surface for a one-component system. The dotted lines on the primitive surface connect points of common tangency (neutral equilibrium), and the construction of a derived surface from such points is illustrated also. The orthogonal projection of the plane triangle on the S-V plane, representing the triple point, is shown. bc represents binodal curves.

The fundamental principles can, with respect to the stability of phases, conveniently and easily be visualized by reviewing certain pertinent features of the Gibbs energy-entropy-volume thermodynamic surface. For purposes of exposition, consider the special case of a pure substance when effects produced by gravity, electricity, capillary tensions or distortion of solid phases are absent, or may be neglected.

For all possible infinitesimal changes of state involving only expansion work in a closed system, the first and second laws of thermodynamics state that

$$dE = TdS - pdV. \tag{A1}$$

Integration of (1) gives the equation

$$E = E(S,V). \tag{A2}$$

Thus, the relations between the energy, entropy, and volume may be represented by a surface in space whose rectangular coordinates are represented by the volume, energy, and entropy of the system (Fig. 35). This surface, called the *primitive surface*, represents equilibrium states of a homogeneous phase. At any point on the primitive surface, the equilibrium temperature and pressure are defined by the slopes of the surface at that particular point:

$$\left(\frac{\partial E}{\partial S}\right)_V = T, \left(\frac{\partial E}{\partial V}\right)_S = -p \tag{A3}$$

and the tangent plane at the point given by

$$E = E_0 + T_0(S - S_0) - p_0(V - V_0). \tag{A4}$$

The intersection of the tangent plane with various other planes and the coordinate axes give various thermodynamic functions. For example, its intersection with the E axes gives F_0, the Gibbs free energy of the substance at the point P_0.

States of the system consisting of two or more phases in equilibrium (heterogeneous equilibrium) can also be represented in E-S-V space, since the system as a whole has a definite energy, entropy, volume, free energy, etc., as well as temperature and pressure. In heterogeneous equilibrium, the substance in each of its aggregation states has the same temperature, pressure, and molal free energy. Thus, planes tangent to points on the primitive surface representing, for example, one mole of the substance in each of these aggregation states will have the same slope $(-p,T)$ and the same intercept in the E-axes (free energy). Hence, these points have a common tangent plane.

In the case of two phases (say liquid and vapor) in equilibrium at a definite temperature and pressure, there are two points on the primitive surface, one representing one mole of liquid and the other representing one mole of vapor at this pressure and temperature which have a common tangent plane. Lying along the straight line connecting the points are the states of mixtures of liquid and vapor in equilibrium at this particular temperature and pressure. By taking all of the lines determined by such tangent planes for varying temperatures and pressures, another surface may be generated called the *derived surface* which represents states of heterogeneous equilibrium. One may conceive of this derived surface as being produced by rolling a double tangent plane (tangent to the primitive surface at two points) on the primitive surface and by connecting the successive pairs of *conjugate points* of tangency by a series of straight lines (Fig. 35).

In the case of three phases in equilibrium (i.e., liquid, vapor, solid), there are three points on the primitive surface with a common tangent plane, and states of the substance at the triple point (mixtures of all three phases in equilibrium) are presented in E-S-V space by points in a plane triangle (Fig. 35). The derived surfaces, therefore, include all of the plane triangles representing three phases in equilibrium, and all of the developable surfaces representing two phases in equilibrium. It is a continuous ruled surface (generated by the motion of a straight line), but it is *not* cylindrical.

Properties of the Thermodynamic Surfaces Which Indicate the Stability of Thermodynamics Equilibrium

Consider a specified amount of a pure substance A, immersed in a large medium M, at constant pressure p_0 and temperature T_0 with an initial energy, entropy, and volume (E',S',V') and which undergoes the following change of state

$$A(E',S',V')=A(E'',S'',V'')$$

under conditions where the action of the system on the media is substantially reversible. It can easily be shown from the first and second laws of thermodynamics that the following relation must hold:

$$(E''-T_0S''+p_0V'')\leqq(E'-T_0S'+p_0V'). \quad \text{(A5)}$$

With reference to the thermodynamic surface, the terms enclosed in parentheses are the vertical distances of the points (E'',S'',V'') and (E',S',V') representing the final and initial states of the system above a plane passing through the origin having the slopes $-p_0,T_0$. Geometrically, it defines the conditions under which changes in state can occur spontaneously: where this vertical distance representing the final state is *less* or at most *equal* to the distance representing the initial state. Changes in state which result in an increase of this distance are, therefore, *not* possible. This principle is crucial to an understanding of phase changes, for it clearly delineates the regions in E-S-V space (and, therefore, the equilibrium of such states) where phase transitions are possible.

In examining the possible equilibrium states of a pure substance in a medium at temperature T_0 and pressure p_0 (represented by points on the primitive surface), two possible categories of change must be considered. The behavior of systems may be related to (1) *continuous changes*—that is, changes considered with respect to nearby or adjacent states, and (2) *discontinuous changes*—that is, in relation to states at a finite distance from the point in question. The *curvatures* of the primitive surface determine the former, while the *over-all relation* of the tangent plane to the surface determines the latter.

Stable Equilibrium

If the primitive surface falls *above* the fixed tangent plane, *except* at the single point of contact (representing the initial state), the state of the system represented by that point is "absolutely stable" toward a phase change when in a medium of constant pressure and temperature. That is, there are no points in E-S-V space for which Eq. (5) is satisfied, and there are, therefore, no possible changes in state which can occur. Any "unnatural" perturbations of the system (such as, a local fluctuation in density) would necessarily lead to changes in state represented by points in E-S-V space where the distance of any such point above the tangent plane would be greater than that of the original point. Since Eq. (5) has shown that such changes in state are *not* possible, natural processes will occur which will return

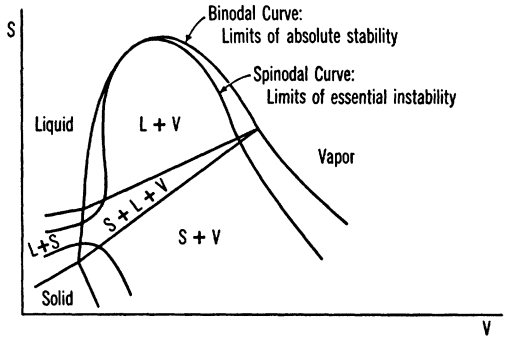


FIG. 36. Orthogonal projection of the limits of absolute stability (binodal curve) and limits of essential instability (spinodal curve) on the S-V plane for a substance having one solid phase.

the system to its original state. It can be shown that that part of the primitive surface representing such stable states of equilibrium is concave upward in both of its principal curvatures.

The points of tangency of the rolling double-tangent plane representing two phases in equilibrium trace the *binodal curves* (Fig. 36) or the limits of *absolute stability*. The tangent plane to points on the primitive surface which fall *outside the binodal curves* has the primitive surface entirely above it except at the single point of contact. That portion of the primitive surface outside the binodal curves represents states of *absolute stability*. This part of the primitive surface (referred to as the *surface of absolute stability*) together with the derived surfaces constitute the *surface of dissipated energy*. The tangent plane to a point on the surface of *absolute stability* is always below the surface of dissipated energy except at the point of tangency.

Solutions in such states of equilibria would be absolutely stable with regard to the *formation* of new phases. The mechanical analog of such a system may be represented by a marble in a hemispherical bowl fitted with a cover, with the conditions of restraint that the marble cannot sink through the bowl or be otherwise removed from it [Fig. 37(a)].

Neutral Equilibrium

If the primitive surface does not fall anywhere below the fixed tangent plane but meets it at more than one point, the equilibrium of such states of the system is considered *neutral*. In such cases, if the system in such an equilibrium state is changed from its original state to a new state represented by another point of tangency to the primitive surface, the distances of both points above the fixed tangent plane (at constant T and p) obviously will be equal (zero). Therefore, such systems will have no tendency to pass into one of the other states (as represented by other points of common tangency) or to return to their original state if so displaced. However, such systems are stable with respect to continuous changes in state similar to systems in stable equilibrium.

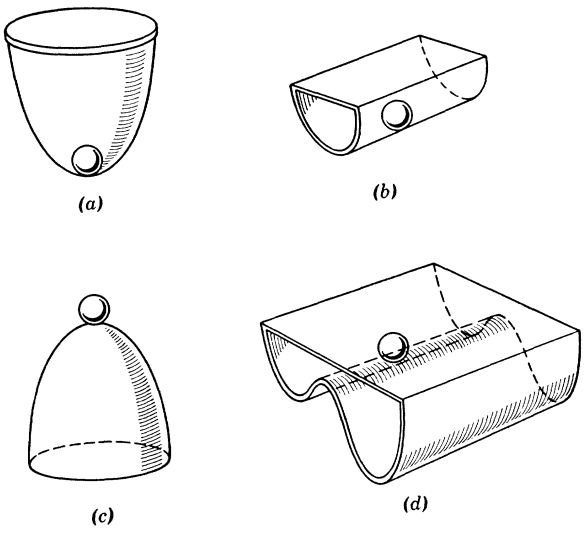


FIG. 37. Diagrammatic illustration of the four types of equilibria based on their relative stability.

A marble in a horizontal trough is an example of such a state of neutral equilibrium [Fig. 37(b)]. Although the displacement of the marble up the sides of the container eventually results in the return of the marble to its original position at the bottom of the trough, there are a number of positions of the marble along the bottom of the trough where no spontaneous tendency to change exists and where no spontaneous tendency for a return of the marble occurs if such horizontal displacements are made. Solutions in such states of neutral equilibrium are, therefore, also stable with regard to the *formation* of new phases.

Unstable Equilibrium

If the primitive surface be continuous, there must necessarily exist regions between the binodal curves where the curvature of the primitive surface is concave *downward* in at least one of its principal curvatures. Points *on* the binodal curves (representing states of neutral equilibrium) separate such states of stability and instability with respect to *discontinuous* changes. The lines on the primitive surface dividing the portion which is concave *upward* in both of its principal curvatures from the portion which is concave downward in one or both of its principal curvatures represent the limit of *essential instability* or the *spinodal curves* (Fig. 36).

In such regions, it is obvious that, where part of the surface falls below the fixed tangent plane, it is possible to change the initial state of the system such that the point representing the final state is now below the fixed tangent plane. In this case, according to Eq. (5) natural processes occur which cause the system to continue to change (represented by moving the point further from the tangent plane) until a state is reached which is entirely different from the initial state [Fig. 37(d)]. Such states of equilibrium are *unstable* with respect *both* to continuous and discontinuous changes. That part of the primitive surface which lies inside the spinodal curve represents such states of *unstable* equilibrium. This type of equilibrium is rarely—if ever—attained in practice, and solutions in such a precarious state of equilibrium would hardly remain so for very long periods.

Metastable Equilibrium

The primitive surface which lies *between* the spinodal and binodal curves is concave upward in both of its principal curvatures and is *stable* with respect to *continuous changes* (adjacent states). However, it is so situated with respect to the over-all thermodynamic surface that other states do exist a finite distance away, where the primitive surface falls *below* the tangent planes drawn through points on the primitive surface

which lie between the binodal and spinodal curves. Such systems which are *stable* with respect to *continuous changes* (adjacent states) and *unstable* with respect to *discontinuous* changes (distant states) are said to be in *metastable equilibrium*. If the conditions necessary for such a discontinuous change *were not* present, the equilibrium would remain stable *indefinitely*. But, for example, if small portions of the same substance in one of the more stable states of aggregation, represented by points below the tangent plane, are introduced or otherwise caused to form by very small disturbances (perhaps ones that cannot be detected experimentally), the equilibrium would be destroyed and a change of state (a phase change) would occur [Fig. 37(d)]. Solutions in metastable equilibrium, therefore, although *capable* of remaining stable indefinitely, *can* form new, more-stable phases under certain conditions without changing the entire system.

With reference to the thermodynamic surface, it is thus apparent, since points *on* the surface of dissipated energy represent *stable* or *neutral* equilibrium and since points *below* the surface of dissipated energy have no physical significance, that points on the primitive surface which lie *above* the surface of dissipated energy representing states of *unstable* or metastable equilibrium delineate the regions in E-S-V space from which phase changes can occur at constant temperature and pressure.

On the basis of these conditions, it can be shown that a single analytical expression representing both the necessary and sufficient conditions of equilibrium for a system in a medium of temperature T_0 and pressure p_0 is

$$\delta(E-T_0S+p_0V)\equiv\delta F=0 \qquad \text{(A6)}$$

for all variations of the state of the system. The system is absolutely *stable* with respect to first-order changes if δF is a minimum, and it is *unstable* if δF is a maximum. Finally, if Eq. (6) holds, but for *some finite changes*, the inequality $\Delta F<0$ exists, the state is *metastable*.

Although the foregoing discussion is limited to systems of one component, Gibbs (reference 91, pp. 100–115) has extended his treatment to a study of the internal stability of homogeneous fluids of many components.

Mechanisms and Time Dependence of Phase Transformations

The discontinuous change of metastable or unstable systems resulting in the formation of the initial fragments of a new, more stable phase is called *nucleation*. When such a phase-change occurs in the interior of a metastable or unstable system in the absence of structural impurities, it is referred to as *homogeneous nucleation*, whereas a phase-change initiated *by* and *on* foreign inclusions extraneous to the system is called *heterogeneous nucleation*.

Homogeneous Nucleation

Classical theories of homogeneous nucleation have assumed that phase-changes occur by the formation of intermediaries as a result of local fluctuations of certain properties (such as density) in initially homogeneous phases in metastable or unstable equilibrium.[46,92–94]

These intermediaries consisting of clusters of molecules (or ions) of the initial phase vary in size (and possibly composition, shape, and structure). They are referred to as "embryos" and are considered to exist as a true heterogeneous system with the mother phase. The cluster of critical size, composition, structure, etc., capable of *further* spontaneous growth (with a net decrease in free energy) and, therefore, capable of initiating the formation of the new phase, is called a *nucleus* (Fig. 38).

The embryos and nuclei are considered to arise by the stepwise addition of single molecules, i.e., a *bimolecular mechanism*.[46] The *order* of the reaction, therefore, is equal to the number of molecules in the nucleus.

As first proposed by Volmer and Weber,[93] the theory considered that nuclei can exist in a state of unstable equilibrium with the mother phase, making it possible, at least in principle, to define the nucleus in terms of reversible thermodynamics. Considering surface effects, the free energies of formation of spherical clusters can be calculated as a function of the radius (Fig. 38). It is obvious from Fig. 38 that the over-all free energy goes through a maximum which corresponds to the critical size cluster (nucleus) of radius, r^*, which can grow spontaneously with a net decrease in free energy. From fluctuation theory, the probability of nuclei formation and, therefore, also of their concentration can be approximated since they are proportional to $\exp(-\Delta F^*/kT)$, where ΔF^* is equal to the free energy or the work of formation of a nucleus corresponding to the maximum as shown in Fig. 38. The condensation velocity or nucleation rate is then evaluated by computing the collision frequency of single molecules with the nuclei. This free-energy barrier is, therefore, similar to the activation energy of ordinary chemical reactions in permitting the derivation of a *nucleation rate*.††

Becker and Döring[45] treated the problem differently. They assumed a size distribution of "embryos" and evaluated the rate of condensation by the solution of a set of equations relating how the number of embryos of any particular size changed with time. By assuming a steady state, the mechanism of change was, therefore, simply the kinetic process of unit condensation and unit evaporation.

In both of the cases, expressions for nucleation rate were quite similar [$J\sim\exp(-1/\ln S^2)$], where J is the nucleation rate and S the supersaturation ratio p/p_e where p is the actual vapor pressure and p_e the equilibrium vapor pressure), and indicated a very marked

†† In more-complicated systems, such as occurs in the case of inorganic crystallization, the various-sized embryos may have a number of compositional and structural differences which also involve free-energy changes, and, in particular instances, there may be important effects owing to strain energy as well. Thus, over-all free-energy barrier to nucleation will be provided by a combination of the chemical-, interfacial-, and strain-free energies.

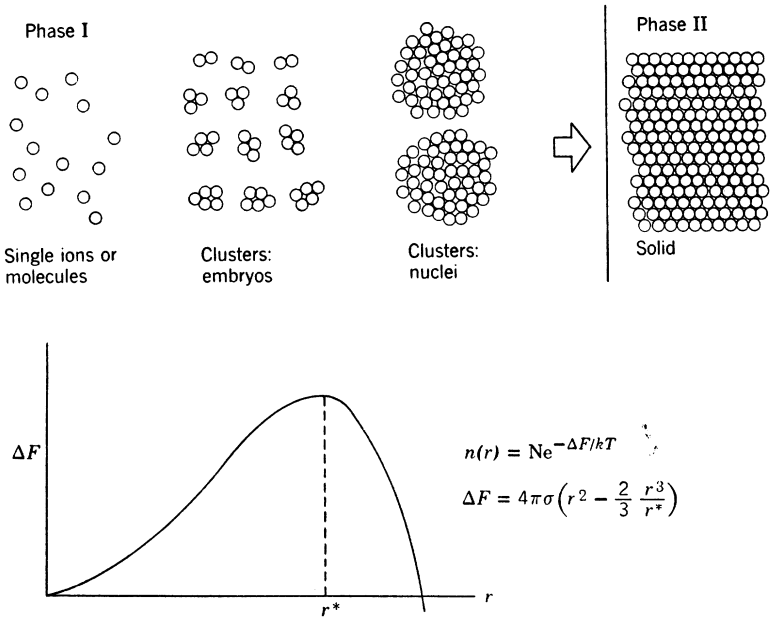


FIG. 38. Schematic illustration indicating mechanism of nucleation as proposed principally by Volmer *et al.*[46,93,94] and by Frenkel.[92]**

dependence of nucleation rate upon the degree of supersaturation, particularly near the critical supersaturation ratio (i.e., where the nucleation rate rather suddenly becomes sufficiently large for ready measurement). For example, in the case of water vapor condensing to liquid drops, the time that must elapse for the appearance of the first drop at a supersaturation ratio of 4, is 0.1 sec, whereas increasing the supersaturation ratio to 5 decreases the time to 10^{-13} sec, and decreasing the ratio to 3 increases the time to 10^3 years![46]

Although based on the theory of Volmer and Weber (fluctuation theory), it is possible that homogeneous nucleation can occur at any level of metastability. Certain theoretical considerations (as well as experimental findings) indicate that this is not possible, except at the limit of essential instability or from systems in *unstable* equilibrium rather than in *metastable* equilibrium. The difficulties arise conceptually from the definitions of equilibrium (a state independent of time), of metastability (stable with respect to continuous changes in state, but unstable with respect to certain discontinuous changes in state), and of what constitutes a discontinuous change in state, and from the applicability of $e^{-\Delta S/k}$ to predict the probability of fluctuations which are large enough to be considered a new phase. Thus, Frenkel[92] considers that the density fluctuations which lead to the formation of embryos (heterophase) transcend the limits usually considered in the ordinary statistical theory of homogeneous systems wherein ordinary fluctuations (homophase) lie "within the limits compatible with the preservation of a given aggregation state" (Frenkel,[92] p. 375).

** While this is usually considered the mechanism of nucleation for most systems, there is recent evidence that, in the nucleation of solid-state transformations[95] the nucleii arise by a cooperative phenomenon and not by discrete atomic jumps.

From these considerations, it would appear that nucleation from true metastable systems occurs only as a result of the introduction of heterogeneities and not spontaneously. This would also seem to be Gibbs' interpretation of the stability of metastable systems, since he states:

". . . the mass in question must be regarded as in strictness stable with respect to the growth of a globule of the kind considered, since W, the work required for the formation of such a globule of a certain size (viz, that which would be in equilibrium with the surrounding mass), will always be positive. Nor can smaller globules be formed, for they can neither be in equilibrium with the surrounding mass, being too small, nor grow to the size of that to which W relates. If, however, by *any external agency* [Ital.: Ed.] such a globular mass (of the size necessary for equilibrium) were formed, the the equilibrium has already (page 243) been shown to be unstable, and with the least excess in size, the interior mass would tend to increase without limit except that depending on the magnitude of the exterior mass" (Gibbs,[91] pp. 255–256).

Heterogeneous Nucleation

The discontinuous change in state which results in nuclei formation by the introduction of a foreign inclusion may be the result of a number of different effects. Thus, either by virtue of nonspecific surface forces or by very specific interactions between certain groups on the surface and the molecules or ions of the initial phase, such heterogeneities may adsorb or bind the molecules or ions of the initial phase on their surface; by acting either as a core whose surface is now composed of the molecules of the initial phase or by binding the molecules or ions in a particular steric array, they may form

a cluster of critical size, shape, and configuration necessary for nucleation. Since the critical-size cluster (assuming no structural or compositional changes) varies with the supersaturation ratio, nucleation rates for heterogeneous nucleation will, as in the case of homogeneous nucleation, be strongly dependent upon and markedly dominated by the degree of metastability of the system.

With regard to crystallization, it is of interest to note the findings of Turnbull and Vonnegut[63] who have proposed a crystallographic theory of crystal nucleation. The theory emphasizes the importance of *geometric* and *structural* factors of nucleation catalysts with regard to their catalytic potency and ability to initiate phase-changes. Based on a number of their own observations as well as on experiments of others, they have postulated that those substances acting as effective nucleation catalysts have atomic arrangements and lattice spacings on certain low-index planes which are very similar to those of the crystal being nucleated.

The potency of the catalyst is formulated to be directly proportional to the reciprocal of the disregistry between the lattice parameters of the catalytic surface and the forming crystal on these planes. Thus, in the formation of ice crystals from supersaturated droplets of water vapor for example, the most potent nucleation catalyst was found to be AgI[96,97] whose lattice structure and atomic arrangement are remarkably similar to ice. Similarly, in the case of the nucleation of hydrated sodium-sulfate ($Na_2SO_4 \cdot 10H_2O$) by sodium-tetraborate ($Na_2B_4O_7 \cdot 10H_2O$) crystals, it was noted that both crystals belong to the same space group and that the disregistry was very small between the two types of crystals on certain basal planes.[98] That some heterogeneities will not act as catalysts at all is probably related to such specific interactions and geometrical factors.

Although many considerations such as specific interactions, types of chemical bonds, etc., as well as relatively nonspecific factors such as surface dislocations and defects, influence the potency or ability of the catalyst in any particular case, the importance of *geometric* and *structural* factors should be kept in mind because of their possible implications for the theory of biological mineralization which is presented.

Other Related Considerations

Whereas *nucleation*—referring to the process of *forming* the infinitesimally small fragments of the new phase—can *in concept* be separated from the subsequent *growth* of these fragments into macroscopic particles of the new phase, *nucleation rate*—which is usually determined by measuring the appearance of the macroscopic particles of the new phase—*operationally* is not completely independent of the kinetics of such growth.

Therefore, the *operational* definition of *nucleation rate* is not completely consistent with the *theoretical* concept of nucleation rate, since the latter strictly speaking refers only to the rate at which the nuclei are forming and not to the subsequent growth of the nuclei to crystals, nor to the phenomenon of recrystallization.

Furthermore, measurements of nucleation rate, particularly in the case of heterogeneous nucleation have given little or no evidence of the molecular or atomic sequence of the nucleation phenomena itself—i.e., the mechanism of nuclei formation—which is of particular importance in the case of biological mineralization, where certain of these steps may be enzymatically controlled and regulated, for example.

Moreover, in the case of crystallization, the number and size of crystals is determined by the relative rates of crystal growth and nucleation rate once nucleation has been initiated. Considered independently, crystal growth may vary in just the opposite fashion as does nucleation rate with respect to certain variables such as temperature. In addition, the growth of crystal nuclei is dependent not only on diffusion rate (i.e., supersaturation ratio) but also on the presence of surface defects (e.g., growth by screw dislocations) even at low-supersaturation ratios.[99]

An interpretation of the entire phenomenon of the crystallization of a specific mineral would, therefore, require not only a study of nucleation phenomena but also considerations of the factors influencing crystal growth, crystal size, crystal habit, etc., for a particular crystal structure. Furthermore, the conditions influencing *recrystallization* (the growth of large crystals at the expense of smaller ones), might also be quite important in the over-all process even after the solid state had been achieved.

BIBLIOGRAPHY

[1] J. E. Eastoe and B. Eastoe, Biochem. J. **57**, 453 (1954).

[2] J. E. Eastoe in *The Biochemistry and Physiology of Bone*, G. H. Bourne, editor (Academic Press, Inc., New York, 1956), p. 81.

[3] Author's unpublished observations.

[4] W. F. DeJong, Rec. trav. Chim. **45**, 445 (1926).

[5] D. Carlström, Acta Radiol. Suppl. 121 (1955).

[6] W. F. Neuman and M. W. Neuman, Chem. Revs. **53**, 1 (1953).

[7] P. Cartier, Bull. soc. chim. biol. **30**, 65 (1948).

[8] M. J. Dallemagne and H. Brasseur, Acta Biol. Belgica **2**, 440, 445 (1952).

[9] H. Brasseur, M. J. Dallemagne, and J. Mélon, Bull. soc chim. France **16**, 109 (1949).

[10] M. J. Dallemagne, C. Fabry, and A. S. Posner, J. physiol. (Paris) **46**, 325 (1954).

[11] A. S. Posner and A. Perloff, J. Research Natl. Bur. Standards **58**, 279 (1957).

[12] D. Carlström, Biochim. et Biophys. Acta **17**, 603 (1955).

[13] S. B. Hendricks and W. L. Hill, Proc. Natl. Acad. Sci. U. S. **36**, 731 (1950).

[14] J. H. Weikel, Jr., and W. F. Neuman, U. S. Atomic Energy Report, University of Rochester, UR-228 (1952).

[15] R. Kunin, K. L. Elmore, and D. L. Johnson, TVA Report, unpublished. Data reported by W. F. Neuman and M. W. Neuman in *The Chemical Dynamics of Bone Mineral* (The University of Chicago Press, Chicago, 1958), p. 39.

[16] W. F. Neuman and M. W. Neuman, *The Chemical Dynamics of the Bone Mineral* (The University of Chicago Press, Chicago, 1958).

[17] M. J. Dallemagne and J. Mélon, Arch. de Biol. **57**, 79 (1946).

[18] P. W. Arnold, Trans. Faraday Soc. **46**, 1061 (1950).
[19] W. E. Brown, J. R. Lehr, J. P. Smith, and A. W. Frazier, J. Am. Chem. Soc. **79**, 5318 (1957).
[20] A. S. Posner (personal communication, 1958).
[21] J. W. Gruner and D. McConnell, Z. Kristallog. **97**, 208 (1937).
[22] D. McConnell in *Metabolic Interrelations*, Transactions 4th Conference, E. C. Reifenstein, Jr., editor (Josiah Macy, Jr., Foundation, New York, 1952).
[23] L. Huber and C. Rouiller, Experientia **7**, 338 (1951).
[24] C. Rouiller, L. Huber, E. Kellenberger, and E. Rutishauser, Acta Anat. **14**, 9 (1952).
[25] W. J. Schmidt, Handbuch biol. Arbeitsmethoden, **A5**, T10, 435 (1934).
[26] J. Wolff, *Das Gesetz der Transformation der Knochen* (Quarto, Berlin, 1892).
[27] R. A. Robinson, J. Bone and Joint Surg. **34A**, 389 (1952).
[28] R. A. Robinson and M. L. Watson, Anat. Record **114**, 383 (1952).
[29] R. A. Robinson and M. L. Watson, Ann. New York Acad. Sci. **60**, 596 (1955).
[30] S. Fitton-Jackson, Proc. Roy. Soc. (London) **B146**, 270 (1957).
[31] H. Sheldon and R. A. Robinson, J. Biophys. Biochem. Cytol. **3**, 1011 (1957).
[32] R. Robinson, *The Significance of Phosphoric Esters in Metabolism* (New York University Press, New York, 1932).
[33] E. Freudenberg and P. Györgi, Biochem. Z. **118**, 50 (1921).
[34] A. B. Gutman and T. F. Yü, in *Metabolic Interrelations*, Transactions 1st Conference (Josiah Macy, Jr., Foundation, New York, 1950), p. 11.
[35] A. E. Sobel and M. Burger, Proc. Soc. Exptl. Biol. Med. **87**, 7 (1954).
[36] A. E. Sobel, Ann. N. Y. Acad. Sci. **60**, 713 (1955).
[37] P. S. Rubin and J. E. Howard in *Metabolic Interrelations*, Transactions 2nd Conference (Josiah Macy, Jr., Foundation, New York, 1951), p. 155.
[38] B. S. Strates, W. F. Neuman, and G. J. Levinskas, J. Phys. Chem. **61**, 279 (1957).
[39] B. S. Strates and W. F. Neuman, Proc. Soc. Exptl. Biol. Med. **97**, 688 (1958).
[40] M. J. Glimcher, A. J. Hodge, and F. O. Schmitt, Proc. Natl. Acad. Sci. U. S. **43**, 860 (1957).
[41] M. B. Engel, N. R. Joseph, and H. R. Catchpole in *Metabolic Interrelations*, Transactions 5th Conference, E. C. Reifenstein, Jr., editor (Josiah Macy, Jr., Foundation, New York, 1953), p. 105.
[42] I. Gersh in *Connective Tissues*, Transactions 2nd Conference (Josiah Macy, Jr., Foundation, New York, 1952), p. 11.
[43] F. McLean and M. R. Urist, *Bone, An Introduction to the Physiology of Skeletal Tissue* (The University of Chicago Press, Chicago, 1955).
[44] F. McLean in *Metabolic Interrelations*, Transactions 2nd Conference (Josiah Macy, Jr., Foundation, New York, 1950), discussion p. 101.
[45] R. Becker and W. Döring, Ann. Physik **24**, 719 (1935).
[46] M. Volmer, *Kinetik der Phasenbildung* (Edwards Brothers, Ann Arbor, 1945).
[47] W. F. Neuman in *Metabolic Interrelations*, Transactions 2nd Conference (Josiah Macy, Jr., Foundation, New York, 1950), p. 32.
[48] Author's unpublished results.
[49] D. S. Jackson (personal correspondence, 1958).
[50] C. C. Solomons and J. T. Irving, Biochem. J. **68**, 499 (1958).
[51] B. F. Martin and F. Jacoby, J. Anat. **83**, 351 (1949).
[52] P. Doty and T. Nishihara in *Recent Advances in Gelatin and Glue Research*, G. Stainsby, editor (Pergamon Press, London, 1958), p. 92.
[53] P. J. Flory and R. R. Garrett, J. Am. Chem. Soc. **80**, 4836 (1958).
[54] W. Cochran, F. H. C. Crick, and V. Vand, Acta Cryst. **5**, 581 (1952).
[55] F. H. C. Crick, Acta Cryst. **6**, 685 (1953).
[56] Many of the low-angle *x*-ray diffraction studies were carried out in cooperation with Dr. A. Posner, Am. Dental Assn., Research Division, National Bureau of Standards, and with Dr. L. Bonar, Department of Biophysics, Massachusetts Institute of Technology.
[57] R. S. Bear, Advances in Protein Chem. **7**, 69 (1952).
[58] O. E. A. Bolduan, T. P. Salo, and R. S. Bear, J. Am. Leather Chem. Assn. **46**, 124 (1951).
[59] K. Kühn, W. Grassmann, and U. Hofmann, Naturwissenschaften **44**, 538 (1957).
[60] W. Grassmann, U. Hofmann, K. Kühn, H. Hörmann, H. Endres, and K. Wolf in *Connective Tissue*, Symposium organized by Council for International Organizations of Medical Sciences, R. E. Tunbridge, editor (Blackwell Scientific Publications, Oxford, 1957), p. 157.
[61] D. Turnbull, J. Appl. Phys. **21**, 1022 (1950).
[62] D. Turnbull, J. Chem. Phys. **20**, 411 (1952).
[63] D. Turnbull and B. Vonnegut, Ind. Eng. Chem. **44**, 1292 (1952).
[64] V. DiStefano, W. F. Neuman, and G. Rouser, Arch. Biochem. Biophys. **47**, 218 (1953).
[65] M. Polonovski and P. Cartier, Compt. rend. **232**, 119 (1951).
[66] R. J. Block, M. K. Horwitt, and D. Bolling, J. Dental Research **28**, 518 (1949).
[67] R. J. Block, Ann. N. Y. Acad. Sci. **53**, 608 (1951).
[68] D. B. Scott, Ann. N. Y. Acad. Sci. **60**, 575 (1955).
[69] H. R. Perkins and P. G. Walker, J. Bone and Joint Surg. **40B**, 333 (1958).
[70] J. Shatton and M. Schubert, J. Biol. Chem. **211**, 565 (1954).
[71] S. M. Partridge and H. F. Davis, Biochem. J. **68**, 298 (1958).
[72] K. Meyer, E. Davidson, A. Linker, and P. Hoffman, Biochim. et Biophys. Acta **21**, 506 (1956).
[73] M. A. Logan, J. Biol. Chem. **110**, 375 (1935).
[74] M. Schubert and D. Hamerman, J. Histochem. Cytochem. **4**, 159 (1956).
[75] J. Einbinder and M. Schubert, J. Biol. Chem. **185**, 725 (1950).
[76] H. P. Gregor, Trans. New York Acad. Sci. Ser. II, **18**, 667 (1956).
[77] E. S. Boyd and W. F. Neuman, J. Biol. Chem. **193**, 243 (1951).
[78] D. Burton and R. Reed, Discussions Faraday Soc. **16**, 195 (1954).
[79] D. Carlström, A. Engström, and J. B. Finean in *Fibrous Proteins and Their Biological Significance*, Symp. Soc. Exptl. Biol. **9**, 85 (1955).
[80] R. A. Robinson and D. A. Cameron, J. Biophys. Biochem. Cytol. **2**, Suppl. 253 (1956).
[81] S. M. Clark and J. Iball, Nature **179**, 94 (1957).
[82] S. M. Clark and J. Iball, Progr. in Biophys. and Biophys. Chem. **7**, 225 (1957).
[83] G. Wallgren, Acta Paediate. **113**, Suppl. (1957).
[84] T. W. Speckman and W. P. Norris, Science **126**, 753 (1957).
[85] H. Fernández-Morán and A. Engström, Biochim. et Biophys. Acta **23**, 260 (1957).
[86] M. L. Watson and R. A. Robinson, Am. J. Anat. **93**, 25 (1953).
[87] Author's unpublished results.
[88] C. Gregoire, J. Biophys. Biochem. Cytol. **3**, 797 (1957).
[89] T. Tsujii, D. G. Sharp, and K. M. Wilbur, J. Biophys. Biochem. Cytol. **4**, 275 (1958).
[90] N. Watabe, D. G. Sharp, and K. M. Wilbur, J. Biophys. Biochem. Cytol. **4**, 281 (1958).
[91] J. W. Gibbs, *The Collected Works of J. Willard Gibbs* (Longmans, Green and Company, New York, 1928), Vol. I.
[92] J. Frenkel, *The Kinetic Theory of Liquids* (Clarendon Press, Oxford, 1946, and Dover Publications, New York, 1955).
[93] M. Volmer and A. Weber, Z. physik. Chem. **119**, 277 (1926).
[94] M. Volmer, Z. Elektrochem. **35**, 555 (1929).
[95] M. Cohen, Transactions Metallurgical Soc. AIME **212**, 171 (1958).
[96] V. J. Schaefer, Chem. Revs. **44**, 291 (1949).
[97] B. Vonnegut, J. Appl. Phys. **18**, 593 (1947).
[98] M. Telkes, Ind. Eng. Chem. **44**, 1308 (1952).
[99] F. C. Frank, Advances in Phys. **1**, 91 (1952).

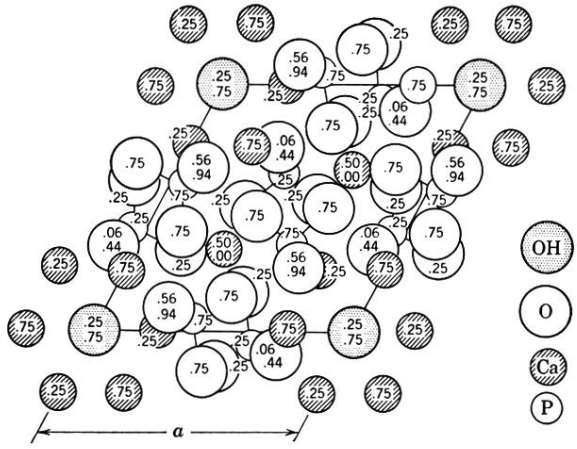


FIG. 1. Atomic arrangement of the constituents of hydroxyapatite projected on the 001 plane. The numbers refer to the fractional height in the unit cell of the atoms in the plane perpendicular to the paper (c-axis), as reported by Carlström[5] [from A. S. Posner, Clin. orthoped. **9**, 5 (1957)].

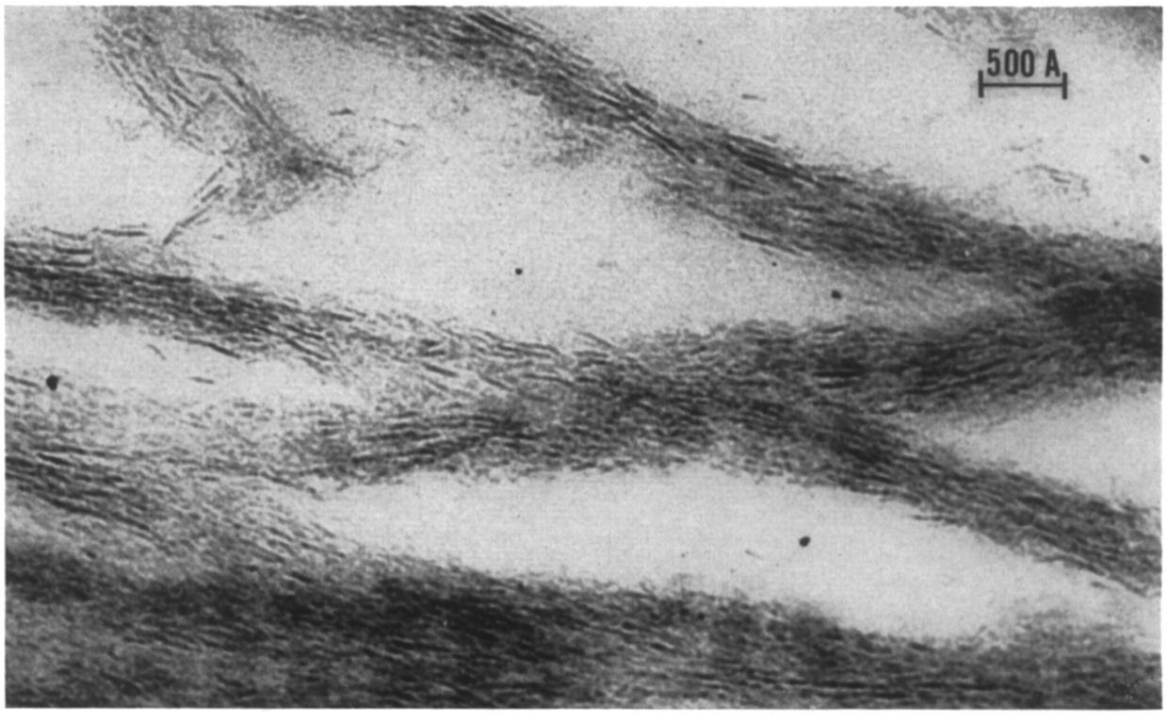


FIG. 10. Higher magnification of an area in Fig. 9. The crystals appear to be rod-shaped, approximately 200 to 400 A long and 15 to 40 A wide. ×225 000.

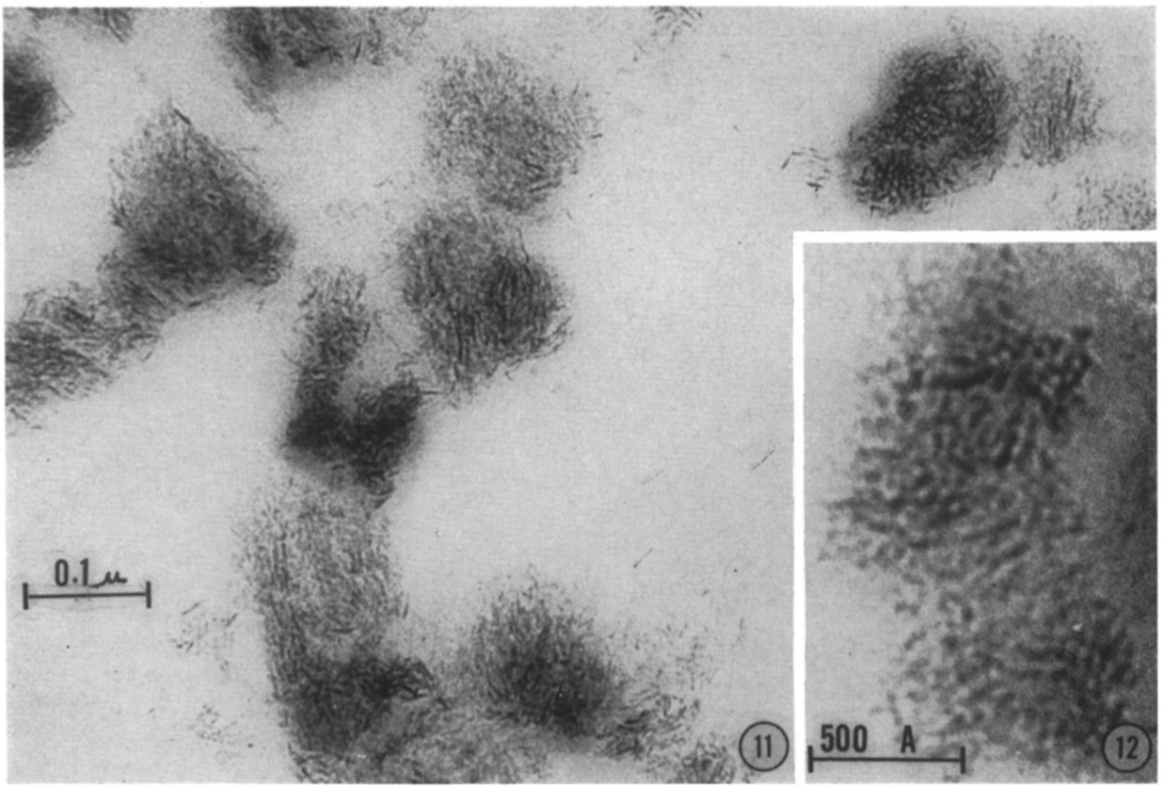
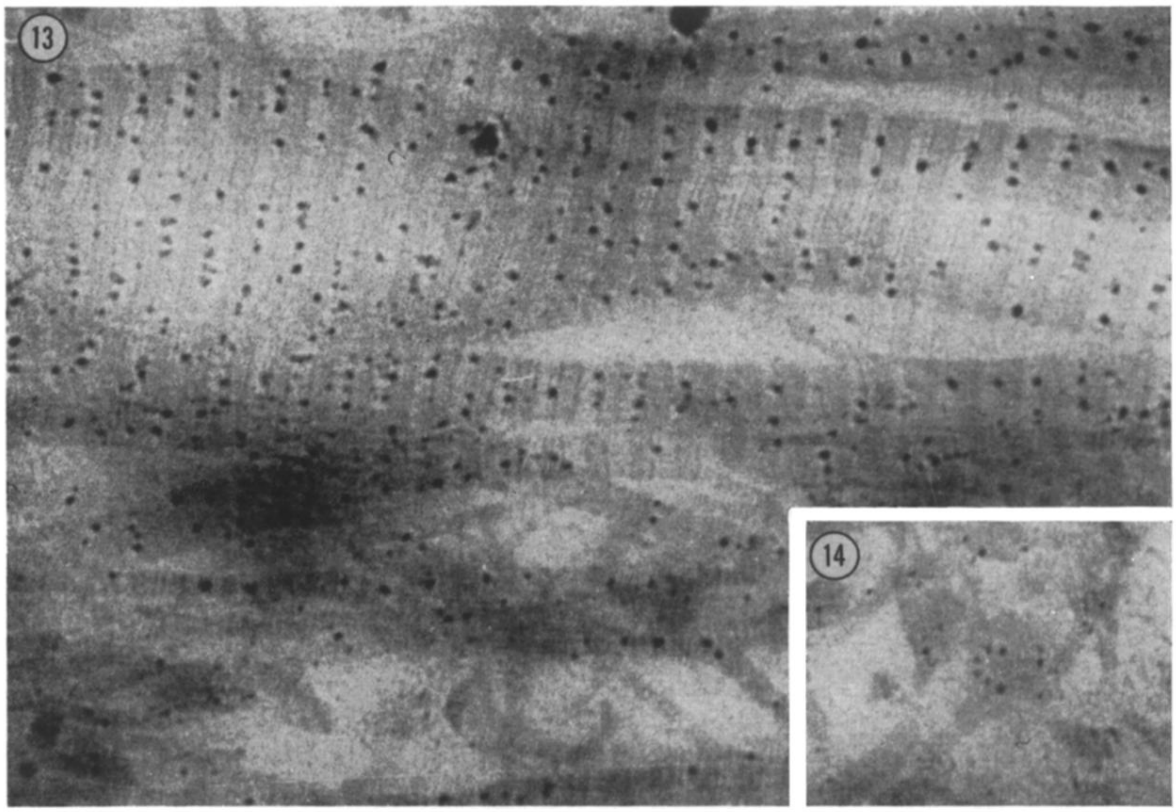


FIG. 11. Higher magnification of cross sections of fibrils in fish bone. The apatite crystals are obviously within the collagen fibrils. ×165 000.

FIG. 12. Cross section of two fibrils in an area similar to Fig. 11. The crystals have appearance of rods viewed on end and appear to be hexagonally packed. ×430 000.



FIGS. 13 and 14. Electron micrographs of avian embryonic bone in the earliest stages of calcification. Note the regular and periodic arrangement of the dense apatite crystals within the collagen fibrils [from S. Fitton-Jackson, Proc. Roy. Soc. (London) **B146**, 270 (1957); reproduced from originals kindly supplied by the author]. ×100 000.

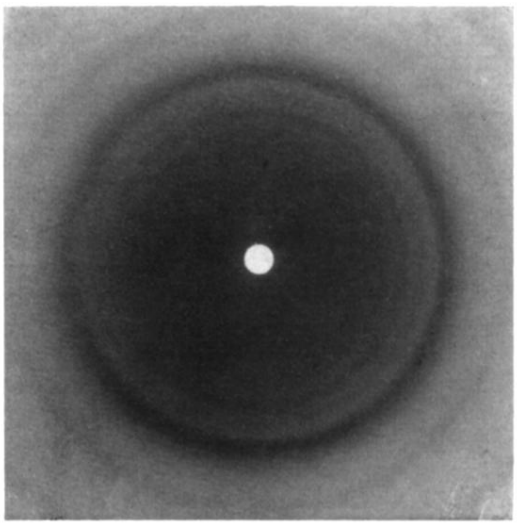


FIG. 15. X-ray diffraction pattern of *in vitro* calcified collagen. Note the lack of crystal orientation (evidenced by complete rings) and the broadness of the diffraction lines attributable primarily to small crystal size.

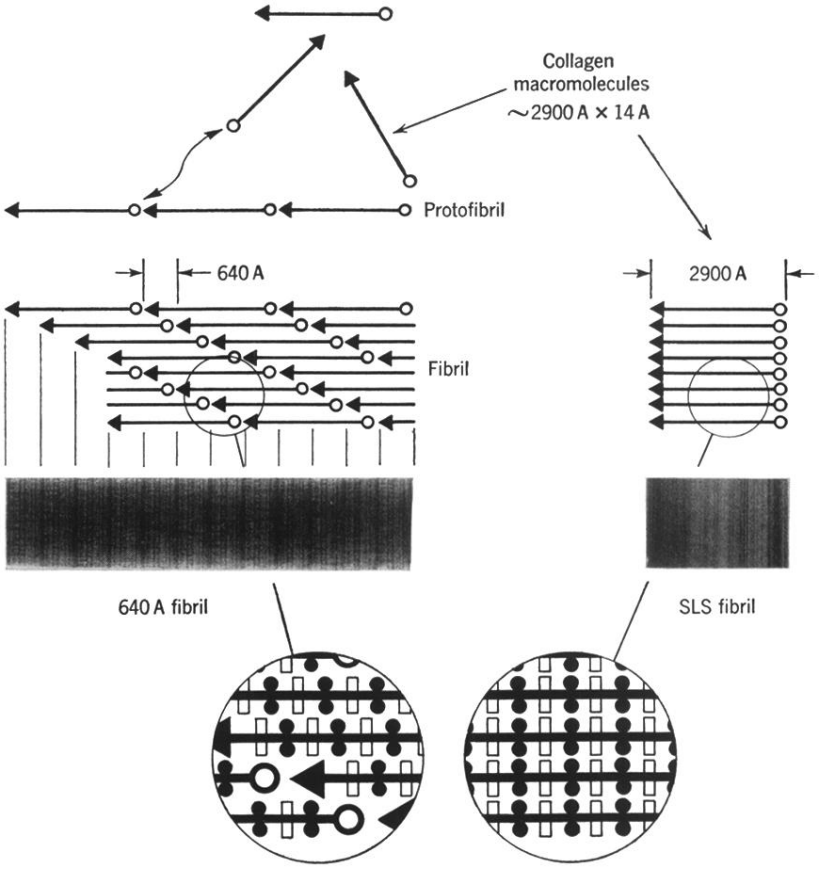


FIG. 17. The steric differences between the reactive side-chain groups of adjacent macromolecules in the native and SLS forms of collagen diagrammatically portrayed.

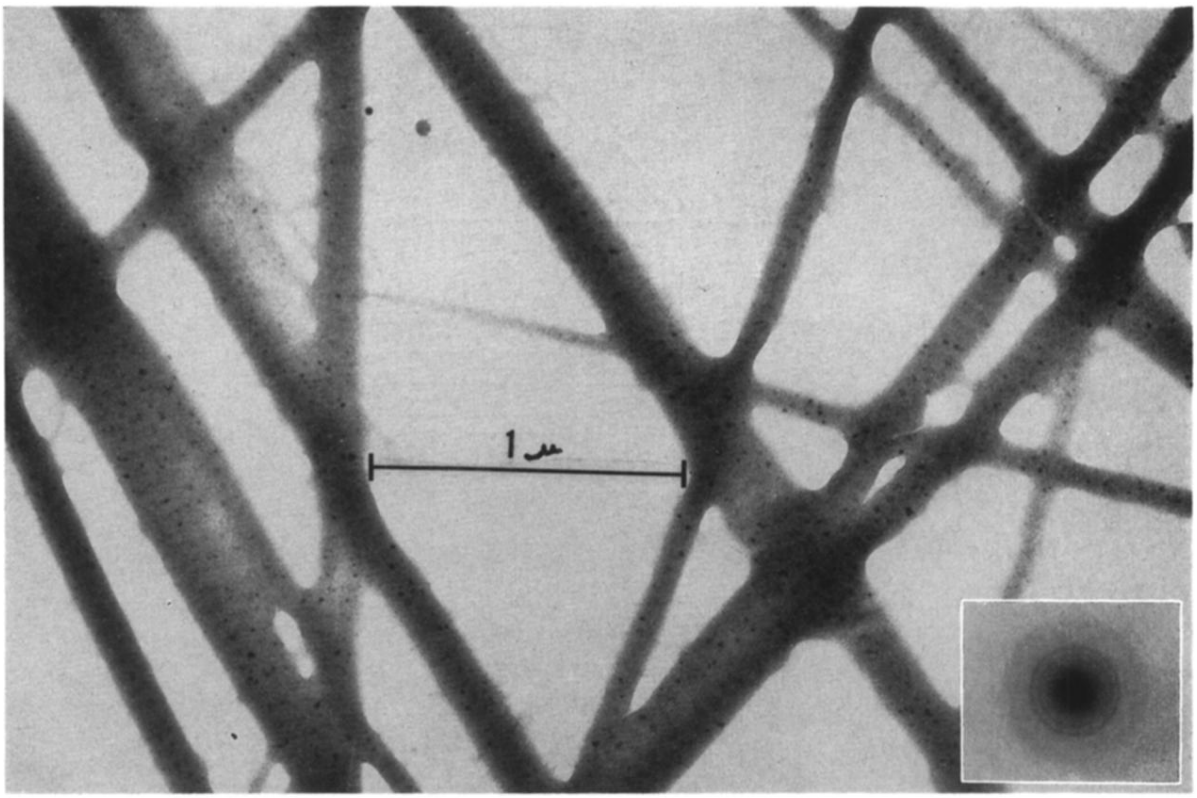


FIG. 18. Unstained, unshadowed preparation of an early stage of *in vitro* calcification of collagen fibrils. Note the regular and periodic distribution of the crystals along the collagen-fibril axis corresponding to the intraperiod fine structure of the fibrils and occurring primarily once per axial period.

FIG. 19 (lower right-hand corner). Selected-area electron-diffraction pattern of preparation similar to Fig. 18, showing the characteristic apatite reflections. There is no evidence of preferred crystal orientation, even though the diffraction pattern was obtained from an area where the fibrils were well oriented. ×42 000.

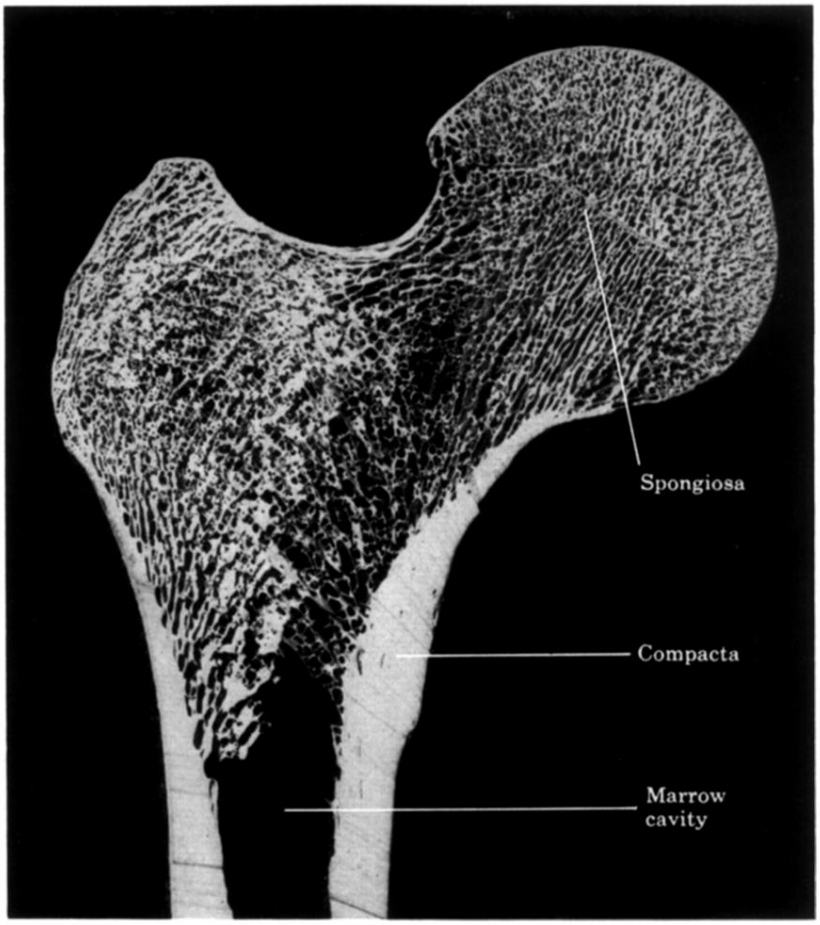


FIG. 2(a). Coronal section of the upper end of a human femur [from D. W. Fawcett, in *Histology*, R. Greep, editor (The Blackiston Company, New York, 1954), p. 133; reproduced from original kindly supplied by the author].

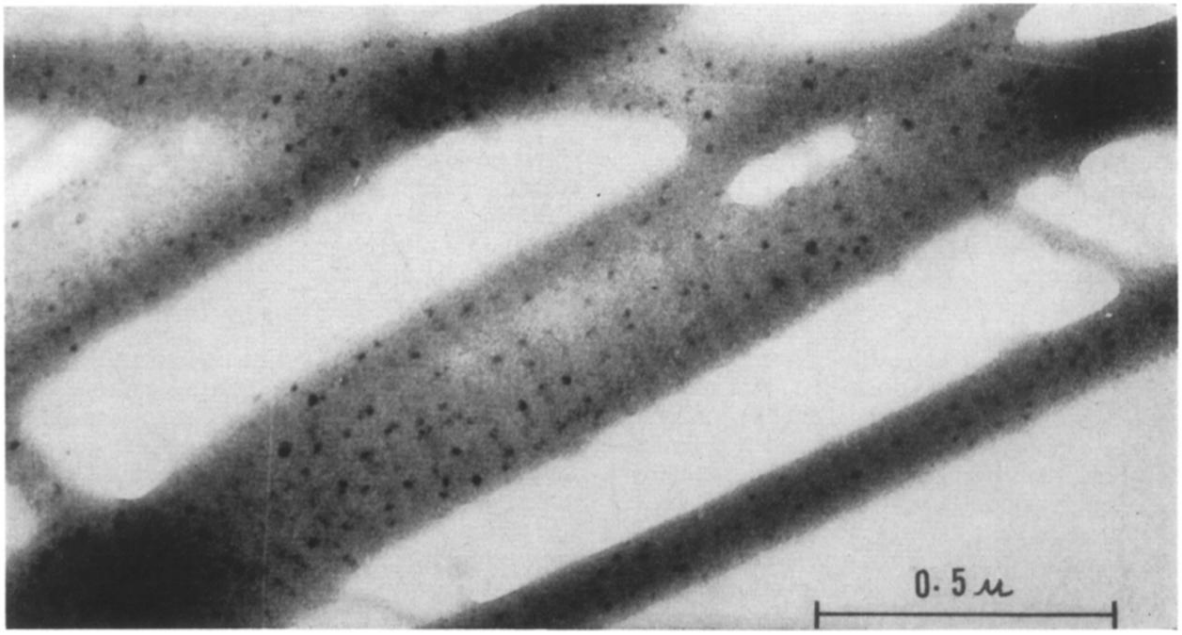


FIG. 20. Higher magnification of an area in Fig. 18. ×80 000.

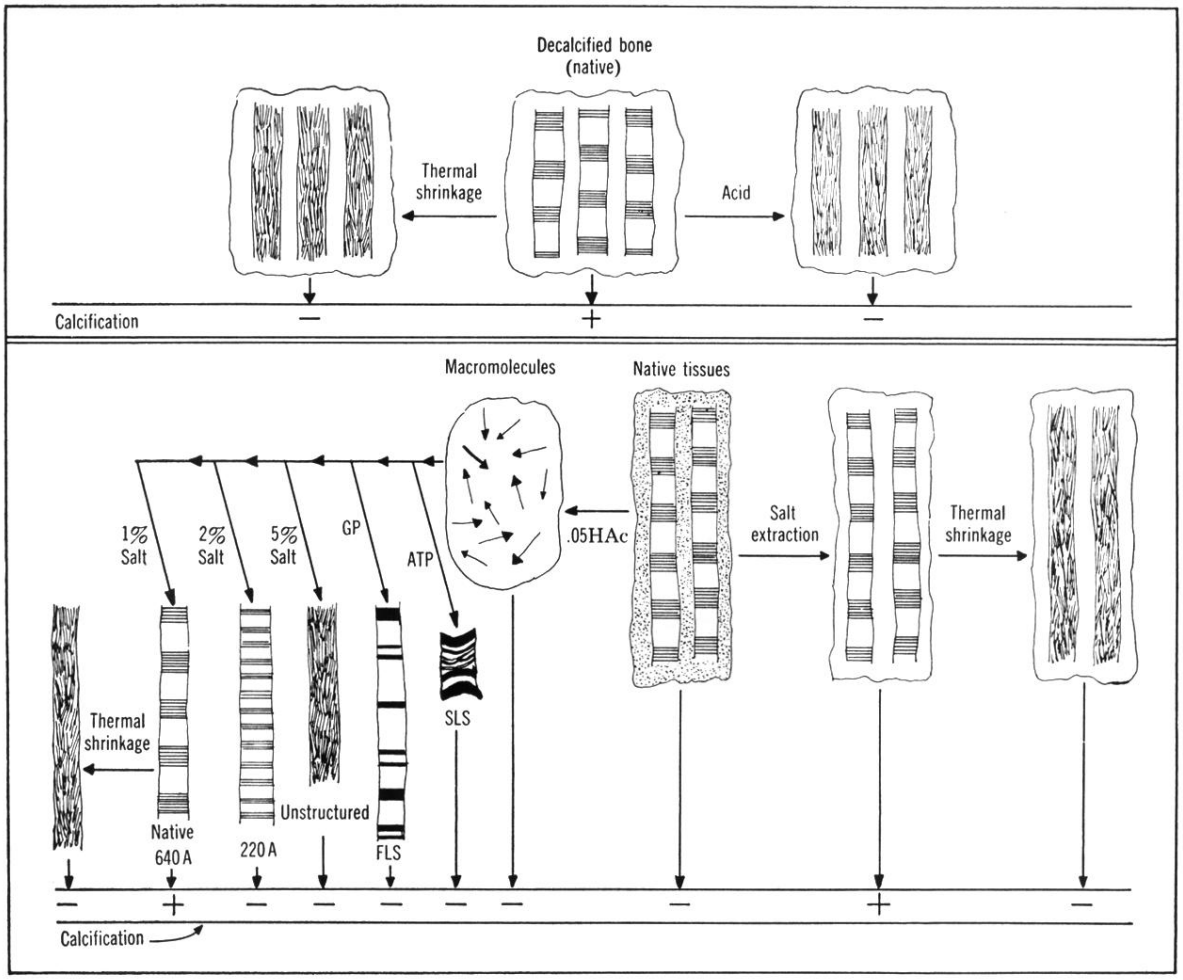
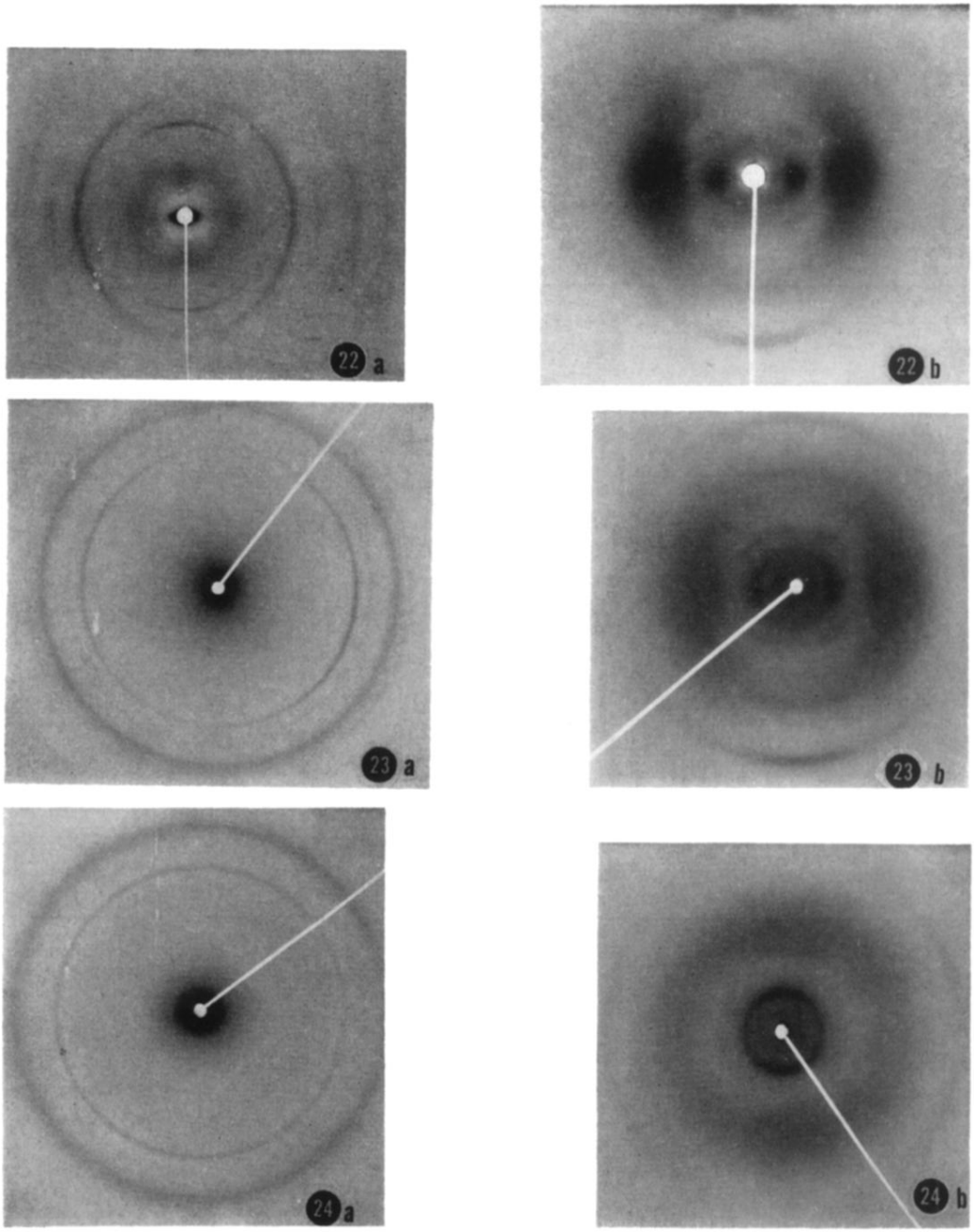


FIG. 21. Composite and diagrammatic illustration of the experiment demonstrating the specificity of the macromolecular aggregation state of native-type collagen fibrils in calcification, and the possible role of the ground substance (heavily stippled regions surrounding fibrils in native tissues) in inhibiting and controlling it. Enzymatic treatment of the native tissue is not shown, since it is not yet certain whether depolymerization is itself effective, or whether depolymerization and subsequent removal from the tissue is necessary.



FIGS. 22, 23 and 24. X-ray diffraction patterns of longitudinally oriented native, calcified fish bone [Fig. 22(a)], rat femur [Fig. 23(a)], and embryonic metatarsal rudiment [Fig. 24(a)], showing apatite orientation; same specimens [Figs. 22(b)–24(b)] decalcified, showing collagen orientation.

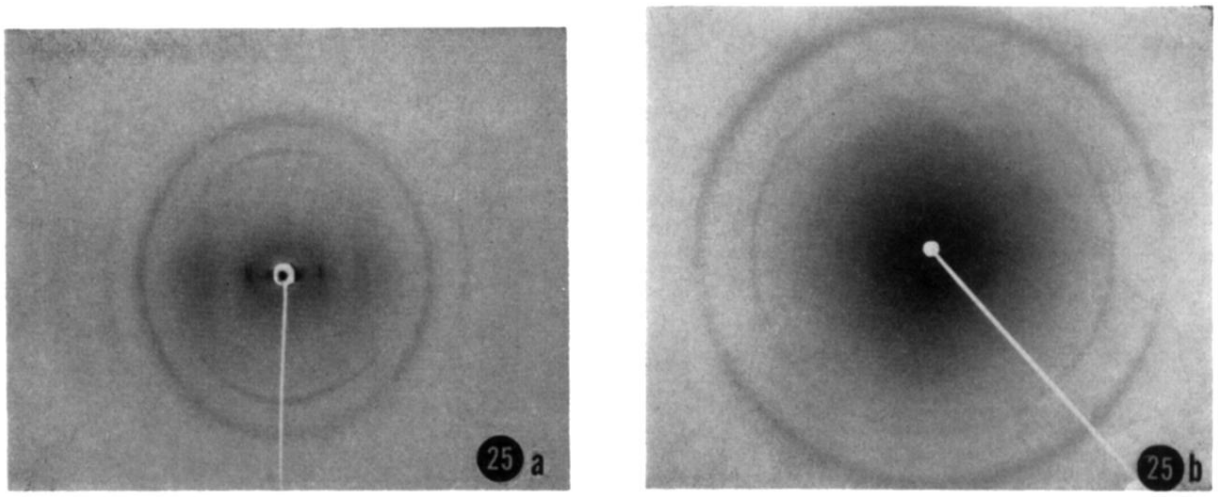


FIG. 25 X-ray diffraction patterns of *in vitro* (a) recalcified fish bone and (b) rat-femur bone. Note that, although there is *some* orientation of the apatite crystals as evidenced by the arcing of the 00*L* reflections, it is *not* as prominent as in the original native, calcified bone [compare Fig. 22(a) with Fig. 25(a), and Fig. 23(a) with Fig. 25(b)].

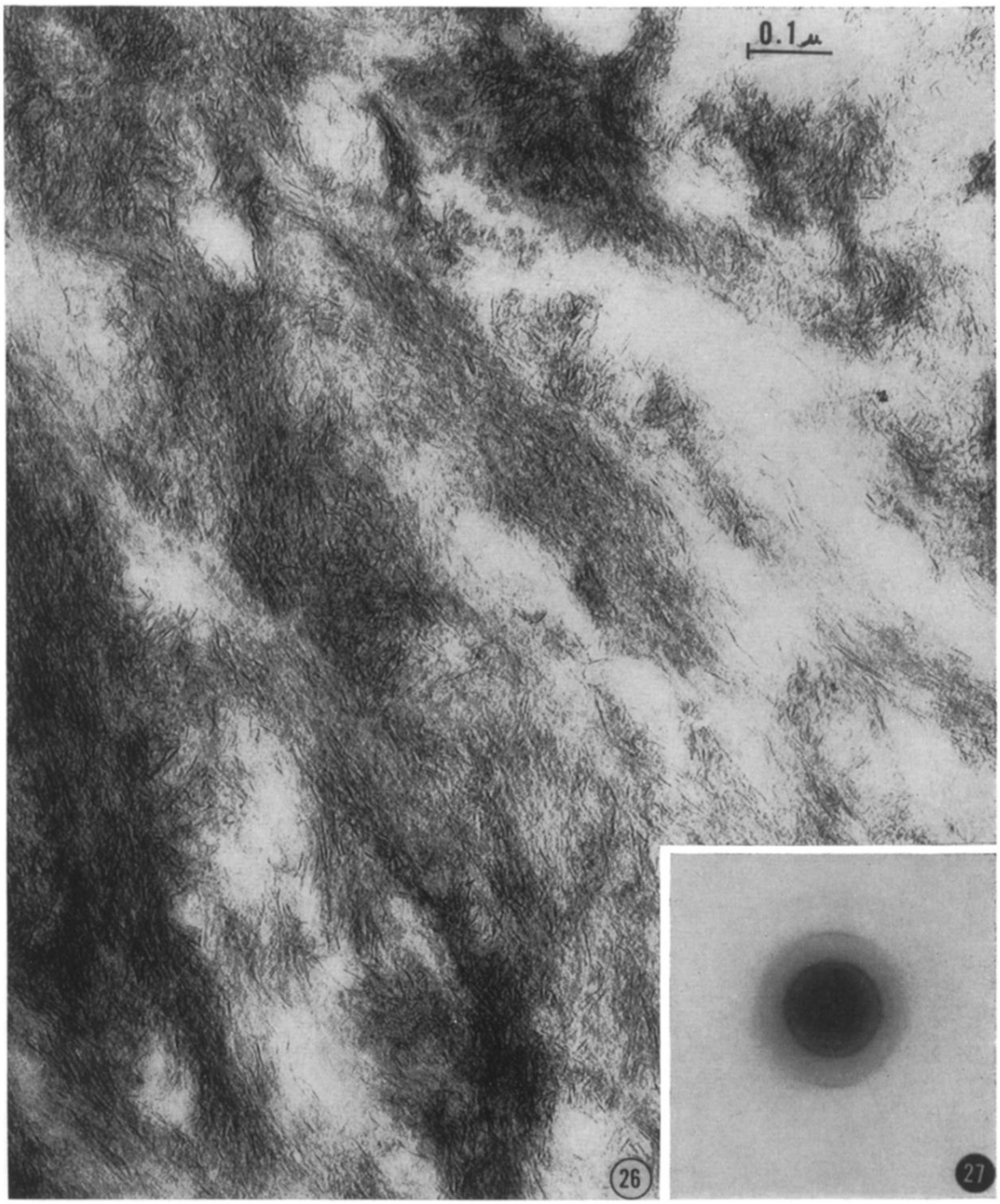


FIG. 26. Electron micrograph of a section of the metatarsal rudiment of embryonic chick bone (16 days). Despite the general randomness of the collagen fibrils, the crystals are oriented in local regions corresponding to the individual collagen-fibril directions. The size and shape of the crystals are similar to those in adult bone. ×40 000.

FIG. 27. Selected-area electron diffraction of specimen shown in Fig. 26. The preferred orientation of the crystals is evident by the arcing of the 002 and 004 reflections.

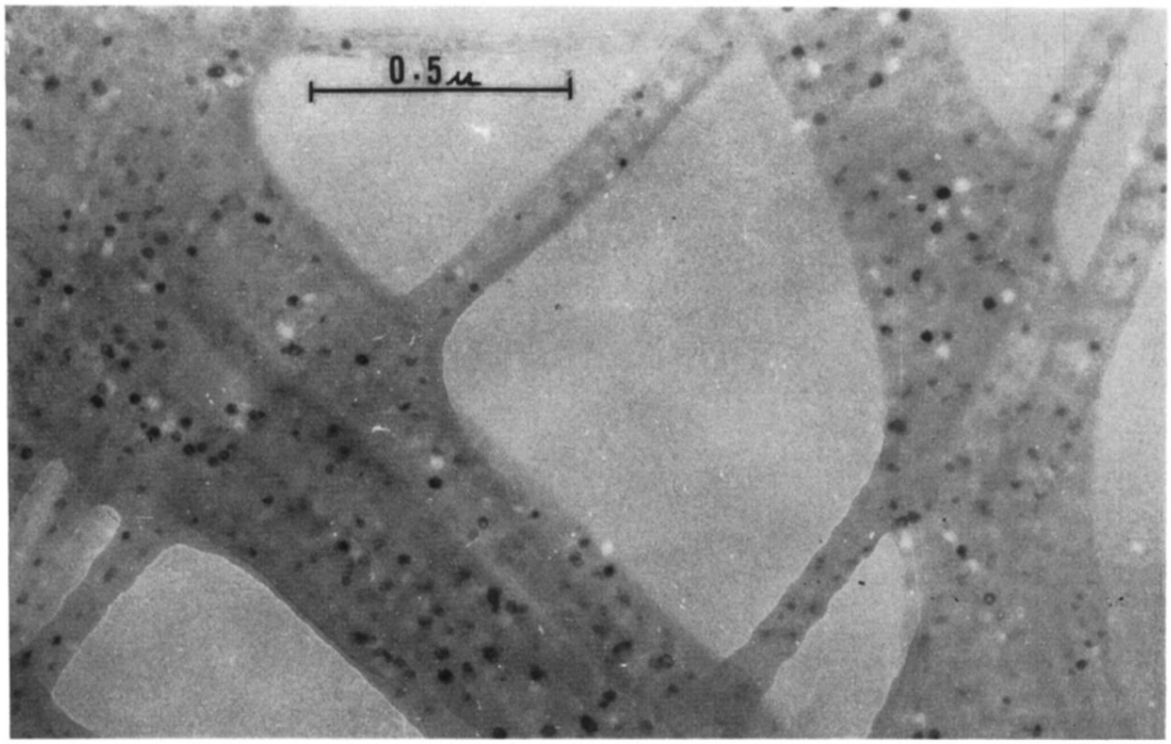


FIG. 28. Unstained, unshadowed preparations of *in vitro* calcified collagen at an early stage. Although the crystals are situated regularly spaced in certain regions of the fibrils, the haphazard arrangement of the crystal reflections (white spots) indicates lack of preferred *orientation* of individual crystals with the fibrils with which they are associated. ×70 000.

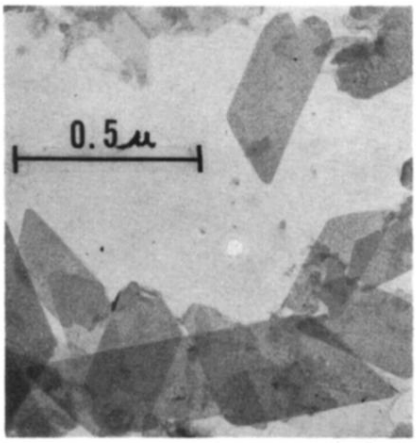


FIG. 29. Electron micrograph of *in vitro* apatite crystals precipitated under conditions similar to those of the *in vitro* collagen-nucleation studies. Note large hexagonal-type crystals. ×50 000.

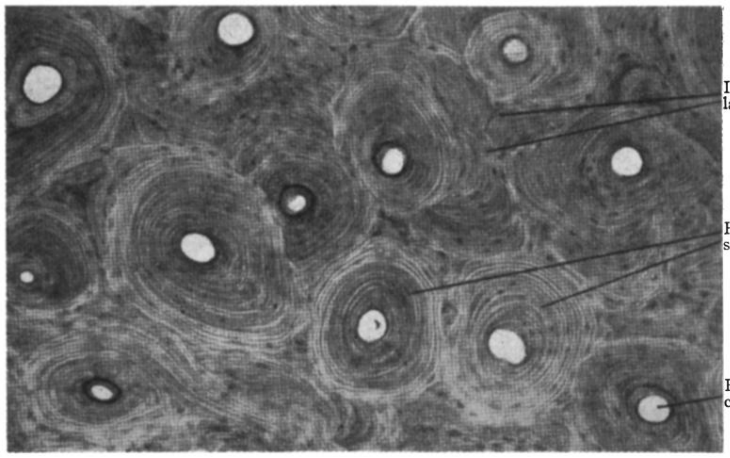


FIG. 3. Cross section of decalcified compact bone ×80. Note the alternating dark and light layers in the Haversian systems and interstitial lamellae attributable to consecutive changes in collagen-fiber orientation [from D. W. Fawcett in *Histology*, R. Greep, editor (The Blackiston Company, New York, 1954), p. 134; reproduced from original kindly supplied by the author].

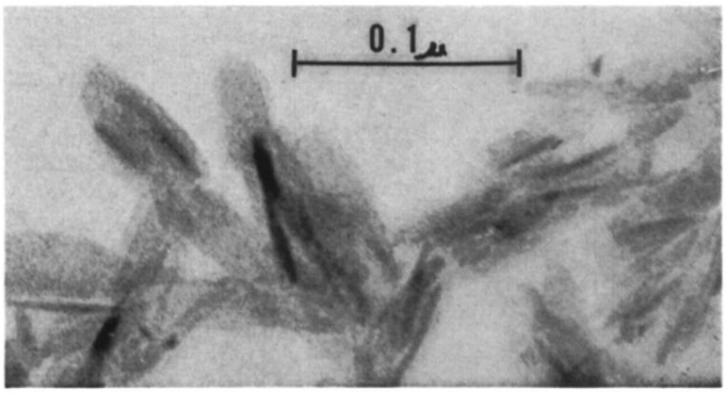


FIG. 30. Under altered physicochemical conditions, crystal size decreases markedly, but crystal habit remains the same. ×300 000.

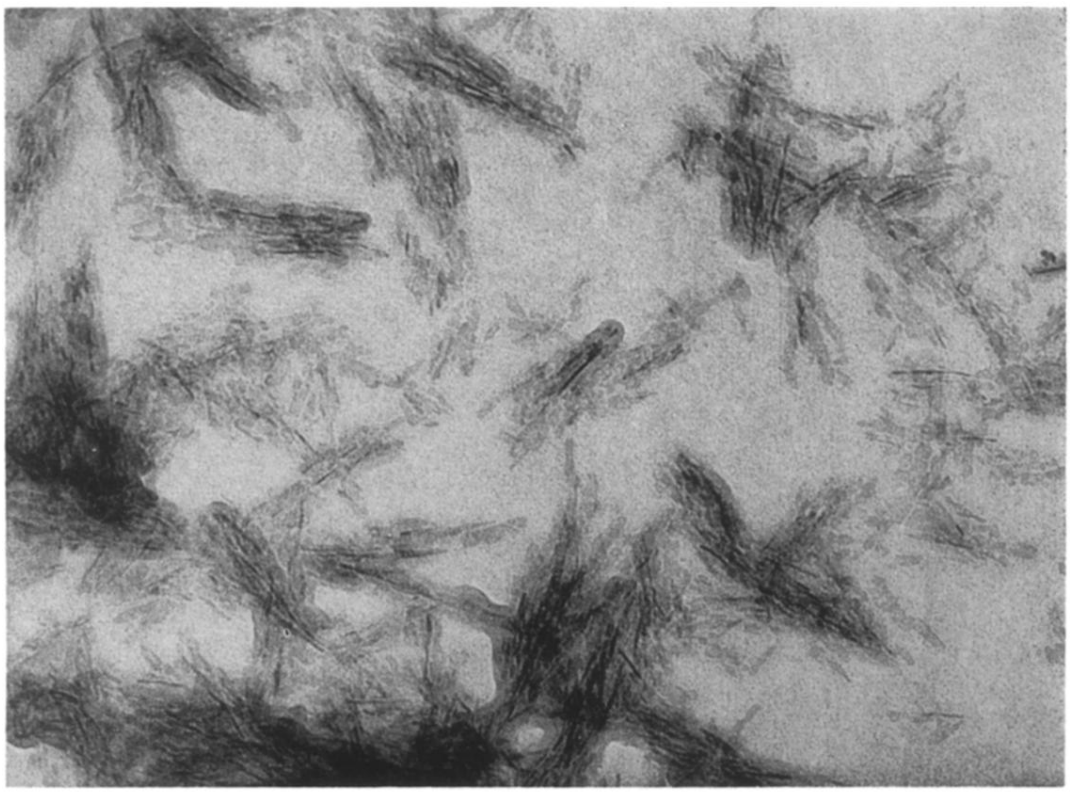


FIG. 31. Further alteration in the physicochemical environment produces not only a further decrease in crystal size but also, for some, a change in the crystal habit to a rod-like appearance. ×180 000.

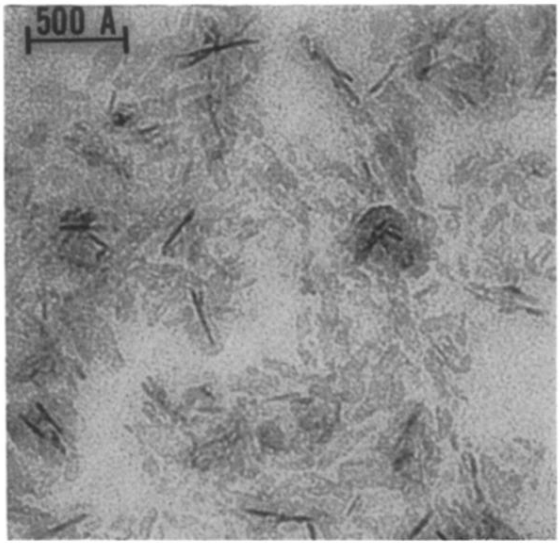


FIG. 32. Higher magnification of an area in a sample similar to Fig. 31, where the "plates" predominate. ×260 000.

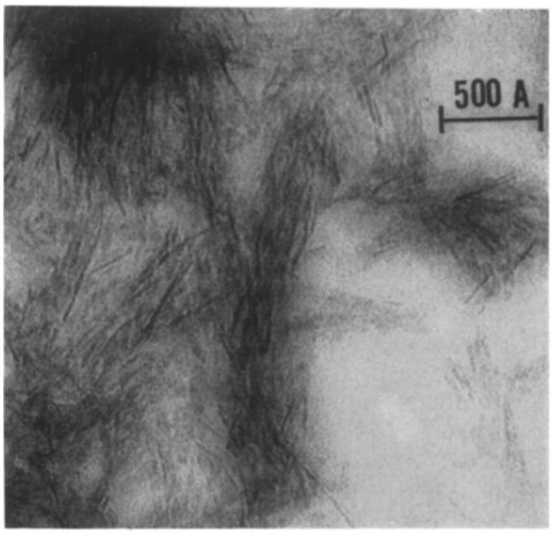


FIG. 33. Higher magnification of an area in a sample similar to Fig. 31, where the rod-like crystals predominate. ×260 000.

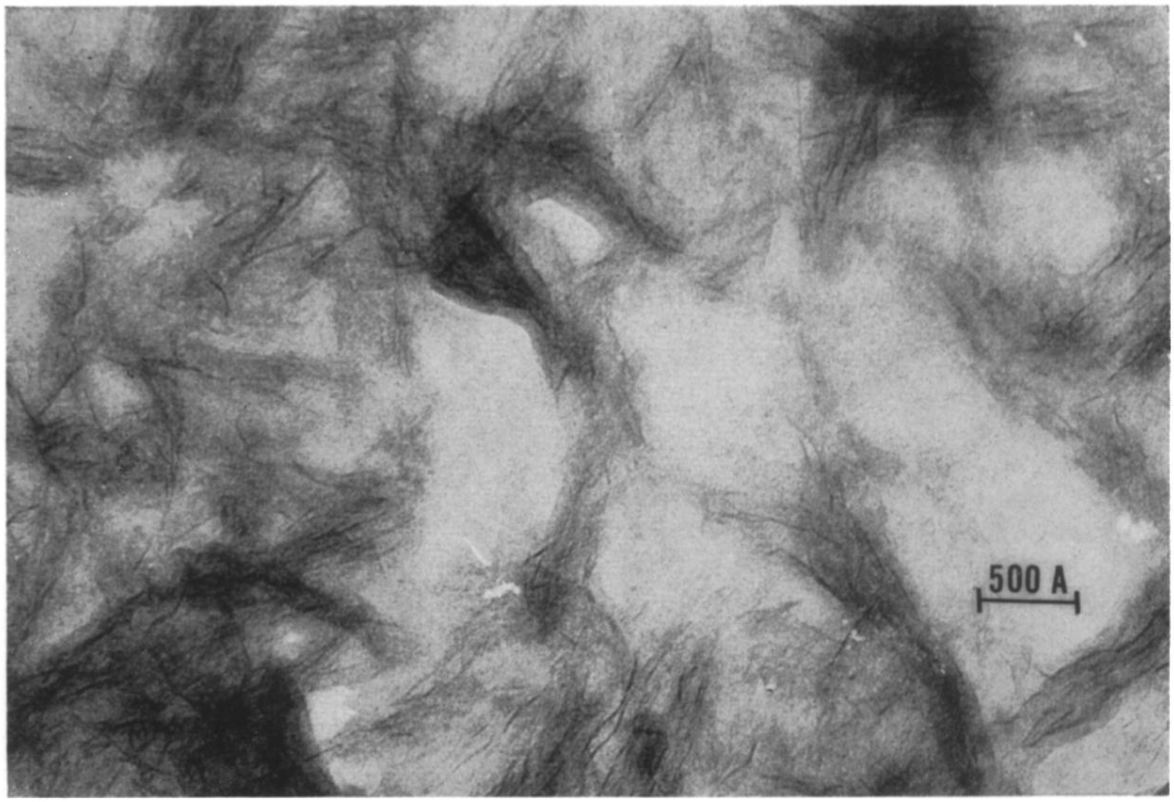


FIG. 34. *In vitro* precipitated apatite crystals. Note the rod-like appearance without evidence of plate formation, except for the minimal background electron density. Crystals vary from 8 to 20 A in width, and many appear to be "bent" similar to areas of lightly calcified bone. ×260 000.

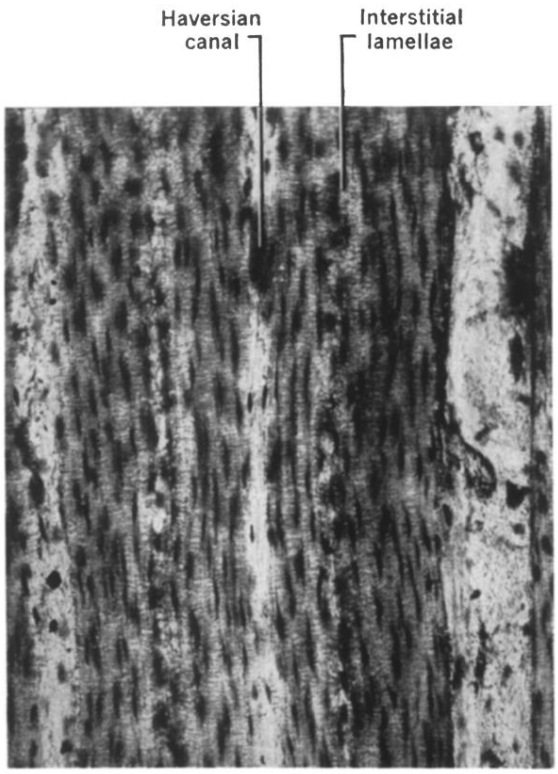


FIG. 4. Longitudinal section of compact bone illustrating Haversian systems (original magnification ×120) [from J. P. Weinmann and H. Sicher, *Bone and Bones, Fundamentals of Bone Biology* (C. V. Mosby Company, St. Louis, 1947); reproduced from original kindly supplied by the authors].

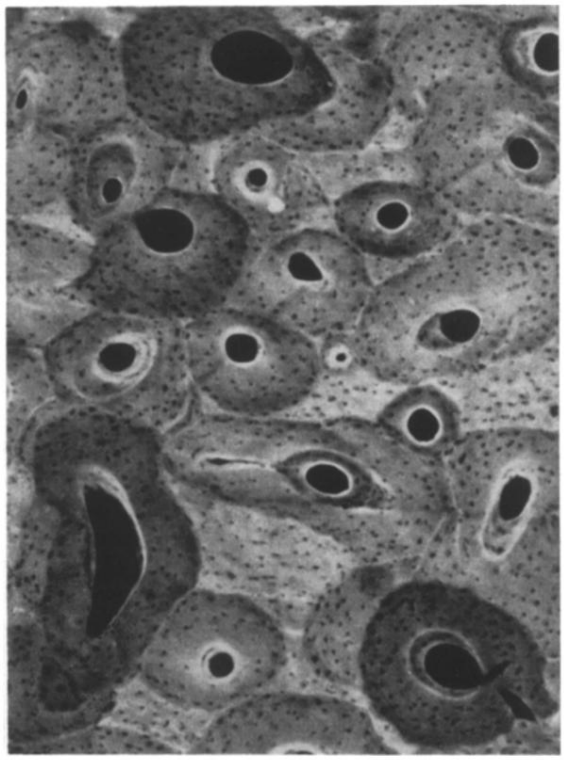


FIG. 5. A microradiogram of a thin cross section of normal cortical bone. Note the variation in density (indicating inorganic-crystal concentration) within any one Haversian system, as well as within the section as a whole. Reproduced from original kindly supplied by A. Engström, Karolinska Institutet, Stockholm, Sweden.

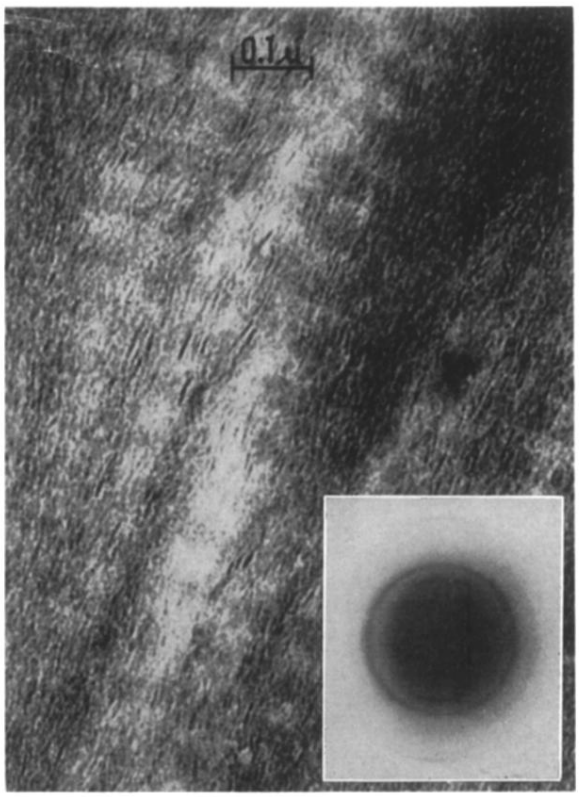


FIG. 6. Electron micrograph of a longitudinal section of compact bone. Note the accentuation of the characteristic axial repeat of the collagen fibrils in some areas by the small inorganic crystals, whose long axes approximately parallel the collagen-fiber axis. ×100 000.

FIG. 7 (lower right-hand corner). Selected-area electron diffraction of the specimen in Fig. 6, showing characteristic pattern of apatite. Arcing of the 002 and 004 reflections indicates that the crystallographic *c*-axes of the crystals are oriented parallel to the collagen-fiber axis, and, therefore, correspond to the long axes of the crystals.

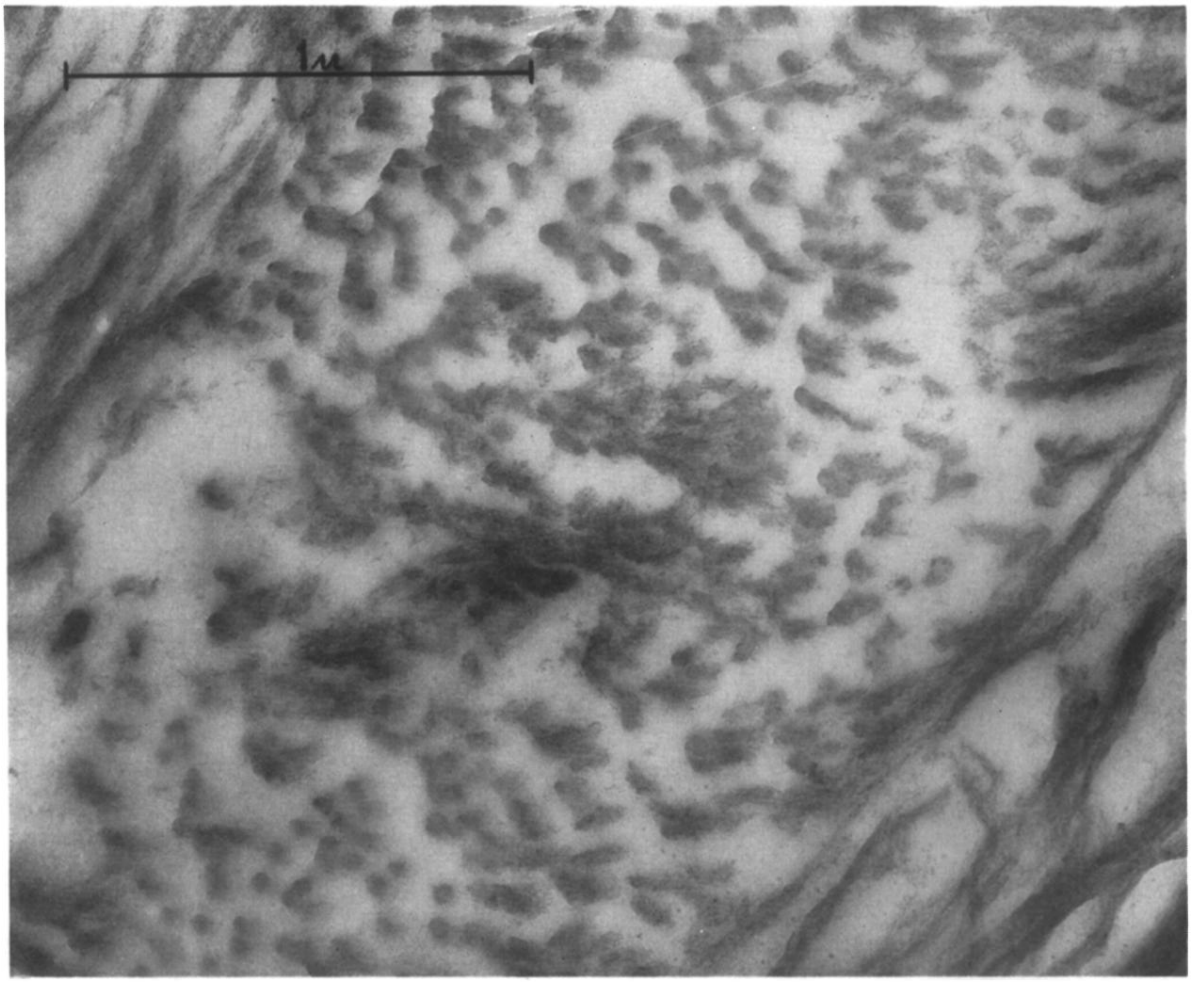


Fig. 8. Electron micrograph of a cross section of fish bone. Note the alternating direction of the collagen fibers in consecutive layers and the packing of the fibrils. Even at this low magnification it is obvious that the dense inorganic crystals are *within* the fibrils and not in the intervening ground substance. ×58 000.

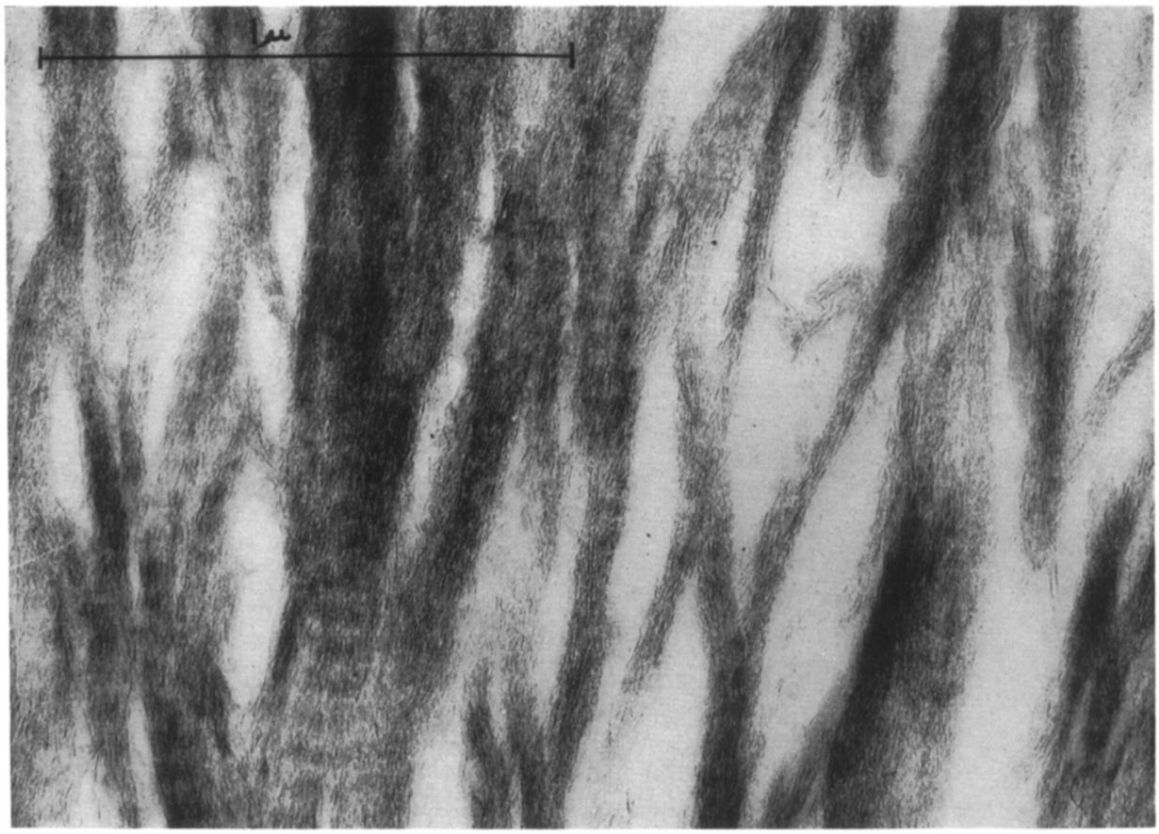


FIG. 9. Electron micrograph of fish bone in a region of loosely packed fibrils such as in Fig. 8. Despite variations in individual fibril direction, long axes of inorganic crystals are parallel to the individual collagen fibrils with which they are associated by virtue of their position within the fibrils. ×75 000.