ORGANIZATION OF EXTRACELLULARLY MINERALIZED TISSUES: A COMPARATIVE STUDY OF BIOLOGICAL CRYSTAL GROWTH

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I. INTRODUCTION

The formation of minerals by organisms is a widespread phenomenon. The types of minerals formed, the sites of deposition, the organizational motifs in which they are found, and the functions they perform, are all extremely diverse.[1] Thus, to date, any underlying principles that organisms may use in the process of mineralization, are poorly understood. Despite the enormous amount of information already available on biomineralization, the field is still primarily at the stage of "data gathering". Even the relatively simple documentation of the approximate number of different mineral types formed by organisms is still far from complete.[2] The far more difficult process of identifying, characterizing, and understanding the molecular organization of the all-important biological macromolecules that are closely associated with mineral deposits, is just beginning. However, this most formidable task will undoubtedly be greatly facilitated by the powerful newly developed molecular biological techniques for manipulating genes and their products. We can, therefore, anticipate a great surge of information on these macromolecules in the near future. The gathering, processing, and comprehension of these data will inevitably be influenced by contemporary concepts of mineralization processes and mechanisms. This comparative review of different mineralized tissues therefore deliberately emphasizes the structural and organizational aspects of these tissues and not the biochemistry of the macromolecular constituents. It will hopefully provide a useful frame of reference for assessing in the future, the functions of the important macromolecules through which the cells exercise this control.

Five very different mineralized tissues are reviewed. Most significantly all the tissues are analyzed in a uniform and systematic manner, in order to achieve what I regard as the major objective of this review: to show that the processes involved in the mineralization of these tissues are all part of one continuum[2,3] in which the degree of control exercised over crystal growth varies. Thus, insights gained from the understanding of an aspect of crystal formation in one tissue can be applied to another tissue. A second objective of this review is therefore to provide a convenient reference source for information on the mineralized tissues with which the reader may be less familiar.

The five different mineralized tissues that I have chosen to analyze differ widely in the degree to which they control various aspects of crystal growth. The five tissues are the mollusk shell nacreous layer, mammalian tooth enamel, mammalian bone, foraminifera with radial and granular shell wall structures, and extracellularly mineralized algae. Each tissue is reviewed in a different section in the order listed above. The tissues in which crystal growth is well controlled are arbitrarily dealt with first. This provides the reader with a more precise understanding of the various topics discussed and a better appreciation of the significance of those aspects of crystal formation that are absent in tissues in which crystal growth is less well controlled. Each section covers the same list of topics, which is shown

Table 1
THE LIST OF TOPICS COVERED FOR EACH OF THE FIVE MINERALIZED TISSUES REVIEWED

- Organizational motif
  - Mineral-organic distribution
  - Mineral-organic proportions
  - Tissue site of mineralization
  - Preformed organic structural framework
- Mineral phase
  - Mineral type
  - Crystal shape
  - Crystal size
  - Orientation of crystallographic axes
- Organic phase
  - Framework macromolecular constituents
  - Acidic macromolecules
  - Macromolecular conformations and ion-binding properties
  - Matrix-mineral relations
- Stages of mineral formation
  - First-formed precipitate
  - Growth of crystals
  - Mineral maturation
- Inferences with regard to crystal formation
  - Crystal nucleation
  - Crystal growth modulation

in Table 1. This uniform "treatment" avoids biasing the review of each tissue to the more popular topics discussed in the literature and helps to highlight the gaps in our knowledge. Most of the topics listed in Table 1 are self-explanatory. In the section on organic phase, the differentiation of the macromolecules associated with the mineral phase into "frame work" and "acidic" follows the reasoning of Weiner et al.[3] and Weiner [4] and is outlined briefly below. The framework macromolecules, when present, tend to be somewhat hydrophobic as well as highly cross-linked and hence usually remain insoluble after the mineral is dissolved by some mild process. The biochemical nature of these macromolecules varies greatly from tissue to tissue. The acidic macromolecules are present in all mineralized tissues for which data are available. There are at least two quite different classes of acidic macromolecules; (glyco-) proteins rich in aspartic and/or glutamic acid and in some cases, certain phosphorylated amino acid residues as well, and secondly proteins rich in serine and sometimes glutamic acid (or glutamine) and glycine. The latter are associated with relatively large amounts of polysaccharide and are possibly all of the proteoglycan type. I do not attempt to review all that is known about the biochemistry of the macromolecules in each tissue, but limit the discussion to identifying the class to which the macromolecules are most likely to belong.

In the last section of the topics discussed entitled "Inferences with Regard to Crystal Formation" (Table 1), the discussions on crystal nucleation and crystal growth modulation are separated, as these two stages of crystal formation need not necessarily be linked to each other.[5,6] For each tissue an attempt is made, based on my assessment of our current state of knowledge, to identify the nucleation and modulation process utilized by the cells through the proxy of their specialized macromolecules to control crystal growth. The following is a brief description of the different nucleation and modulation processes that I differentiate between. Many of the ideas and concepts are taken from Addadi and Weiner.[6] For more detailed discussions of crystal nucleation and modulation in the biological context, see Williams,[7] Mann,[8] and Simkiss.[9]

A. Nucleation

Nonspecific nucleation — Nucleation occurs on a random inorganic particle suspended in solution, or on a molecule or macromolecule deliberately introduced into the crystallizing solution, or on a nonspecific surface. The crystal so formed has no well-defined structural relationship to the nucleating substrate.

Stereochemical surface nucleation — The in vitro experiments performed by Addadi and Weiner[6] show that oriented crystal growth can occur on a charged surface as a result of the stereochemical requirements associated with optimally completing the coordination polyhedron around a cation bound to the surface. The crystal orientation is fixed in one direction; namely, perpendicular to the plane of the substrate. It is assumed, but not yet proven, that the crystal axes in the plane of nucleation are not aligned with some structural parameter of the nucleation surface.

Epitaxial nucleation — The substrate uniquely orients the crystal in space by first binding ions to its surface in a highly specific configuration. Epitaxial nucleation can be identified by demonstrating that the crystal formed is spatially oriented in a specific manner to the substrate and by showing that the lattice dimensions of the interacting surfaces match.

B. Crystal Growth Modulation

This refers to any interference in the crystal growth process following nucleation by foreign molecules or macromolecules.

Absent — Once nucleated, the crystal grows as it would in a pure saturated solution. Crystal shape and size is determined, among other things, by the space available for growth.

Adsorption on growing surfaces — Foreign molecules or macromolecules in solution can bind with higher affinity to some crystal faces as compared to others.[10] If this occurs during crystal growth, the morphology of the affected crystals will change. Addadi and Weiner[6] show that when calcium-loaded acidic proteins in the β-sheet conformation are dissolved in solution, they will preferentially interact with crystal faces which have certain stereochemical properties and as a result they specifically affect the morphology of the growing crystals.

Prepositioned modulation surfaces — Crystal growth in a specific direction can be inhibited when the crystal comes into contact with a prepositioned surface. This will occur if the functional groups of the surface molecules are able to bind to the crystal structure and thus disrupt any further growth.

It should be emphasized that the processes of crystal nucleation and modulation listed above do not necessarily include all the possible ways by which organisms form minerals. For a stimulating review of some other possible ways that organisms may regulate mineral formation, see Mann.[8] An important example of a widespread phenomenon in biomineralization that probably does not fall into the realm of processes discussed in this review, is the formation of minerals within lipid membrane-bound vesicles. It is quite conceivable that the factors that govern mineralization in this unique chemical environment are quite different from those associated with macromolecular structures.[11] Another prominent phenomenon in biomineralization that this review may not be directly relevant to is the formation of amorphous minerals. These constitute a major group of biologically formed minerals.[1,2] The fact that they lack long-range atomic order, may mean that the matrix macromolecules that regulate their formation and possibly even their stabilization, function differently from those involved in crystalline mineral formation. For a more detailed discussion of biogenic amorphous mineral formation, see Williams[7] and Mann.[8]

The cells that orchestrate the whole mineralization process are only alluded to indirectly in this review. This does not in any way reflect on the importance of the roles they play in controlled biomineralization. Throughout this discussion it is assumed that the cells exercise direct control over crystal formation by introducing the macromolecules into the minerali-

zation site in an appropriate order at the appropriate times and by closely regulating the influx of the various ions necessary for precipitation to occur.

II. MOLLUSK SHELL NACREOUS LAYER

The nacreous layer is one of seven different microarchitectural constructions used by mollusks to form their shells.[12] Mollusk shell structures have been investigated for more than a century (reviewed by Wise[13]) and the first definitive study is that of Schmidt[14] in 1923. From the organizational point of view, the nacreous microarchitecture is the simplest of all the seven forms. Its remarkably uniform and geometrically regular arrangement of organic and mineral phases (see next section) makes this shell layer particularly attractive for studying the organization of the shell constituents at the molecular level. In this respect it is certainly the best characterized mollusk shell microarchitectural arrangement and from the ultrastructural point of view, possibly the best understood mineralized tissue to date. For a recent comprehensive review of mollusk shell formation in general, see Wilbur and Saleuddin.[15] Note also that the information presented is restricted to nacre in bivalves, gastropods, and the cephalopod, *Nautilus*.

A. Organizational Motif

Mineral-organic distribution — The nacreous microarchitecture is made up of uniformly thick laminae or layers of mineral (Figure 1A) separated by much thinner layers of organic material[16-18] (Figure 1B). Each mineral layer is composed of a pavement or mosaic of single crystals (Figure 2). Some organic material is also present at the junctions between adjacent crystals[18,19] and possibly even within the mineral phase itself.[20,21] Broken sections of bivalve nacreous layers reveal a characteristic "brick-wall construction",[14,16,17] whereas in gastropods the texture is characterized by a regular stacking of the crystals[13,22] (see Wise[13] for excellent illustrations). The reason for this difference is discussed in Section E.

Mineral-organic proportions — The organic constituents of nacreous shell layers comprise between 1 and 5% by weight of the dry shell.[23]

Tissue site of mineralization — The mantle is the organ responsible for the formation of all the different elements that together constitute the shell. The nacreous layer is, as a rule, formed by certain cells located on the dorsal epithelium of the mantle. These cells synthesize and secrete the organic macromolecular constituents into the extracellular space between the shell surface and the outer epithelium.[15,24] Here they self-assemble to form the structural framework in which the crystals subsequently nucleate and grow.[25] The exact relationship of the cells to the underlying shell structure is not known.

Preformed structural framework — Bevelander and Nakahara[25] and Nakahara[26,27] have shown that a number of these interlamellae sheets are formed prior to the onset of crystal growth and are in place at distances from each other that approximate the width of formed mineral layers during crystal growth (Figure 3B). Watabe[28] has interpreted the micrographs of Nakahara[27] differently. He envisages the regularity of the shell structure arising from a rhythmic alternating deposition of mineral and matrix. Although there is no conclusive evidence to refute one or other of the explanations, there are good precedents in mollusks (for example, in the formation of the radular teeth of the chitons[29,30]) for entire organic structures to be formed prior to the introduction of mineral; an observation more consistent with Nakahara's[26,27] interpretation.

B. Mineral Phase

Mineral type — The mineral of all nacreous shell layers is aragonite;[12,14] one of the three crystalline polymorphs of $CaCO_3$ that crystallizes in the orthorhombic crystal system.

Crystal shape — All the crystals in the mature shell have basically the same shape,

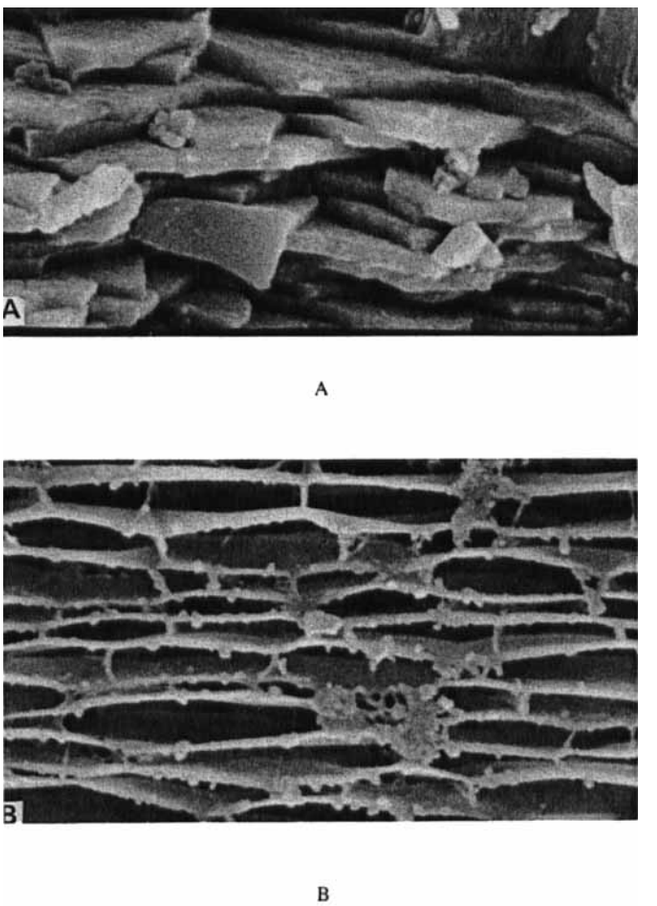


FIGURE 1. (A) Fractured section of the bivalve, *Mytilus californianus* nacreous layer showing uniformly thick layers of aragonite. (Magnification × 10,000.) (B) Same section as in Figure 1A prepared by polishing, etching, and then critical point drying. This shows the presence of interlamellae organic matrix sheets between the mineral layers as well as thin vertical sheets which separate individual crystals within a layer. (Magnification × 10,000.) (Courtesy of G. R. Clark II, Kansas State University.)

namely flat irregular polygons[31] which are usually six-sided[13] (Figure 2). Significantly, the boundaries between the polygons are linear and smooth (Figure 2). This is in contrast to the boundaries between calcite crystals in foraminiferal shells (see Section V.B) where the interface is sinuous or serrated, suggesting that intergrowth between crystals has occurred.

Crystal size — For a given species the thickness of the crystals is remarkably uniform (Figures 1, 2, and 3). In different species, the crystal, and hence mineral layer, thickness does differ.[19] The range of crystal thicknesses observed in different species is between 0.4 and 1.2 μm.[19,27,32] The diameters of the crystal polygons are much more variable than their thicknesses. In fact, the polygons vary significantly in diameter even within a single mineral layer.[31] The range of maximum polygon diameters in different species is from a few microns up to about 13 μm.[32]

Orientation of crystallographic axes — In all nacreous layers the c axes of the aragonite crystals are oriented perpendicular to the plane of the organic sheets.[14,17,19] The a and b crystallographic axes are therefore in a plane parallel to the organic sheets. X-ray diffraction

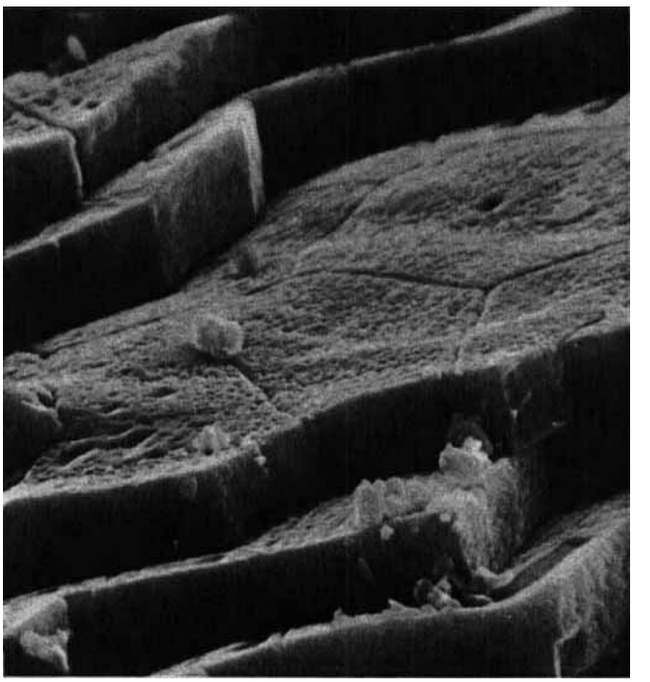


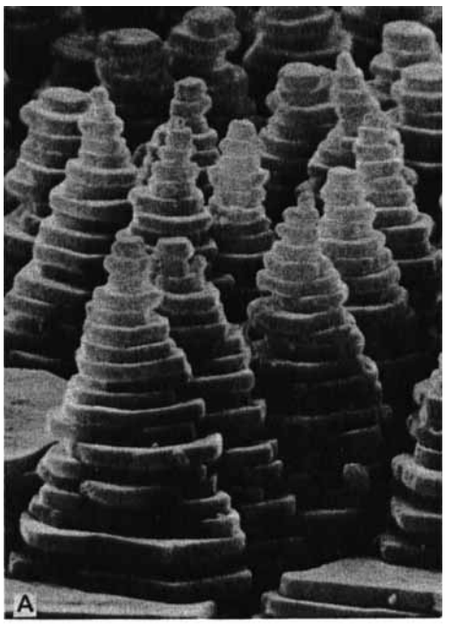
FIGURE 2. Fractured section of the bivalve, *Mytilus californianus* nacreous layer showing the polygonal-shaped crystals which make up an individual mineral layer. (Magnification × 7,000.)

patterns of gastropod nacreous layers show that there is no preferred orientation of the a and b axes of the crystals present in an area of approximately a few thousand square microns — the usual diameter of X-ray beams.[13] In contrast, some bivalves and the cephalopod, *Nautilus*, do show some preferred orientation of the a and b axes.[13] This may be barely discernible (e.g., *Mytilus californianus)*, fairly well defined (e.g., *Nautilus repertus)*, or even perfect such that the X-ray diffraction pattern is close to that of a single crystal (e.g., *Pinctada margaratifera* and *Neotrigonia margaritacea).*[33,34]

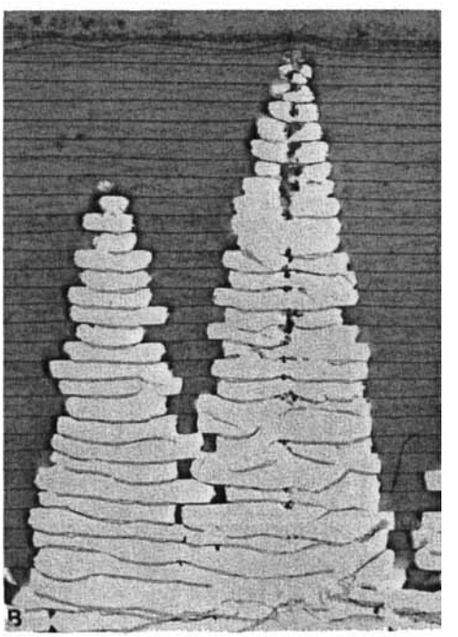
C. Organic Phase

Transmission electron micrographs of stained sections of individual matrix sheets show that these may be composed of up to five different layers[27] (Figure 4). The surface layers are electron dense and are composed primarily of a complex assemblage of acidic macromolecules. The three core layers together constitute a relatively insoluble complex — the organic matrix framework.[35,36] For a more detailed discussion, see Weiner and Traub.[36] In some matrix sheets, the framework layers appear to be absent or present in very small amounts[26] (Figure 4B).

Framework macromolecular constituents — The thin layer at the core of the matrix sheet is composed of chitin.[27] Although Nakahara[27] has not identified the chitin layer in bivalve nacreous layers using TEM, chitin is apparently present in all mollusk shells.[37] The quantitatively abundant constituents of the framework complex are proteins characteristically rich in glycine and alanine.[18,38] The whole complex is cross-linked,[39,40] and as a result very little is known about the biochemistry of the individual macromolecules. In TEM the sheets have a lace-like texture with the pore shapes varying in different taxonomic groups.[18]



A



B

FIGURE 3. (A) Scanning electron micrograph of the growing surface of the nacreous layer of the gastropod, *Monodontia labio*, showing how new crystals form at the same site in consecutive layers. The crystals rapidly achieve their maximum thickness and then grow laterally to eventually form a uniformly thick mineral layer. (Magnification × 4,500.) (B) Transmission electron micrograph of an embedded and sectioned portion of the same growing surface shown in Figure 3A. The evenly spaced organic matrix layers are clearly seen, as well as three additional layers which are presumably in the process of formation. It is not known to what extent this latter structure is affected by sample preparation procedures. (Magnification × 4,500.) (Courtesy of H. Nakahara, Josai Dental University, Japan.)

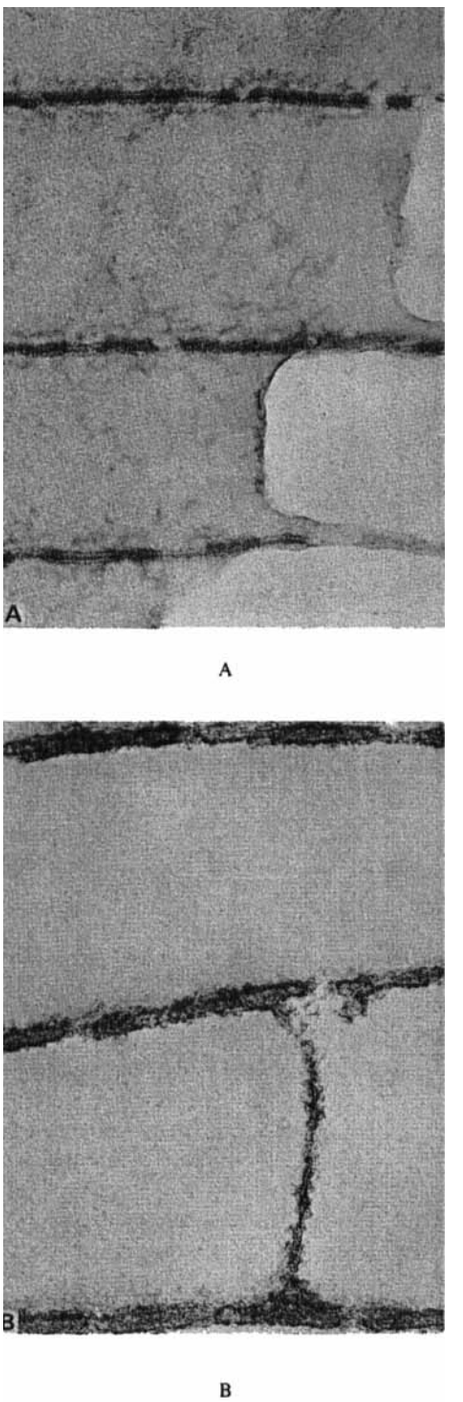


FIGURE 4. (A) Decalcified and double stained section of the nacreous organic matrix of the gastropod, *Tegula pfifferi*, showing the five-layered structure of the interlamellar sheets referred to in the text. (Magnification × 75,000.) (B) Decalcified and double stained section of the nacreous organic matrix of the gastropod, *Turbo cornutus*, showing that the vertical organic matrix sheet (intercrystalline matrix) may only be composed of a double layer. (Magnification × 90,000.) (Courtesy of H. Nakahara, Josai Dental University, Japan.)

Acidic macromolecules—These are present in the surface layers of the matrix sheets,[35] and at least some of them are therefore in direct contact with the mineral phase.[3,35,36] These hydrophilic macromolecules rapidly dissolve when the mineral is removed using EDTA.[21] Chromatographic analyses show that many macromolecules are present.[41,42] At least two quite different classes of macromolecules can be identified.[4,43] Proteins rich in aspartic acid (usually 25 mol percent or more) which are possibly associated with relatively small amounts of polysaccharide constitute the major class of acidic matrix components.[44] Some of these have been purified using reversed phase liquid chromatography.[45] Analyses of partial acid hydrolysates show that sequences in which aspartic acid residues are separated by one or a few particular amino acids are common.[46] Other repeating amino acid sequences which, in certain species, are unique to the nacreous layer have also been identified.[47] The second class of acidic macromolecules are composed of proteins rich in serine (about 20 mol percent) and often glutamic acid and/or glutamine (about 14 mol percent), as well.[4,43] These appear to be associated with relatively large amounts of polysaccharide.[43] Some of the polysaccharides are probably bound to the protein by means of *O*-glycosidic linkages, known to be present in the EDTA soluble fraction of the bivalve, *Mercenaria mercenaria*.[3]

Macromolecular conformations and ion-binding properties — X-ray[48] and electron diffraction[49] patterns of the insoluble framework constituents show that the chitin is in the β-form and the proteins adopt the antiparallel β-sheet conformation. The d-spacings of the protein reflections are very similar to those obtained from insect silk-fibroin.[48] Furthermore, the mean orientation of the chitin fibrils is perpendicular to the mean orientation of the protein polypeptide chains.[48,49] Thus, the three layers of the insoluble core have a plywood-like construction which probably contributes significantly to its mechanical properties.[36] The acidic macromolecules of the surface layers are avid calcium binders.[21,40,50-52] Upon binding calcium, the aspartic acid-rich proteins adopt the β-sheet conformation.[43] The serine-rich protein-polysaccharide complexes also concentrate calcium, but nothing is known about their conformations.[43] Infrared spectroscopy shows that the carboxylate groups of the acidic proteins are major calcium binding ligands.[43] Sulfate groups in the EDTA soluble fraction of the clam *Mercenaria mercenaria* are known to bind calcium.[21,51] These are presumably associated with the polysaccharides. The carbohydrate groups of the proteins and the sulfate groups of the polysaccharides (?) probably associate with calcium in quite different ways.[52]

Matrix-mineral relations — X-ray [48] and electron diffraction[49] patterns obtained with the beam perpendicular to the matrix sheets and parallel to the aragonite c axes, show that the mean orientation of the chitin fibrils and the protein polypeptide chains of the core layers are well aligned with the a and b axes of the aragonite crystals. This specific matrix-mineral relation has been observed in a cephalopod, a gastropod, and a bivalve and is presumably common to the nacreous layers of all mollusks.[49] Nothing is known about the relative orientations of the acidic macromolecules in relation to the crystals, although it is reasonable to assume that at least some of them (in the nucleation site?) also have a well-defined relation to the adjacent crystal lattice.[36]

D. Stages of Mineral Formation

First-formed precipitate — Nothing is known about the first-formed precipitate, although it has been postulated that it would probably be amorphous.[52]

Growth of crystals — The first identifiable crystals of aragonite using SEM are elongated along their c axes (unpublished observation). They grow rapidly to their maximum height[32] as determined by the prepositioned next matrix layer and then grow laterally until they merge with adjoining crystals.[25,26,32] An outstanding study by Wise[13] documents the various crystal growth stages of several gastropods, bivalves, and the cephalopod, *Nautilus*. In gastropods, the nucleation sites of each successive layer are located directly above those of the previously formed layer [53] (Figure 3). In bivalves, there are no such obvious relations.[13] The growing

crystals are usually elongated in some direction. In bivalves and *Nautilus*, a preferred orientation of the crystals over large areas is observed whereas in gastropods there is no preferred orientation.[13] These observations are consistent with the X-ray diffraction information pertaining to the preferred orientations of the a and b crystallographic axes.[13,48] Wada[32] noted that the growing crystals of two bivalves have different shapes in different seasons, which correlated with the rate of shell deposition. It is not known whether the lateral boundaries of each crystal are determined by a prepositioned matrix sheet or by adjacent growing crystals coming into contact with each other. In the latter case, the organic material present between crystal polygons may be trapped by the growing front.[25] These vertical intercrystalline matrix sheets in one species of gastropod do not appear to have any internal five-layered organization such as is observed in the interlamellar matrix sheets[26,27] (Figure 4B). Insufficient information is available to distinguish between these possibilities.

Mineral maturation — There is no evidence showing that the crystallinity or any other property of the mineral changes with time, although this has not been specifically excluded.

E. Inferences with Regard to Crystal Formation

1. Crystal Nucleation

Crystal nucleation is well controlled by the organic matrix— The specific spatial relations between the core macromolecules and the a and b axes of the associated crystals strongly suggests that crystals form by epitaxy upon the matrix substrate.[49] Until it is shown, however, that the lattice dimensions of the two interacting surfaces of the mineral and the matrix at the site of nucleation are matched; this cannot be confirmed.

Nucleation occurs at a specific site on the matrix surface— Perhaps the best evidence for this is the "stack-of-coins" mode of growth of gastropod nacre which is a result of the fact that nucleation of crystals in successive layers occurs at the same location.[13] An examination of the growth stages of individual crystals in nacre shows that the nucleation sites must be on one or other matrix surface at a point below the center of the crystal polygons. In fact, Crenshaw and Ristedt[54] showed by histochemical staining that the surface properties of the matrix at this site differs from the surrounding areas — an observation consistent with the existence of a specific nucleation site location.[36] A speculative discussion of the nucleation site of mollusk nacre is given in Weiner and Traub[36] in which it is proposed that acidic proteins are constituents of the nucleation site. These adopt the β-sheet conformation and bind calcium in such a way as to induce oriented aragonite crystals to form by epitaxy.

2. Crystal Growth Modulation

The thickness of individual crystals is determined by the location of a prepositioned matrix layer—The most striking feature of mollusk nacre is the remarkably uniform thickness of individual mineral layers. This property must therefore be exceedingly well controlled. The observations of matrix layers preformed at appropriate distances prior to crystal formation in gastropods, supports this conclusion.[26,27] Presumably, the acidic macromolecules on this matrix surface inhibit further crystal growth in that direction. One implication of this "scenario" of crystal growth is that the two surfaces of an individual matrix sheet should be different — one surface containing a nucleation site and the other primarily functions to prevent further crystal growth in the direction perpendicular to the c axis.

Crystals grow primarily by lateral enlargement—Once they have attained their maximum thickness, the surfaces of the matrix sheets over which the crystals grow laterally are also presumably covered by macromolecules which prevent crystal growth in the c axis direction. As the shapes of the growing crystals are different for different species[32] (see illustrations in Wise[13]), some modulation of growth by molecules in solution adsorbing onto growing crystal faces aligned parallel to the c axis, may occur. We do not know what determines the lateral boundaries of the crystal polygons.

III. MAMMALIAN TOOTH ENAMEL

Tooth enamel is one of the most complex and highly ordered mineralized tissues known. Whereas most mineralized tissues are built up of one or two repeating units (crystals or groups of crystals and their associated matrix), enamel is sometimes composed of three such repeating units — two sets of rods or prism-shaped structures and interrod enamel[55] (see next section). Furthermore, many of the organic macromolecules which are present during the early stages of mineralization are broken down and removed during the later stages.[56] This added complexity superimposed on the already complex ultrastructure makes the prospect of studying enamel formation a most formidable task.

Fortunately, the growth stages of teeth in the process of forming are fairly well separated from each other and as a result, can often be analyzed separately. Continuously growing teeth are even more convenient as even in the adult animal all the stages of mineralization are present in one tooth. For this reason the continuously growing incisors of rats have been extensively studied.

In this section I will emphasize the processes involved in the formation of the mineral phase, rather than the processes involved in the breakdown and removal of the bulk of the organic macromolecules during the later stages of tooth formation. The latter phenomenon although not unique to enamel, is not known to be commonplace in biomineralization. It should be noted that organic matrix loss on a scale comparable to enamel is thought to occur in mineralized coral skeletons.[57]

In any discussion of enamel it is important to stipulate the developmental zone from which the sample analyzed was derived. Probably the most practical definition of developmental zones or stages in enamel is that of Robinson et al.[58] for the rat incisor. It is, however, applicable to forming teeth in general:[59] zone 1, from the root apex to a point half-way towards the opaque boundary; zone 2, from this mid-point to the opaque boundary; zone 3, the enamel located beyond the opaque boundary — up to a point where it becomes too hard to cut with a scalpel; and zone 4, mature enamel too hard to cut with a scalpel. The problem is that not all literature reports use these zone definitions. In this section I will refer to these zones wherever possible. In the absence of specific information, I will use the terms *forming*, *maturing*, and *mature* to roughly correspond to zones 1 and 2, zone 3, and zone 4, respectively. For an excellent overview of the ultrastructural aspects of enamel formation, see Warshawsky.[60]

A. Organizational Motif

The basic organizational motif of many, but certainly not all enamel structures is shown in Figures 5 and 6. The two sets of rods are in the same plane and the interrods are perpendicular to them. Each rod and interrod is composed of hundreds of elongated crystals.[55,61] In rat incisors the whole structure is tilted so that the plane in which the rods are located forms an angle of up to 30° to the outer surface of the tooth.[62] This ultrastructural arrangement represents the major bulk of the enamel. The zone adjacent to the dentin layer and the area immediately below the tooth surface have somewhat different microarchitectures.[55,63,64] For a more detailed description of rat enamel, see Skobe[64] and for variations in enamel microarchitecture between species, see Boyde.[55,63]

Mineral-organic distribution — The crystals that make up the rods and interrods are intimately associated with organic material. Two basically different types of organic material are differentiated by their appearance in stained sections in the TEM. The one form is generally described as granular or stippled and has no conspicuous ultrastructural form.[65] It is most abundant in forming enamel in the spaces not yet occupied by the rods and interrods. The second form of organic material is more closely associated with the crystals themselves[66] and as a result upon demineralization using appropriate fixatives, this organic material is

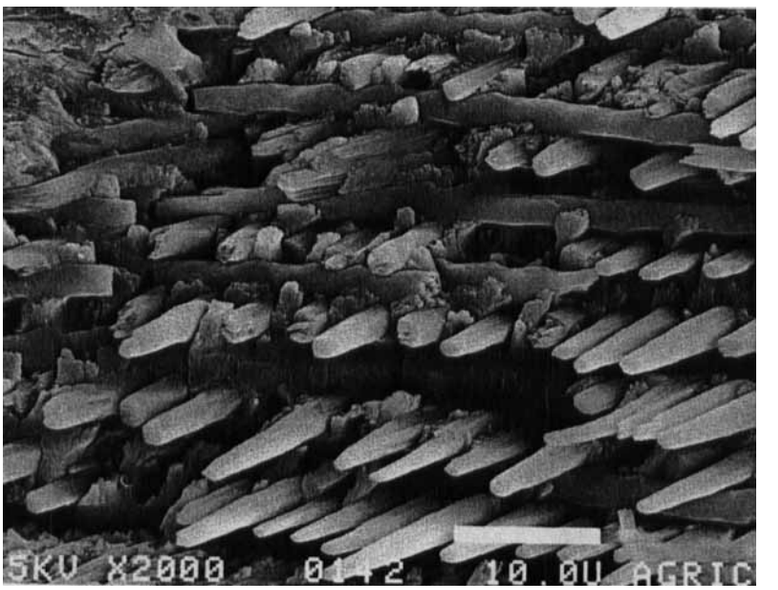


FIGURE 5. Scanning electron micrograph (SEM) of the enamel of relatively mature (zone 3) rat incisor showing the two sets of rods in the horizontal plane of the photograph, and the interrod enamel perpendicular to the rods. (Magnification × 2,000.) (Courtesy of A. Jodaikin, Weizmann Institute, Israel.)

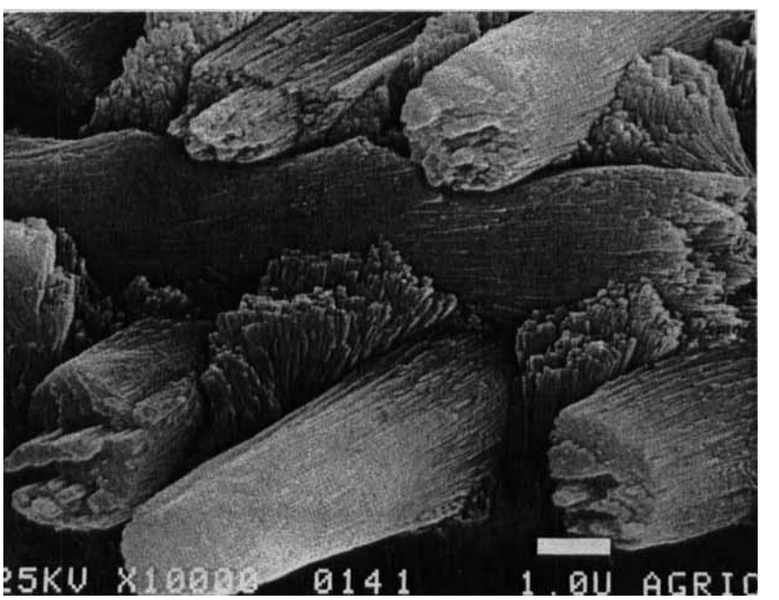


FIGURE 6. Scanning electron micrograph of the rat incisor enamel (zone 3) showing how the rods (in the horizontal plane of the photograph) and the interrods are composed of well-aligned, highly elongated crystals. (Magnification × 10,000.) (Courtesy of A. Jodaikin, Weizmann Institute, Israel.)

Table 2
THE RELATIVE PROPORTIONS OF MINERAL, ORGANIC COMPONENTS AND WATER IN DEVELOPING ANIMAL AS COMPILED BY GLICK [70]

Stage of mineralization	Mineral		Organic		Water	
	Wt. %	Vol. %	Wt. %	Vol. %	Wt. %	Vol. %
Early developing (zones 1 and 2?)	32	15	30	32	38	52—58
Late developing (zone 3?)	70	44	2	3	28	52—58
Mature (zone 4?)	95	87	1	2	4	11

observed to envelope each of the individual crystals.[67-69] These envelopes or sheaths are usually described as "tubules".[69]

Mineral-organic proportions — Because components of the organic material are removed during the development of the enamel[55] and at the same time the crystals are increasing their mass, the measurement of mineral-organic proportions is exceedingly complex. Glick[70] has tabulated some of this data from a variety of sources in a most informative way. Table 2 represents part of his tabulation. It is pertinent to note that in the earliest formed matrix (probably zone 2) the mineral, by volume, is only a small component when compared to the organic constituents and water. Furthermore, it has been suggested that during development the organic material is replaced by water, which in turn is replaced by mineral.[59,71,72]

Tissue site of mineralization — The ameloblasts, the cells responsible for the formation of enamel, envelope the outer surfaces of the forming tooth.[73] The cells are elongated and closely packed, showing a hexagonal cross-section. The part of the cell in contact with the mineralizing zone, called the Tomes process,[74] is highly invaginated. The newly formed crystals are aligned more or less perpendicular to the surfaces of the Tomes process.[75,76] An anorganic preparation of the surface of newly formed crystals in the fetal teeth of kittens shows a remarkably regular arrangement[77] (Figure 7) which in many ways must reflect the ordered arrangement of the Tomes processes themselves. It is of interest to note that the indented areas in Figure 7 are the most distal ends of the rods, whereas the elevated areas are the ends of the interrods.[77] Each rod is formed by one ameloblast and each interrod represents the combined activity of two or more ameloblasts.[55,76,78]

Preformed structural framework — A puzzling issue in enamel formation is whether or not a preformed structural framework exists into which the crystals grow. TEM observations of the mineralizing front of individual rods in mammalian enamel generally show that the crystals abut or are very close to the plasma membrane of the Tomes processes,[75,79] giving the impression at least, that no room exists for a preformed extracellular framework to exist. In sharks, however, preformed tubules have been observed in the zone close to where mineralization is taking place.[80-82] Decker [83] did notice in rat enamel that in undemineralized TEM sections, some tubules close to the cell processes do not contain crystals, whereas at greater distances from the cell, all tubules contain crystals. He did not, however, cite these observations as evidence for a preformed structural framework, but thought that they resulted from an artifact of tissue preparation.

B. Mineral Phase

Mineral type — The mineral of enamel was first identified as belonging to the apatite group by Hoppe[84] in 1862. It is now known to be hydroxyapatite, which crystallizes in the hexagonal system. In addition to calcium and phosphate, significant quantities of carbonate (1.95 to 3.66 weight percent CO_2) are also present.[85]

Crystal shape — All mammalian enamel crystals are exceptionally long relative to their cross-sectional dimensions (Figure 6). In fact, Daculsi et al.[86] recently observed crystals more than 100 μm long; an observation consistent with an earlier suggestion that individual crystals

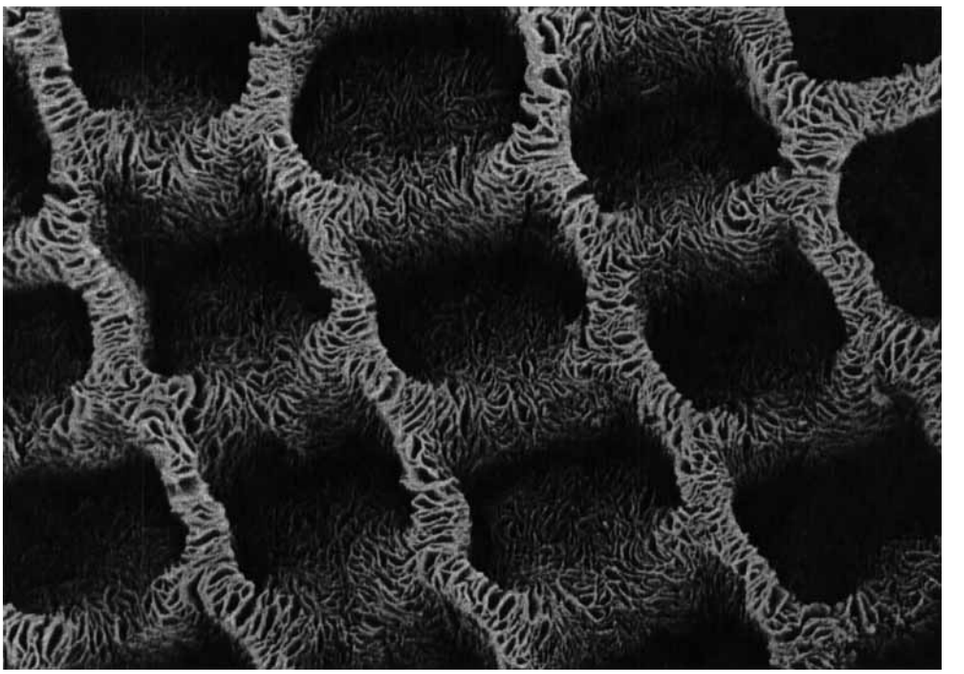


FIGURE 7. Scanning electron micrograph of an anorganic preparation of the developing surface of the enamel of a cat tooth, showing a series of indented areas (holes) which were occupied by the Tomes processes of secretory ameloblasts. The shapes of the holes represent the gross configurations of the Tomes processes. (Courtesy of T. Sasaki, Second Department of Oral Anatomy, Showa University, Japan.)

are continuous from the dentino-enamel junction to the tooth surface.[87] The cross-sectional morphology of the crystals varies with tooth development (see later section). In zones 1 to 3 the individual crystals are well separated from each other and have very regular hexagonal morphologies (particularly in zone 3).[88] The faces expressed are all (100) faces.[89] One pair of crystal faces has a larger surface area than the others, causing the crystals to be somewhat flattened.[90] It is generally thought that the cross-sectional shape is hexagonal and not rectangular as has been suggested,[60,87] as lattice images prove that crystals observed with their c axes exactly perpendicular to the electron beam, do have hexagonal outlines.[89] This assumes that the c axis is parallel to the long axis of the crystal. Hirai[91] has reported otherwise (see below). In mature enamel (zone 4), additional crystal growth results in adjacent crystals fusing and as a result having very irregular cross-sectional morphologies.[89,92] It is intriguing to note that the cross-sectional shapes of forming shark enameloid crystals are equilateral hexagons and not the flattened hexagons observed in mammals.[80-82] A central dark line has been repeatedly described in enamel crystals.[93-96] It is apparently not an artifact of the method used for observation, although its physical significance is still not clear. If indeed, as has been suggested,[75,96] this represents the site of initial crystal growth, it is of paramount importance to fully understand this phenomenon.

Crystal size — The lengths of enamel crystals are difficult, if not impossible to measure in sectioned material. By dispersing the crystals of forming human enamel and then allowing them to settle on a grid for TEM examination, Daculsi et al.[86] showed that the crystals are very long, even exceeding 100 μm. The cross-sectional dimensions of maturing crystals (prior to crystal-crystal contact) are more reliably measured in carefully oriented thin-sections. Nylen et al.[90] report that rat enamel crystals are 500 to 600 Å wide and 250 to 300

Å thick. Bovine enamel is reported to have crystals 400 Å wide and 150 Å thick.[93] In their measurements of human enamel crystals, Daculsi et al.[86] report cross-sectional dimensions of 683 ± 34 Å and 263 ± 22 Å.

Orientation of crystallographic axes — The c axes are generally thought to be aligned with the long axes of the crystals and these, in turn, are preferably oriented with the long axes of the rods and interrods.[90] It is of interest to note that Hirai[91] reported that the c axis actually can diverge by as much as 2.2° from the long axis of the crystal. Within a rod, the c axes can vary in orientation by approximately ±25° about their mean orientation and the interrod c axes orientations can vary by as much as ±45° or so about the mean.[62] In apatite, the a axes are oriented perpendicular to the c axis in individual crystals. Within a rod, the a axes of the assemblage of crystals have no overall preferred orientation, although clusters of neighboring crystals can be locally oriented with respect to each other.[75]

C. Organic Phase

The organic macromolecular constituents of enamel are relatively difficult to characterize because they degrade into fragments and are removed from the tissue during development[59] — a process which has been well documented and will not be reviewed here. A significant advance in characterizing the intact macromolecules prior to degradation, was made by Termine and co-workers[97] who introduced a simple and reproducible technique to separate the two major classes of proteins present in enamel — the amelogenins and the enamelins, as defined by Eastoe.[98] Most of the amelogenins from forming enamel can be extracted with a denaturing agent (guanidine hydrochloride), whereas the enamelins can only be extracted by dissolving the mineral.[97] This empirical fact, supported by direct immunochemical observations,[99] strongly suggests that the enamelins are intimately associated with the mineral phase whereas the amelogenins are not.[97] The implication is that the amelogenins are primarily responsible for the stippled material observed in the TEM,[78] and the enamelins are thought to be constituents of the tubules.[58] Both classes of organic macromolecules are present in forming enamel and both classes degrade during maturation.[97] The amelogenins are, however, preferentially removed from the tissue as compared to the enamelins which persist even into mature enamel[97] (zone 4). See recent reviews by Fincham and Belcourt,[101] as well as Robinson and Kirkham.[59]

Structural framework constituents — In most mineralized tissues the structural framework is identified as the insoluble fraction which remains after the removal of the mineral. The framework molecules are usually somewhat hydrophobic and are ordered to some degree.[34] Significantly, not all mineralized tissues have such a structural framework[3] and it is possible, but certainly not proven, that enamel belongs in this category. It should be noted that some enamel organic material is insoluble after EDTA decalcification, although it is hydrophilic and not hydrophobic in nature.[102,103] The *amelogenins* are certainly candidates for fulfilling some indirect structural function[104] during crystal formation. They are hydrophobic (containing 25 to 30 mol percent Pro, 15 to 20% Gln, 8 to 12% Leu, and 6 to 9% His),[105] they do constitute the bulk of the forming enamel protein and they are not closely associated with the mineral phase.[97] Their stippled or granular appearance in TEM does not appear to relate in any obvious way to the crystal fabric and to date it has not been shown that the amelogenins adopt a regular ordered conformation (Jodaikin, Worms, Weiner, and Traub, unpublished results). The complete amino acid sequences of the major and possibly even the only amelogenin macromolecule is known from pig,[106] cow,[107] and mouse,[108] but provides no direct clue to its structure or function. It contains up to 180 amino acids and some of its characteristic features are the frequent appearance of double residues such as Pro-Pro, Gln-Gln, His-His, etc. and the repeat of a triplet Gln-Pro-Leu. Perhaps one of the most important facts about the amelogenin protein which suggests that it does have a specific, structure-dependent function, is that the N-terminal residues are highly conserved in three mammalian species.[106-109]

Acidic macromolecules — In contrast to the amelogenins, little is known about the acidic macromolecular constituents of enamel, the enamelins. Asp, Ser, and Glu each constitute about 10 to 12 mol percent of the average amino acid composition, and Gly constitutes 15 to 20%. The molecular weight range of the enamelins is between 50,000 and 70,000 daltons, with two quantitatively abundant components having molecular weights of 52,000 and 56,000. These proteins are also associated with relatively large amounts of polysaccharides.[97,103] It is of interest to note that the enamel of the teeth of the blue shark contains only enamelins (referred to as enameloid). The amelogenins appear to be absent.[110]

Macromolecular conformations and ion-binding properties — X-ray diffraction has been used to study the enamel organic matrices from the forming and maturing zones. Most of these studies show that at least a subset of the matrix macromolecules give rise to an X-ray diffraction pattern which contains a prominent 4.7 Å reflection. This reflection is indicative of the presence of proteins adopting the β-sheet conformation.[111-114] The recent studies of Jodaikin and co-workers[115] show that the 4.7 Å reflection is present in samples subjected to a wide variety of preparation procedures, thus dispelling doubts raised about the validity of the earlier observations.[116] Furthermore, the 4.7 Å reflection was strongest in zone 3 as compared to zones 1 and 2, suggesting that the enamelins are responsible for the reflection as in this zone the amelogenins are only a minor constituent. Very little is known about the conformation of the amelogenin protein(s), nor about the specific ion-binding properties of enamel matrix macromolecules.[101] Smales[117] made the intriguing observation in the TEM that the tubules around individual crystals have a double helical structure. This is in apparent contradiction to the X-ray diffraction data, unless the helical structure is itself composed of a β-strand (Jodaikin, Traub, and Weiner, unpublished data).

Matrix-mineral relations — Jodaikin et al.[118] report that the polypeptide chains of the proteins that adopt the β-sheet conformation (enamelins) have a mean-preferred orientation approximately perpendicular to the mean orientation of the c axes of the hydroxyapatite crystals of individual rods. This is based on X-ray diffraction patterns of partially decalcified rat incisor teeth which includes both mineral and matrix reflections. This study represents the first determination of just one of the matrix-mineral relations in enamel. It is of interest to note that this relation does not correspond to the optimal lattice match that could occur between hydroxyapatite (unit cell dimensions of 9.4 Å × 6.9 Å) and a β-sheet protein with approximately the same dimensions.

D. Stages of Mineral Formation

First-formed precipitate — The nature of the mineral phase of the first-formed precipitate is not known.[119] All X-ray diffraction or electron diffraction patterns of the earliest formed mineral show poorly crystalline hydroxyapatite to be present.[62,74,119] These studies do not exclude the possibility that some other transient mineral phase is formed first.

Growth of crystals — It is well established that the most recently formed crystals, or perhaps more accurately the forming ends of growing crystals are long, flat, ribbon-like plates.[87,90,120] Additional growth is primarily due to thickening of these plates. Significantly, all the crystals in a given area are nearly the same size and shape and at least in zones 1, 2, and 3 there is no evidence of fusion between adjacent crystals.[90] This, however, does occur in zone 4, during the final stages of mineralization.[89] Daculsi and Kerebel[121] observed that the number of crystallites per unit area decreased markedly with increasing distance from the Tomes processes. One possible explanation for this is that at certain stages during development the growing crystals split into "daughter" crystals. Split crystals have been directly observed.[86]

Mineral maturation — The most recently formed mineral in enamel is less well ordered or more poorly crystalline than the more mature mineral phases. This has been convincingly demonstrated by X-ray diffraction.[90,119]

E. Inferences with Regard to Crystal Growth

To date, no clear-cut evidence is available showing that enamel crystals in mammals grow into a preformed organic matrix, as has apparently been demonstrated for shark enamel. The formed crystals are, however, observed to each be surrounded by an organic envelope or a tubule. Bearing in mind that enamel is certainly one of the most exquisitely ordered mineralized tissues known, it is therefore difficult for me to conceive that the macromolecules that make up the tubules adsorbed onto the crystals after they were formed (see Nylen[122] and the discussion that follows and a review of this topic in Frank[123]). I will therefore assume in the following discussion that the tubules, at least, do constitute preformed structures into which the crystals grow. A definitive study on this most important topic would be very helpful.

Crystal Nucleation

The site of nucleation — Following the observation of Daculsi et al.[86] that the crystals in enamel are so long that it is quite conceivable that they extend from the dentino-enamel junction to the forming surface,[87] two possibilities arise with regard to the location of the nucleation site(s): each crystal is nucleated once at or close to the dentino-enamel junction or each crystal is the product of multinucleational events during growth. The two possibilities have quite different implications with regard to the degree of control exercised over nucleation. The multinucleation site hypothesis would require an extraordinarily ordered substrate in order to account for the resultant crystallites merging into a "single" crystal (from the crystallographic point of view). Precedents do exist. For example, the nacreous layers of the mollusks, *Pinctada margaratifera* and *Neotrigonia margaritacea* form sheets of aragonite composed of thousands of individual crystals. These are all so well aligned that their X-ray diffraction patterns are similar to those of single crystals.[34] The single-site hypothesis for enamel crystal nucleation is much simpler to envisage, but less compatible with observations showing that the newly formed crystals are long, thin plates quite different in shape from the more mature crystals.

The role of the tubule in nucleation — Without direct information on the exact nucleation site location, it can only be inferred indirectly that from the stereochemical point of view, the (100) faces of hydroxyapatite are most likely to be involved in nucleation.[6] These faces are parallel to the crystal c axis and therefore abut the inner surface of the tubule. Thus, the nucleation site or sites are more likely to be associated with the tubule inner surface rather than the tubule base, which would be in the plane perpendicular to the crystal c axis.

Crystal orientation relative to the substrate — It would be important to ascertain whether the nucleation event(s) orients the crystal uniquely in 3-dimensions relative to the substrate macromolecules, or only in 2-dimensions. Even if nucleation is indeed on the tubule inner surface, the observation that the crystal c axis is perpendicular to the orientation of the polypeptide chains[118] does not fully resolve this issue. More information is needed to relate the crystal a axes to the substrate components, possibly using electron diffraction.

Thus, the data currently available are compatible with both an epitaxial mode of nucleation as well as a stereochemical one. As recent X-ray and electron diffraction data show evidence for a relatively ordered organic matrix phase and as enamel in general is such an extraordinarily well-organized tissue compared to most others, an epitaxial mode of nucleation seems more likely.

Crystal Growth Modulation

Crystal growth is extremely well modulated—This is probably best inferred from the observation of Nylen et al.[90] that at every stage of growth all the thousands of crystals are extremely similar, both in size and in morphology.

Crystals grow in compartments isolated from each other—This can be inferred from the fact that larger crystals do not grow at the expense of smaller crystals. This would happen if the crystals all had access to the same reservoir of calcium and phosphate ions suggesting that the crystals must grow in isolated compartments. An independent observation in support of the same conclusion is based on a comparison of TEM micrographs of cross-sections of zone 3 crystals with zone 4 crystals (maturing and mature stages). The zone 3 crystals are all separated from each other and have very characteristic cross-sections, whereas the zone 4 crystals have fused with their neighbors and have lost their characteristic shape.[90] The transition from zone 3 to zone 4 may possibly involve the breakdown of the compartments, presumed here to be the same as the tubules.

It seems most likely that modulation of crystal growth is achieved by the prepositioned inner surfaces of the tubules. However, until their existence prior to crystal formation is proven, this conclusion remains tentative at best.

Crystal growth modulation affects one pair of (100) faces more than the others—The fact that the cross-sectional shapes of the mammalian enamel crystals are always extended hexagons with one pair of (100) faces having a larger surface area than the others, suggests that these faces form at a slower rate as compared to the other faces. It is not known how this occurs (see discussion of this same phenomenon in the "Inferences" section on bone). Even more perplexing is the observation that the cross-sectional shapes of the shark enameloid crystals are equilateral hexagons, although it has been suggested[82] that this could be a consequence of their high fluoride content.[124]

IV. MAMMALIAN BONE

Bone is by far the most thoroughly investigated mineralized tissue and yet in many respects still one of the less well understood in terms of its molecular ultrastructure. One reason for this is that the crystals are exceedingly small and their relationships to the organic matrix macromolecules are complex. Furthermore, the stages of mineralization are for most analytical purposes, not separable in space or time. Tooth dentin and mineralizing avian tendon on the other hand are very similar to bone and both do have stages of mineralization that are more easily separated than bone. So, even though this section will deal primarily with bone, and mostly with mammalian bone, some key studies using these other two tissues will be cited. For comprehensive reviews of bone, see Glimcher,[125-127] Miller,[128] and Boskey and Posner.[129]

A. Organizational Motif

The basic organizational motif of bone is dominated by the collagen fibril. Scanning electron microscope observations (SEM) of bone from which the organic components have been removed (anorganic preparations) show that the large majority of mineral crystals are associated in some way with the collagen fibrils.[130] Higher orders of organization (see Currey[131]) reflect the manner in which domains of collagen fibrils and their associated crystals are organized. Woven bone has very little long-range order of the collagen fibril-mineral complexes, whereas in lamellar bone ordered sheets composed more or less of aligned collagen fibrils and mineral, extend for short distances (up to 30 to 100 μm). In parallel-fibered bone, the collagen-mineral complexes have preferred orientations over much larger areas. These basic bone types can also coexist in the same bone to form even more complicated arrangements. For example, fibrolamellar bone is composed of roughly alternating regions of lamellar bone and parallel-fibered bone. The following section will, however, be confined to the basic "building unit" of bone, i.e., the collagen fibril and its associated mineral crystals.

Mineral-organic distribution — The mineral can exist in only three possible "com-

partments'' in relation to the collagen fibrils: (1) within the fibrils; (2) on the surface of the fibrils; and (3) between the fibrils, but not in direct contact with the fibrils.[132] Different bones or the same bone at different stages of mineralization may have mineral in one, two, or all three of the compartments.[133-135] Almost all EM observations of bone and in particular newly formed bone, are consistent with some of the mineral being present on the surface of the fibrils.[127,136,137] More mature bones often have at least some of the mineral located between fibrils, but not in direct contact with the fibrils.[126,134] Evidence supporting the presence of mineral within the fibrils is somewhat less compelling.[138] SEM examination of the mineralized portions of collagen fibers from tendons which are inserted into bone, sometimes have their centers more mineralized than the periphery, strongly suggesting that indeed mineralization does occur within the fibers.[139] Note that these are very large entities compared to the collagen fibrils usually observed in TEM. TEM observations of bone cut perpendicular to the long axes of the fibrils so as to reveal their cross-sections, do show that mineral is present throughout the fibril.[134,136,137] Unless the fibrils are aligned exactly perpendicular to the plane of the thin-section throughout its thickness, it is very difficult to prove conclusively in this way that mineral is indeed located within the fibril. The micrographs do, however, certainly support this possibility. An indirect approach to this same question involved an assessment of the relative volumes occupied by the major constituents of bone, i.e., collagen, water, and mineral.[133] The calculations upon which this is based assume a particular packing arrangement of the tropocollagen molecules and also use as an estimation of the average distance between adjacent tropocollagen molecules, an equatorial X-ray reflection derived from demineralized bone collagen. Based on these measurements, Katz and Li[133] estimate that between 69 and 76% of the mineral is within the fibrils. Bonar et al.[135] and Lees et al.[140] used neutron diffraction to check the collagen equatorial spacing in mineralized bones, and found that they were significantly smaller in dry bones as compared to wet bones and demineralized bones. Their recalculation shows that there is only enough space within the fibril to accommodate, at most, 35% of the mineral and possibly much less.

Mineral-organic proportions — The range of mineral contents of bone is between about 45 and 85% by mass and approximately half of that by volume.[141] Most bones, however, have a mineral content of around 65% by mass.[126] The remainder of the bone comprises collagen and other macromolecules, as well as water. Doty et al.[142] show a useful diagrammatic illustration of the varying proportions of the major components as a function of increasing mineralization. The properties may also vary in different parts of the same bone or in different bones from the same animal and as a function of age of the animal.

Tissue site of mineralization — Bone mineralization occurs in the extracellular space. The cells primarily responsible for bone formation are called osteoblasts. There appear to be two fundamentally different modes of cellular regulation over the mineralization process. The ubiquitous mode is the synthesis, secretion, and self-assembly of the extracellular organic matrix framework which in turn directs crystal formation.[127] A second mode, not always present, involves the release of so-called ''matrix vesicles'' into the extracellular space, which also induce crystals to form. It is conceivable, although by no means proven, that the framework-related mineralization is primarily responsible for crystal that forms in close association with collagen, whereas the matrix vesicles contribute to the mineralization of the intercollagen fibril compartment. For a good discussion of these important questions, see Boskey.[143]

Preformed structural framework — There is good evidence for the existence of a preformed organic framework in bone,[144] in dentin[145] (the predentin), and in calcifying avian tendon.[146,147] In the latter, mineralization starts at the center of the formed tendon and progresses towards the extremities.

B. Mineral Phase

Mineral type — de Jong[148] first identified the mineral in bone as apatite in 1926, and Klement and Trömel[149] determined that it is hydroxyapatite. In a recent review Glimcher[127] described the mineral in bone as "a single phase of poorly crystalline nonstoichiometrical hydroxyapatite, which becomes more crystalline and approaches ideal stoichiometry with time after its initial deposition" (p. 484). The evidence for amorphous calcium phosphate comprising the bulk phase of bone has been refuted.[150] Bone also contains significant amounts of carbonate and citrate, as well as a host of other trace constituents. For reviews on the mineral phase in bone, see Brown and Chow[151] and in dentin see Posner and Tannenbaum.[152]

Crystal shape — A complete description of the shapes of bone crystals is not available. Numerous TEM studies of embedded and sectioned bone report that the crystals are elongated, needle-shaped crystals comparable in habit to inorganically precipitated apatite. Careful tilting of the electron microscope stage shows unequivocally that all the crystals studied are actually thin, flat plates,[134,153-155] with a somewhat irregular shape.[155,156] Cross-sections cut through the crystals of bone and dentin confirm that they are indeed thin and elongated. A small number of these crystals also showed an elongated hexagonal profile.[156] Studies such as these do not preclude the existence of needles. A more direct approach is to disperse the crystals after removing the organic phase. Robinson[157] in 1952 used this method and reported that the crystals are indeed plate-shaped. Using the same approach, Weiner and Price[158] unequivocally confirm Robinson's early observation in a study of six different bones (Figure 8). It should be noted that bone crystals are quite different in shape (and size) when compared to most synthetically precipitated hydroxyapatite crystals (Figures 8 and 9).

Crystal size — Compared to the crystals in most other mineralized tissues or even to inorganically precipitated hydroxyapatite, the crystals in bone are extremely small. TEM of embedded sections provides reliable data for the thickness of the plate-shaped crystals. Almost all the measurements made are between 20 and 40 Å,[153,154] and the impression gained from the micrographs themselves is that the thicknesses are quite uniform. Estimates of thickness using X-ray diffraction line broadening are between 40 and 60 Å.[159,160] Most TEM observations of bone sections cannot be used to measure the maximum widths and lengths of the crystals as their precise orientations relative to the microscope stage are unknown. Dark field imaging using the 002 reflection does provide a means of reliably measuring the crystal dimensions parallel to the c axis.[155] In human, rabbit, and ox bones the ranges measured are from 60 Å to 1130 Å with the means and standard deviations being around 340 Å ± 160 Å for the three species. These measurements are consistent with most other estimates of length.[126,138] Reliable measurements of maximum crystal widths are not available except for Robinson[157] who dispersed an anorganic preparation of human bone crystals on an EM grid and measured the average crystal widths as being 250 Å. The sizes and shapes of the crystals do not change significantly with progressive mineralization of the tissue.[134]

Orientation of crystallographic axes — Periodic lattice images of hydroxyapatite crystals from both bone and dentin show that the c axis is parallel to the plane of the crystal and that the faces expressed are (100) faces. Even crystals that have a highly irregular shape show lattice images indicating that their constituent atoms are regularly spaced.[156]

C. Organic Phase

Framework macromolecular constituents — Type I collagen is the major framework constituent of bone, dentin, and tendon. It comprises some 85 to 90% of the total protein in these tissues. It is very similar, but not identical to type I collagen from nonmineralizing tissues. Some of the more significant differences relate to the degrees of hydroxylation of lysine, the extent of cross-linking, and the amount of glycosylation. It is not known if these differences are responsible for the fact that certain type I collagens mineralize, whereas others do not (recently summarized by Veis[161]). The collagen fibrils constitute a three-

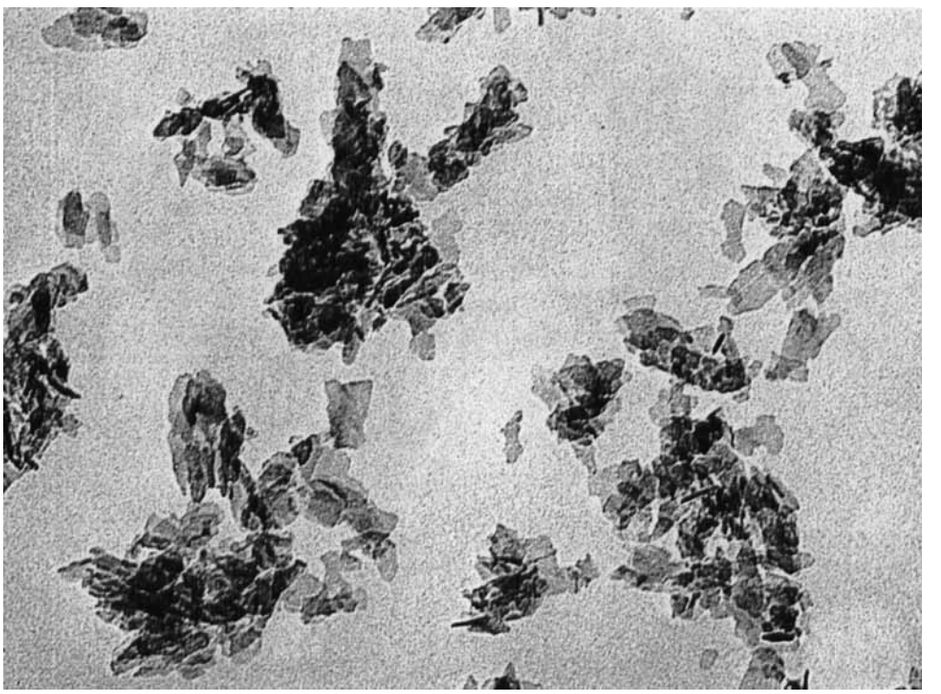


FIGURE 8. Transmission electron micrograph of disaggregated bone crystals from human long bones after removal of the organic matrix. Note that the crystals are very thin (approximately 40 Å), irregularly shaped plates. (Magnification × 110,000.) (Courtesy of S. Weiner and P. A. Price, Biology Department, University of California, San Diego.)

dimensional framework on which and presumably in which crystals form. The distribution of mineral within the fibril is dominated by the spaces created by the internal organization of the collagen macromolecules. Even though the connotation of a "framework" is one of a passive entity with regard to the regulation of mineralization, information available to date by no means excludes a more active role for collagen in mineralization, particularly in controlling intrafibrillar crystal formation.

Acidic macromolecules — The quantitatively abundant acidic macromolecules in bone and dentin have been relatively well characterized compared to other mineralized tissues. The two major classes of acidic macromolecules present in other mineralized tissues are also in bone and dentin, i.e., acidic glycoproteins, some of which are phosphorylated and proteoglycans.[104,127,162] Bone also contains a host of serum proteins.[163] (For reviews of the so-called "noncollagenous proteins", see Boskey and Posner,[129] Linde,[162] Termine,[163] and Veis.[161]) It is interesting to note that almost all the acidic macromolecules present in bone are also present in dentin, with the exception of the highly phosphorylated proteins (also called phosphophoryns).[162] Two-dimensional gel electrophoresis of the acidic macromolecules in bone shows that about 160 components are separated. These can be subdivided into 40 individual protein groups of which 15 are not present in plasma.[164] Clearly, bone contains a very heterogeneous and complex assemblage of acidic macromolecules. Their functions are unknown except for the one protein involved in inducing the formation of new bone (bone morphogenic protein).[165]

Macromolecular conformations and ion binding properties—The molecular conformation of *collagen* is better understood than all other macromolecules associated with mi-

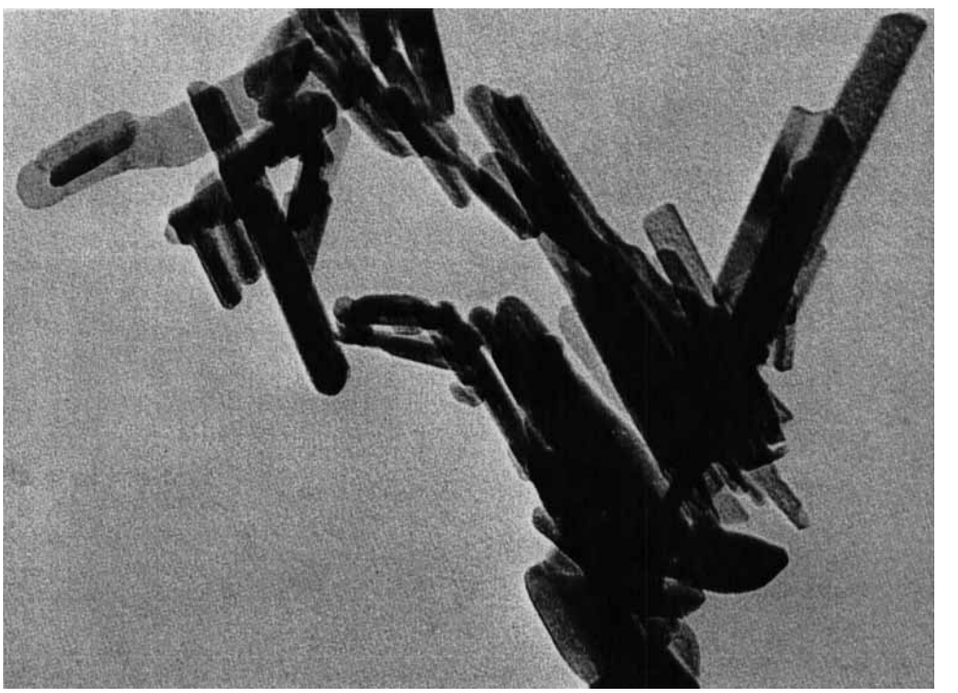


FIGURE 9. Transmission electron micrograph of synthetically prepared hydroxyapatite (Mallinkrodt). These crystals are quite different in shape and size when compared to the bone crystals in Figure 8. (Magnification × 41,500.) (Courtesy of S. Weiner and P. A. Price, Biology Department, University of California, San Diego.)

neralized tissues.[128,166,167] Moreover, the unique staggered arrangement of the triple helical molecules as first enunciated by Hodge and Petruska[168] is also a key to understanding the distribution of the mineral crystals within and on the surfaces of the fibril (see next section). However, despite the large body of information available on collagen structure including its amino acid sequence, there are still uncertainties about the detailed molecular conformation and packing in fibrils.[166]

Collagen does have many specific binding sites for calcium.[169] It has been postulated that the binding of calcium to these sites could promote mineralization of the collagen fibril. However, in the absence of more detailed information on the exact molecular structure of collagen, the configurations of the bound calcium ions are also unknown, particularly in the important "gap" regions where some of the crystals form (see next section).

The *acidic macromolecules* of bone and dentin probably all bind calcium ions. The calcium-binding properties of two classes of these macromolecules have been well examined, i.e., the highly phosphorylated phosphoproteins (called phosphophoryns or PP-Hs) in dentin[104,162,170] and the γ-carboxyglutamic acid containing proteins of bone.[171,172] Significantly, in both cases it was observed that the binding of calcium ions induces a change in the molecular conformation of the protein.[170-174] Similar observations were made for acidic proteins from mollusk shells.[43] It seems likely therefore that in vivo matrix assembly is dependent upon calcium binding and that the initiation (or trigger) for crystal growth is the introduction of the anion. Boyde and Shapiro[175] have in fact observed that in the epiphyseal growth plate calcium binding precedes phosphate binding.

The complete amino acid sequence of acidic proteins are known only for the bone-Gla proteins (osteocalcin).[176] The N-terminal sequences of bovine and porcine osteonectin have

recently been determined.[177] Both bone-Gla protein and osteonectin sequences are remarkably conserved between species. The secondary or tertiary molecular conformations of the acidic macromolecules from bone and dentin are unknown.

Matrix-mineral relations—The fact that the crystals are aligned relative to the collagen fibril such that their long axes (or at least the planes of the plates) are parallel to the collagen fibril axis was observed by Schmidt[178] using polarized light in 1936. Stuhler[179] showed that this corresponded to the crystallographic c axis being aligned with the fibril axis. In cross-sections through the fibrils, no preferred orientation of the crystals is observed.[136] Furthermore, during the early stages of crystal formation, the crystals are seen by TEM to arrange themselves according to the axial periodicity of the collagen fibril (at approximately 670 Å repeating intervals).[160] When Hodge and Petruska[168] proposed the staggered array model for collagen, they also recognized that a possible primary location for the mineral within the fibrils is the gap regions (or holes) which are present as a result of the nonintegral relation between the collagen molecular lengths and the axial intermolecular shifts. The fact that the crystals are located at the level of the gap regions is confirmed by X-ray and neutron diffraction;[180-182] the latter indicating that there is a tendency for the crystals to be displaced towards the N-terminal side of the gap regions.[182] These data do not prove that these early-formed crystals are present in the gaps throughout the fibril and not just on the surface. Measurements of electron density show, however, that there is a twofold excess of mineral in these fibrils over what could be expected to be present even if all the gap regions contain mineral. It is not known for sure where this mineral is located. The inference is that at least the gap regions within the fibril do contain mineral which is consistent with the TEM observations cited earlier (see review by Miller[128]).

All this information provides very little insight into the matrix-mineral relations at a molecular level — the level from which we could begin to infer the basic manner in which collagen either directly or indirectly regulates crystal growth, or for that matter how any of the other matrix macromolecules and vesicles function in mineralization.

D. Stages of Mineral Formation

First-formed precipitate—A large amount of literature exists in which proposals identifying the mineral type of the first precipitate have been made (summarized by Boskey[143]). It has not been conclusively shown that any one of these is in fact the first-formed phase. The earliest formed mineral phase detected to date is poorly crystalline hydroxyapatite.[183] Amorphous calcium phosphate (ACP) is still a distinct possibility. It is only known that ACP does not persist as a bulk phase for any length of time following the onset of mineralization. Furthermore, ACP is the one mineral phase which is known in vivo to form a first precipitate which later transforms into a carbonate apatite. This occurs in the radular teeth of a mollusk.[30] Intriguingly, these apatite crystals also have their c axes oriented in a preferred direction.[30]

Growth of crystals—The small size of the crystals and their rapid rate of formation precludes the possibility of directly observing the growth stages of a single crystal in a manner analogous to observations of crystal growth in mollusks and enamel. The very elegant study of Berthet-Colominas et al.[182] using X-ray and neutron diffraction, however, shows indirectly that the initial mineral does not uniformly fill the gap region. The model that best fits their data is one in which mineralization starts at the N-terminal side of the gap region and progresses towards the C-terminal, while simultaneously increasing in thickness. From the size of the crystals, it is assumed that on the average one crystal occupies one gap region.

Electron-micrographs show that the first mineral crystals tend to form at the periphery and the interior of the fibril in the gap regions and later between the gap regions.[134,137,160,181,187] In general, mineralization of the fibril precedes the appearance of mineral between the fibrils.[127] One confusing observation is that the first observed crystals in the osteoid of bone

are not intimately associated with the collagen fibrils as they do not show preferred orientation, but appear to be randomly distributed clusters of crystals.[184,185]

Mineral maturation—Bone crystals do undergo a marked maturation process as evidenced by the change in many of their properties with age. The degree of crystallinity, density, carbonate content, and calcium content all increase with increasing age of the bone, whereas the HPO_4^- content decreases. The Ca/P ratio tends towards that of stoichiometric hydroxyapatite.[186-188]

E. Inferences with Regard to Crystal Formation

The various compartments in which mineral can be located in bone (within, on, and between collagen fibrils) may all have quite different modes of nucleation and modulation of crystal growth.[134] Very little is known about the crystals that are located between fibrils. Do they have different sizes and shapes as compared to the crystals associated with collagen? Are they as well aligned as the other crystals? Is there a matrix framework which envelopes each of these crystals? For lack of information, the inferences drawn in this section will relate primarily to collagen associated crystals.

Crystal nucleation—Nucleation is directly or indirectly controlled by the collagen fibril framework. The ubiquitous alignment of the crystal c axes with the collagen fibril axis does imply, but does not prove, that the fibril structure is exerting influence over the nucleation process.[127] Without more information on the matrix-mineral relations at a molecular level, we cannot differentiate between the stereochemical type of nucleation process or epitaxial nucleation, where the crystal orientation relative to the matrix is fixed in three dimensions.

There are different classes of nucleation sites.—The observed sequence in which mineralization occurs, namely, crystals, form first on the fibril surface and interior associated with the gap regions, then within the fibril outside gap regions and finally between fibrils, suggests that nucleation at these sites is differentially regulated,[134] possibly by the structure of the site itself. Nothing is known about the organization of the macromolecules at these nucleation sites. It has been proposed that some of the acidic noncollagenous proteins fulfill a function in crystal nucleation.[189,190] It seems doubtful that there is sufficient space to accommodate them as well as a crystal in the gap regions within the fibril. There is also no evidence based on electron density for their presence in the gap regions.[182] In addition, they are unlikely to be able to penetrate into the formed fibril judging from the observation that even a 4000 dalton polymer of polyethylene glycol is unable to penetrate into the interior of the fibril.[133] The most likely sites, therefore, for acidic macromolecules to participate in nucleation, if indeed they do, is on the surface of the fibrils or between fibrils. Immunochemical localization of two such proteins (osteonectin and bone-Gla protein) show that they are associated with collagen fibers.[191]

Crystal Growth Modulation

Growth of two of the (100) faces is slow relative to the other faces—This can be inferred from the plate-like shape of the crystals. This can conceivably be achieved by specific adsorption of small molecules or macromolecules such as the acidic proteins (in a manner analogous to that described by Addadi and Weiner[6]) onto these faces. It is not, however, obvious how two of the six identical (100) faces are selected. Addadi and Weiner[6] pointed out that the (100) faces do indeed have the appropriate stereochemical properties for interaction to occur. On the other hand, the plate-like shape could be the result of the crystals growing in a preformed space with similar dimensions to those of the crystals. For the intrafibrillar crystals the collagen framework could fulfill the latter function, although it is not known where exactly in the fibril crystals of the observed size range could form. A third proposed way of forming plate-shaped crystals is for the initial bone particle to be octacalcium phosphate which itself is plate-like, and then to assume that it can dictate the final crystallite morphology.[152]

Crystal thickness is better controlled than their lengths and widths —The observed variation in crystal thickness measurements appears to be small compared to their lengths and widths, suggesting that this parameter is well controlled. The data are, however, not well substantiated and it is not known whether crystals in different compartments in the same bone have different sizes and shapes — information which could provide valuable clues about crystal growth regulation processes. The observations of uniform crystal thickness could probably best be accounted for by the crystals filling a preformed space.

The axial periodicity of the collagen fibril may be influential in determining the lengths of the majority of crystals—As most crystals do have lengths less than 400 Å or so, the approximate length of the gap regions; as the majority of the crystals in bone are almost certainly not limited to the gap regions, this represents a somewhat paradoxical observation.[128]

Concluding comment — So many fundamental questions still remain unanswered in bone that it is difficult to even begin to analyze this mineralized tissue in a similar manner to that used for enamel and mollusk nacre. From the ultrastructural point of view, bone must be one of the most difficult tissues to understand.

V. RADIAL AND GRANULAR FORAMINIFERA

The foraminifera are marine protozoans which belong to the order, foraminiferida. There are about 34,000 known species, of which some 4000 are living and the rest are extinct.[192] They are found throughout the oceans of the world. The planktonic foraminifera are extremely abundant. They utilize such huge amounts of calcium carbonate to form their shells that they significantly alter aspects of seawater chemistry. Great numbers of their dead shells accumulate on the ocean bottoms above the carbonate compensation boundary and eventually get incorporated into the sedimentary record. As a result, they are very often recovered from drill cores and are therefore widely used as index fossils to determine the age of the rock and the environment in which it was formed.

Foraminifera were first described by Strabo (63 B.C. to 20 A.D.) who observed specimens of a giant species, *Nummulites* in the pyramids of Egypt.[193] There is a vast literature on these organisms dating back more than a century. Recent reviews of living foraminiferal biology have been written by Boltovsky and Wright,[193] Lipps et al.,[192] and Anderson and Bé.[194]

Foraminifera build a variety of shell types; not all of which are mineralized. Of the mineralized shells, only the granular and radial types will be considered in this section. In polarized light, the granular shells show many flecks of color, whereas the radial ones have a typical uniaxial interference figure. (The third common form, not considered here, is the porcellaneous shells which have the characteristic milky-white color of porcelain in normal light).[193,195,196]

A. Organizational Motif

The key to understanding the organizational motif of the shells of granular and radial foraminifera is the manner in which they grow by the addition of new chambers. The shell of the first-formed chamber is composed of a layer of organic material and either a single layer of mineral (''monolamellar'') or two layers of mineral on either side of the organic layer (''bilamellar'') (Figure 10). With the formation of each additional new chamber, a layer of mineral and a layer of organic material is added on to the entire specimen. The result is that the earlier formed chamber walls are composed of a number of mineral and organic layers (lamellae)[197-199] (Figures 10 and 11). Planktonic foraminifera form thin lamellae during the earlier stages of their life while they live close to the ocean surface.[200] Before reproduction a few species have been reported to sink to depths of approximately 1000 m. During this stage, some species secrete a thick crust of large crystals over their entire shell[201] (Figures 10 and 11).

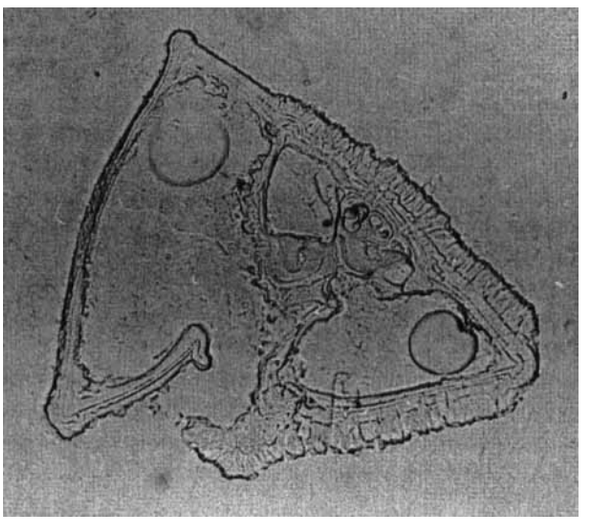


FIGURE 10. Light microscope photograph of an embedded thin section of the shell of the planktonic foraminifer, *Globorotalia truncatulinoides*, showing how the last formed chamber wall (left-handed side) has only two lamellae, whereas the earlier formed chamber walls are composed of several lamellae. The crust composed of large calcite crystals is also visible. The shell diameter is about 500 μm. (Magnification × 150 approximately.) (Courtesy of Prof. Z. Reiss, Department of Geology, Hebrew University, Israel.)

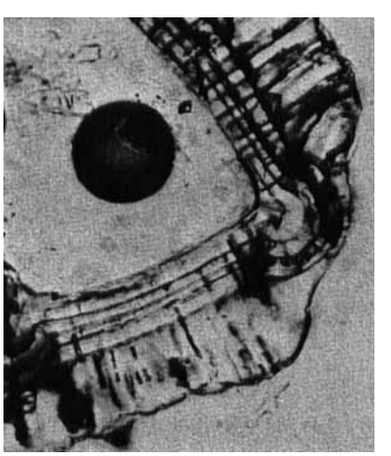


FIGURE 11. Higher magnification light microscope view of the shell of the planktonic foraminifer, *Globorotalia truncatulinoides*, showing the thin inner lamellae and the thick outer crust. (Magnification × 450 approximately.) (Courtesy of Z. Reiss, Department of Geology, Hebrew University, Israel.)

FIGURE 12. Scanning electron micrograph of the fixed, etched, and critical point dried shell of the benthonic foraminifer, *Heterostegina depressa*, showing the lamellae separated by organic matrix layers. Note that the shapes of the crystals are very difficult to discern. (Magnification × 2,400.)

Mineral-organic distribution — The shell walls are essentially composed of layers of mineral and layers of organic material (Figures 12 and 13). These are more easily seen in decalcified thin sections of the shell wall viewed in the TEM.[194,202] The organic layers appear to be continuous, except that in some places they are interrupted by holes or pores.[202,203] Electron microscopy has not revealed any internal structure within an individual organic layer, although it is not clear whether this property has been specifically studied. It should be pointed out that these layers represent only the fraction of organic matter that is insoluble after the mineral is dissolved in EDTA. Unless special precautions are taken, the macromolecules that dissolve upon mineral dissolution are lost. They might well coat the surfaces of the observed layers in a manner analogous to the matrix sheets in mollusk nacre. The EM does show the presence of nonuniformly dispersed organic material between successive layers,[202,203] but it is not known whether this forms an organized framework structure.

Mineral-organic proportions — Very few reliable measurements of the total organic contents of foraminiferal shells are available because a major part of the material is soluble upon dissolution of the shell. Weiner and Erez[204] take this into account and estimate that the shell of one species of a benthonic foraminifer contains 0.1 to 0.2% by weight organic material. The protein content is only about 0.02% by weight. A comparable amount of total protein in planktonic foraminifera shells was reported.[205]

Tissue site of mineralization — The term "tissue" is not appropriate for a single-celled organism. In effect, the process of mineralization does take place in the pseudo-extracellular environment. It has been observed directly in the benthic foraminifera *Rosalina floridana*.[206] New chamber walls are formed by extended pseudopodia that first construct an organic cyst. This differentiates into a compartment (or "anlage") in which mineralization takes place. While mineralization is in progress, the anlage is surrounded on both sides by protoplasm.

Preformed organic structural framework — The compartment or anlage acts as a preformed framework in which mineralization occurs.[196,207]

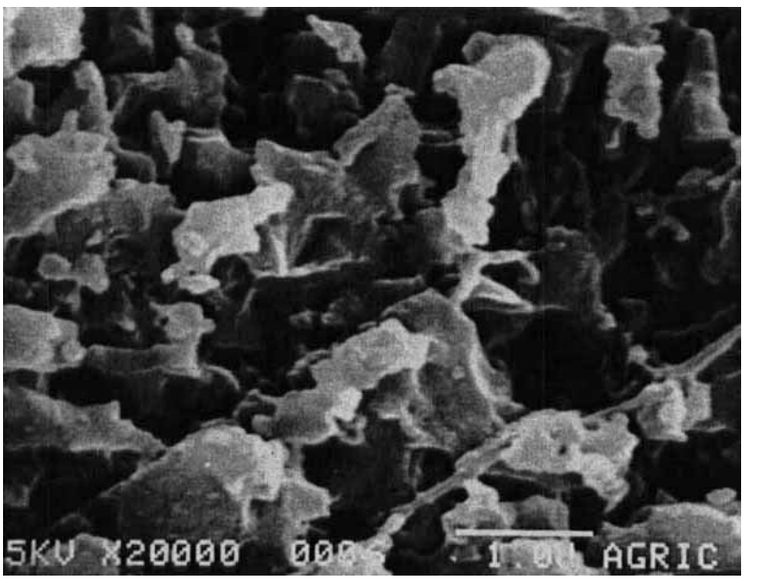


FIGURE 13. Higher magnification view of the shell of *Heterostegina depressa*, showing the organic matrix layers with the juxtaposed crystals. The crystals appear to be truncated by the organic matrix layer, possibly as a result of nucleation off this face. (Magnification × 20,000.)

B. Mineral Phase

Mineral type — Almost all the radial and granular foraminiferal shells are composed of calcite.[208] However, the shells of one phylogenetically distinct group, the suborder *Robertinina*, are composed of aragonite.[209] No known foraminifer has both calcite and aragonite in its shell. The magnesium contents of calcitic shells vary considerably from trace amounts to about 25 mol percent. Interestingly, all the granular species form low magnesium calcites (less than 4 mol percent) whereas the radial types have shells containing both low and high magnesium calcite. All the planktonic foraminifera form shells of low magnesium calcite.[208]

Crystal shape — The shapes of the calcite crystals are very difficult to discern in broken sections of shell walls examined by SEM (e.g., Figures 12 and 13) or in replicas of shell walls observed in TEM.[196] This is reflected in the terms commonly used to describe crystal shapes, for example, anhedral, subhedral, and euhedral, or equant grains, lamellar granular, and microgranular.[194,203] The calcite walls do not appear to possess characteristically shaped crystals. Only the outer layers, exemplified by the crusts of planktonic foraminifera, contain crystals which express the same faces as that of the inorganic calcite cleavage-rhombohedron (e.g., Figure 31 in Anderson and Bé[194]). Micrographs of some etched crystals do show plate-like or needle-like forms.[196,199,210] These are, however, thought to result from the etching of single crystals.[196] In fact, it has been shown by crushing shells into constituent grains that each grain gives an electron diffraction pattern of a single crystal.[203] EM observations of shell walls of certain planktonic foraminifera (e.g., *Globorotalia truncatulinoides)*[194] and benthic foraminifera (e.g., *Lenticulina calcar)*[196] show that the crystal boundaries are very irregular (described as "sinuous" or "sutured").[195,203,211] This suggests to me that adjacent crystals have penetrated into each other. This contrasts with crystal boundaries in, for example, mollusk nacre, which are smooth and linear (see mollusk nacre Section II.B). The shapes of the crystals of aragonite of the species *Hoeglundina* are more easily discerned. In cross-section they are roughly hexagonal and are elongated in the plane perpendicular to the cross-section. The boundaries between adjacent crystals are also "sutured".[203,212]

Crystal size — Based on the previous discussion of crystal shape, it is not surprising that crystal size is also highly variable[211] and very difficult to measure. The impression gained from EM micrographs is that crystal sizes vary to some extent even within a single lamella and certainly between lamellae. In planktonic foraminifera, crystal sizes (significantly reported as "diameters") increase during shell thickening from about 0.2 μm up to 15 μm in the crust.[194] Crystal sizes in benthonic foraminifera are estimated to range between 0.5 and 4.0 μm.[203] The aragonite crystals of *Hoeglundina* also vary significantly in size, being approximately 0.1 μm wide and 0.4 μm long.[203,212]

Orientation of crystallographic axes — The radial and granular shell types are differentiated by the orientations of the c axes of their crystals. In a radial shell the c axes are aligned more or less perpendicular to the shell wall surface, explaining why a uniaxial interference figure is observed in polarized light.[196,213] In the granular shell there is a tendency for the crystals to form so that their (104) faces (the face of the cleavage rhombohedron) are more or less parallel to the shell surface.[196] This observation has been confirmed using X-ray diffraction.[213] In granular and radial calcitic foraminifera, the relative orientations of the a axes of different crystals are not generally known — a surprising situation bearing in mind the importance ascribed to crystallographic orientation of the c axes in taxonomic classifications. One selected area diffraction pattern from the radial wall of *A. becardii* shows a "strong preferred orientation of the calcite c axes perpendicular to the test surface, with perhaps a weak additional preferred orientation of the a axes" (p. 287).[211] Crystal etch patterns of the aragonitic species, *Hoeglundina* indicate that the crystals are probably not aligned with respect to each other in the ab plane.[203,212] The same may be true of calcitic species based only on an examination of the cleavage rhombohedron faces of the crystals of the outer lamellae in planktonic foraminifera (for example, see Figure 31 in Anderson and Bé[194]).

C. Organic Phase

The few published reports on aspects of the biochemistry of the organic macromolecules in the shell are based primarily on either histochemistry or amino acid composition analyses.[214-218] The only reasonably comprehensive study of matrix biochemistry is that of Weiner and Erez[204] who used shells of the benthonic species *Heterostegina* that had reproduced in the laboratory and as a result were essentially free of contamination by protoplasm. (During reproduction the protoplasm is divided among the progeny which swim away!)

Framework macromolecular constituents — The major fraction that is insoluble after EDTA dissolution of mineral is composed of heavily sulfated polysaccharides associated with small amounts of protein(s).[204,214,215,217] In *Heterostegina* this fraction is derived primarily from the inner organic lining[202] which separates the shell from the protoplasm.[204]

Acidic macromolecules — The macromolecules that are soluble after EDTA dissolution of the shell of *Heterostegina* are predominantly composed of various acidic proteins rich in aspartic and glutamic acids, as well as acidic protein-polysaccharides, the proteins of which are rich in serine.[204] These appear to be the same two classes present in other mineralized tissues.[4] Amino acid compositional analyses of the total protein fraction from planktonic foraminiferal shells (actually a few thousand years old) also show that they are rich in aspartic and glutamic acids.[216]

Macromolecular conformations and ion-binding properties — Nothing is known.

Matrix-mineral relations — Nothing is known about the spatial relations between the matrix macromolecules and the associated crystals at the molecular level.

D. Stages of Mineral Formation

First-formed precipitate — Nothing is known.

Growth of crystals — Direct observations of mineral formation show that the crystals

grow in a preformed organic matrix composed of a central layer ("primary organic membrane"[202]) bounded on both sides by layers of organic material (reviewed in Anderson and Bé[194]). Angell[207] observed the stages of crystal growth in *Rosalina floridana* using both light and EM. The first discernible crystals form as tiny specks with no obvious regular distribution. Within hours they increase in size and thickness and eventually merge with neighboring crystals to form a continuously mineralized layer. This process is complete after 20 to 24 hr. Significantly, Angell[207] reports that many of the smaller crystals decrease in size and dissolve, whereas the larger ones tend to prevail.

Mineral maturation — Nothing is known.

E. Inferences with Regard to Crystal Formation

Crystal Nucleation

Nucleation in radial foraminifera may be stereochemically controlled—Epitaxial nucleation, which has been proposed,[196] requires a more complex and ordered organic matrix to function as a template for nucleation, as compared to that required for stereochemical nucleation. We do not, as yet, have any relevant information to distinguish between these two possibilities in foraminifera. All options are definitely open.

Nucleation in granular foraminifera is probably of the nonspecific type—Granular shells show no obvious orientation of the crystals. Towe and Cifelli[196] demonstrate that the crystals are not randomly oriented, but tend to lie on one of their (104) faces — the face expressed in the cleavage rhombohedral habit of inorganic calcite. The easiest way to envisage this occurring is for crystals to nucleate in solution or nonspecifically on a matrix surface. Calcite crystals formed in vitro tend to lie on one of their (104) faces.[6] It is far more difficult to conceive that the crystals were nucleated such that they grew in this orientation, particularly when the importance of the "stereochemical effect" observed by Addadi and Weiner[6] is taken into account.

Crystal Growth Modulation

Evidence for crystal growth modulation is absent—The following properties of radial and granular foraminiferal shells indicate that no modulation of the crystals after they are nucleated, takes place: (1) crystals have a large range of sizes even within a single layer; (2) crystal shapes, where recognizable, approximate that of the cleavage rhombohedron characteristic of inorganically precipitated calcite; (3) sutured boundaries between crystals suggest that adjacent crystals are intergrown; and (4) during growth large crystals grow at the expense of small ones, implying again that crystals are not growing in compartments isolated from each other.

Note—A number of very unusual shell structures are formed by certain foraminifera, which may, if better understood, provide important insights into processes of crystal formation. Bellemo[210] reports that in the shells of *Cibicides* the calcite c axes are parallel to the shell wall surface. Following Addadi and Weiner[6] this would imply that the most likely surface for nucleation is perpendicular to the plane of the lamellae. Towe et al.[219] used X-ray diffraction, polarized light microscopy, and overgrowth techniques to confirm that the shell of *Patellina corrugata* is constructed out of a single crystal of calcite. Its mode of formation is a complete puzzle.

VI. EXTRACELLULARLY MINERALIZING ALGAE

Calcareous algae form a remarkably diverse array of mineralized structures. These have been classified in an excellent review by Borowitzka[220] into the following categories: extracellular deposits, intercellular deposits, sheath calcification, calcified cell walls, and

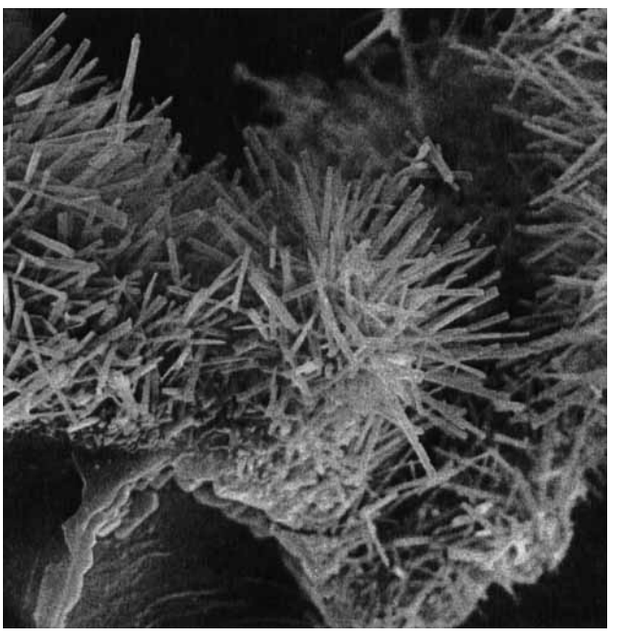


FIGURE 14. Scanning electron micrograph of the extracellularly mineralizing calcareous alga, *Halimeda* (species unknown) showing the thin, unoriented crystals of aragonite. A portion of the cell wall is visible in the upper right hand corner. (Magnification × 3,600.)

intracellular deposits. The order in which they are listed roughly corresponds to increasing ultrastructural complexity and hence probably reflects an increasing degree of control exercised over the crystal growth processes as well. In the following section only the ultrastructurally least complex algae with extracellular and intercellular mineralized deposits will be reviewed. Intercellular deposits are a special case of extracellular deposits.[220] In order to avoid misleading general statements about the very diverse extracellularly mineralizing algae, I will restrict the data presented in this section almost entirely to the Charophyta, Chlorophyta, and Rhodophyta and even within these phyla primarily to the genera, *Chara*, *Halimeda*, and *Liagora*, respectively.

A. Organizational Motif

The characteristic feature of the mineralized structures of these algae is the absence of any obvious organizational motif, as shown in Figures 14 and 15. Upon dissolution of the crystals, no insoluble organic residues have been observed.[221-223] Apparently, no one has separated the spicules from the cells even by simple oxidation of the exposed organic material and then dissolved the mineral to determine whether any relatively soluble organic molecules are released.

Mineral-organic distribution and proportions — These are not known and possibly not relevant.

Tissue site of mineralization — All these mineralized deposits are extracellular (Figure 15). The crystals are either in contact with, or are located in close proximity to the cell wall surface. In *Chara* the crystals are on the outer surfaces of the cell walls. They are deposited in bands which correspond to alkaline regions of the cell surface.[224] In *Liagora* the crystals are located between cells (intercellular space), but are still in open contact with the external

FIGURE 15. Scanning electron micrograph of the extracellularly mineralizing calcareous alga, *Liagora* (species unknown) showing the thin, unoriented crystals of aragonite surrounding the cell. (Magnification × 5,000.)

seawater.[222] In *Halimeda* most of the crystals are intercellular and therefore isolated from seawater.[225]

B. Mineral Phase

Mineral type — Calcite is formed by *Chara*, whereas aragonite is formed by *Halimeda* and *Liagora*. Mixtures of the two polymorphs in the same species are never found.[226] In general, the freshwater algal species deposit calcite. Marine species deposit aragonite in tropical and subtropical surface waters, and calcite in temperate and arctic surface waters.[222,223,227,227a]

Crystal shape — Algally formed aragonite crystals are similar in shape to the needles of aragonite precipitated directly from seawater.[228,229] Significantly, certain species do form crystals of different shapes.[221] Examples from *Halimeda* and *Liagora* are shown in Figures 14 and 15. Algal calcite crystals tend to be more variable in shape than the aragonitic crystals, but basically resemble the characteristic cleavage rhombohedral shape of inorganically formed calcite crystals. Inorganic calcite crystals also vary considerably in shape[230] depending upon the environment of formation.

Crystal sizes — Borowitzka et al.[222] review the size variations for both aragonitic and calcitic crystals. In general, crystal sizes vary considerably even in one tissue. The aragonite needles of *Halimeda* are 0.08 to 0.3 μm wide and up to 4μm long. Those of *Liagora* are 0.5 to 0.7 μm wide and 3 to 6 μm long. The calcite crystals of *Chara corallina* are 6 to 10 μm wide at the base and are 6 to 27 μm long.

Orientation of crystallographic axes — This group of extracellularly mineralized algae characteristically do not show any preferred orientation of their crystallographic axes.[220-222]

C. Organic Phase

As noted above, no insoluble organic residue remains after the mineral phase of these algae is dissolved.[221-223] The possibility that some soluble organic material is intimately associated with the mineral phase, has not been excluded. Nakahara and Bevelander[231] did observe an organic coating on the aragonite crystals of *Halimeda*. Borowitzka,[220] however, suggests that this might be due to nonspecific adsorption onto the formed crystal surfaces. Studies by Böhm[232] elude to the possible role during mineralization of water-soluble, calcium-binding polysaccharides in *Halimeda*. These polysaccharides are not yet known to be directly associated with the mineral phase.

D. Stages of Mineral Formation

First-formed precipitate — Nothing is known.

Growth of crystals — The stages of crystal growth have been best studied in *Halimeda*.[221,223] The first crystals are formed only after the peripheral utricles have fused to seal off a space from the external environment. These are closely associated with the fibrils of the "pilose" layer, but show no well-defined spatial relationship to the fibrils or any preferred orientation. Crystals then rapidly increase in size from a few hundred angstroms in length up to 4 or 5 μm and fill the entire chamber. The final phase of crystal growth in mature segments of the plant involves secondary crystallization around existing aragonite needles.

Mineral maturation — Nothing is known.

E. Inferences with Regard to Crystal Formation

Crystal Nucleation

Crystals form by nonspecific nucleation—The fact that crystals do not form in association with an *organized* substrate, that the crystals show no preferred orientation and that the mineral phase found is the one that would form inorganically in the same environment, all imply that crystal nucleation is of the nonspecific type. We do not, however, know whether these cells do synthesize and secrete into the extracellular space some specific molecule(s) for the purpose of inducing crystal growth.

Crystal Growth Modulation

Modulation of crystal growth is absent in some species, whereas in others adsorption of molecules onto growing surfaces may occur—As the crystals form, they generally resemble their inorganic counterparts in shape and display a wide range of sizes; crystal growth modulation is either minimal or absent in many species. The fact that crystal shapes are different in some species strongly suggests that at least for some species active crystal growth modulation does occur. This may take place by the adsorption of certain ions, molecules, or even macromolecules from solution onto specific crystal faces in a manner analogous to the in vitro experiments performed by Addadi and Weiner.[6]

VI. DISCUSSION

Table 3 summarizes my best assessment of the types of crystal nucleation and crystal growth modulation processes that occur in the five different mineralized tissues reviewed. It is only a reflection of the current state of knowledge, which I am sure, includes a good deal of prejudice and ignorance. Its main value lies in the fact that it represents a systematic and uniform analysis of some very different mineralized tissues. It shows that there is a common basis for understanding the processes involved in forming these tissues, and indicates that they are all part of one continuum,[2] whose end members differ markedly in the degree of control exerted over crystal growth. This is particularly apparent when crystal nucleation and crystal growth modulation processes are analyzed separately. It is also clear that the tissues in which more control is exercised over crystal nucleation, the modulation processes

Table 3
THE AUTHOR'S BEST ASSESSMENT OF THE TYPES OF CRYSTAL NUCLEATION AND CRYSTAL GROWTH MODULATION PROCESSES THAT OCCUR IN THE MINERALIZED TISSUES REVIEWED

	Crystal nucleation			Crystal growth modulation		
		Preformed structures				
	Nonspecific nucleation	Stereochemical surface nucleation	Epitaxial nucleation	Absent	Adsorption on growing surfaces	Prepositioned modulating surfaces
Mollusk nacre			X			X
Mammalian enamel		P	X			X
Mammalian bone		X	P		X	P
Radial foraminifera		X	P	X		
Granular foraminifera	X			X		
Extracellularly mineralizing algae	X			X	X	

Note: X — The most probable process involved.
P — An alternate reasonable possibility.

are also more controlled and vice versa. This is by no means a one-to-one correlation, and there is no reason to believe that the two are necessarily coupled.[6]

Epitaxial nucleation of crystals by organisms upon the organic matrix substrate, is a widely discussed topic in the literature. Information directly supporting epitaxial nucleation is only available for the mollusk shell nacreous layer, and even this has not yet been proven. Stereochemical surface nucleation may well be far more common in biological mineralization than epitaxial nucleation. Unfortunately, there is very little information available on the crystallography of mineralized tissues which could serve to differentiate between stereochemical and epitaxial nucleation mechanisms, such as crystal lattice dimensions matching or not matching the structure of the appropriate macromolecular constituents of the matrix substrate.

Table 3 also underscores the observation that the mineral type deposited is not a reliable guide to the underlying processes involved in forming the tissue. Aragonite is present in the mollusk nacreous layer as well as in the hard parts of some extracellularly mineralized algae— the two extremes of the continuum. By the same token, the deposition of a carbonate mineral or a phosphate mineral is not in itself an indication of a fundamental difference in the mechanisms involved in the mineralization of different tissues.

The terms "biologically induced" mineralization and "organic matrix-mediated" mineralization were introduced by Lowenstam[1] to describe two quite different processes in biomineralization. Biologically induced mineralization is epitomized by the extracellularly mineralized algae, whereas organic matrix-mediated mineralization could refer to any of the four other tissues reviewed. One implication of this review is that organic matrix-mediated mineralization therefore refers to a whole spectrum of processes and that an improved understanding of the underlying principles involved will require a more specific description of the important facets of each system. More appropriate terms to describe all these processes may be "biologically controlled" mineralization[8] and "biologically induced" mineralization. "Organic matrix-mediated" mineralization would refer to only a subset of mineralization processes in which control is exercised by means of the prior construction of an organic framework or matrix into which, or onto which, crystals form. Lowenstam[233] in a recent review of mineralization processes in monerans and protoctista, has independently made the same proposal.

It is also noteworthy that the organic matrices from the four tissues reviewed, in which crystal formation is "biologically controlled",[3,10] all contain acidic macromolecules and from the somewhat incomplete data currently available it also appears that the same two classes of acidic macromolecules are present, i.e., acidic (glyco-) proteins and serine-rich protein polysaccharides (probably all proteoglycans). The framework macromolecules differ biochemically in all four tissues. These observations are all consistent with the previously stated view that the acidic macromolecules perform functions common to all mineralized tissues, namely, regulating crystal formation and that the framework macromolecules primarily fulfill tissue-specific functions.[3,4]

This review also highlights some of the gaps in our knowledge. It, therefore, comes as somewhat of a surprise to observe that the two mineralized tissues that together are the subjects of the overwhelming majority of published papers in this field, mammalian bone and dentin, are also among the least understood in terms of structural and organizational aspects of their organic and mineral phases. This is undoubtably in part a reflection of the fact that these tissues contain the smallest biologically formed crystals known. (Even their size distribution is by no means certain.) As a consequence, the ultrastructural organization, particularly at the molecular level, is very difficult to resolve and significant progress in our understanding of these tissues is severely limited. This is one reason why I think that it is particularly important to place more emphasis on the study of mineralized tissues which are more amenable to investigation and then to apply the techniques and the concepts derived

from these studies to resolving the very formidable problems associated with understanding bone and dentin.

VIII. CONCLUDING COMMENT

The field of biomineralization is poised to tackle the very difficult problems of understanding the functions of the matrix macromolecules in regulating crystal formation. This will require not only an understanding of the biochemistry of these macromolecules, but also a thorough understanding of the molecular organization of these matrix constituents — their precise locations in the structure, their conformations, and the ways in which they interact with the mineral phase. This review shows that much can be inferred about the processes of mineralization from the more easily studied properties of the mineral phase itself. It also provides some of the guidelines for the types of functions that some of these macromolecules may perform in each tissue. Hopefully, it is one step along the road towards a better understanding of the very important phenomenon of biomineralization.

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