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Macromolecules in Mollusc Shells and Their Functions in Biomineralization [and Discussion]

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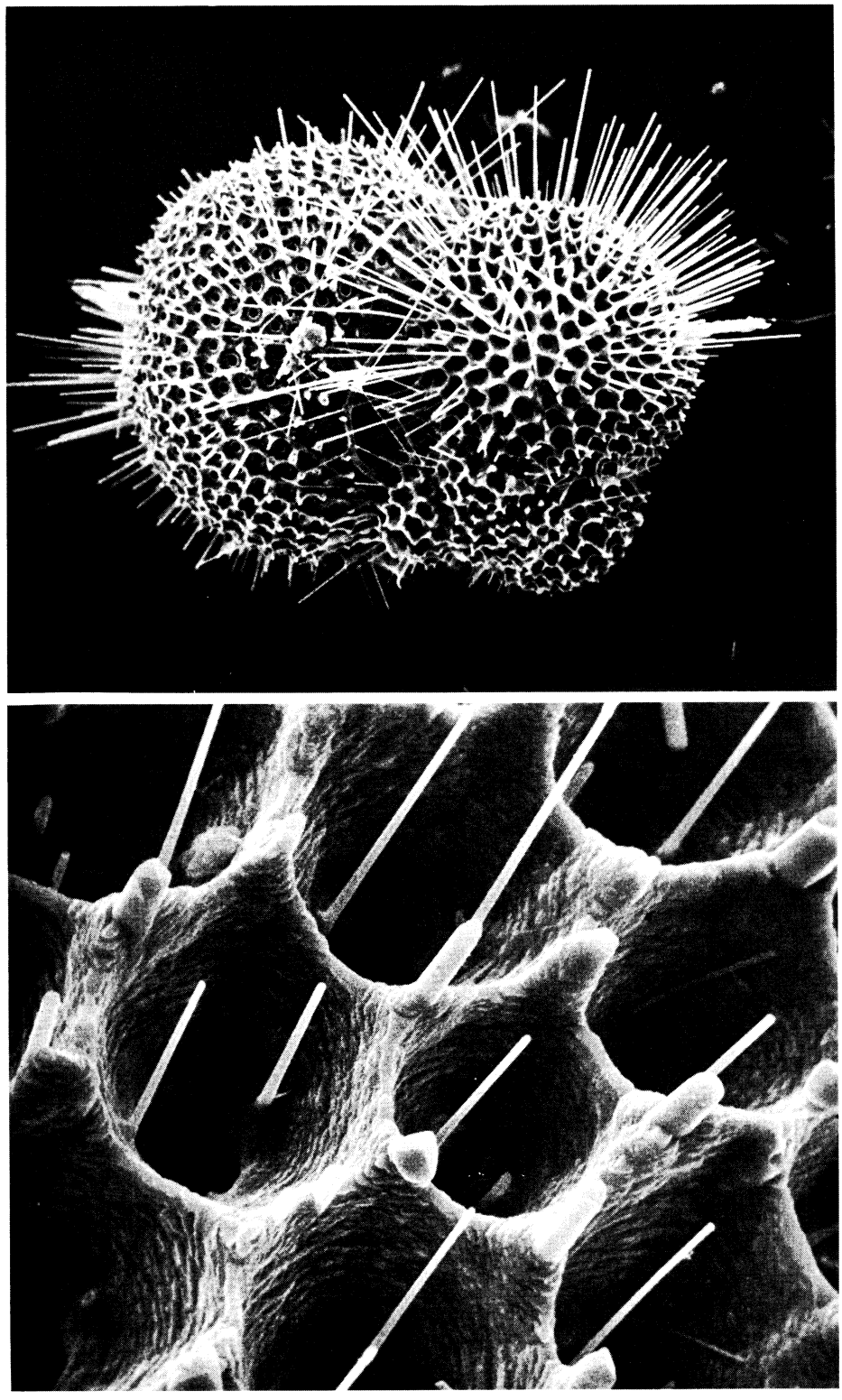

CALCIUM CARBONATES

Globigerinoides sacculifer; Globigerina is found in all tropical to subtropical oceans, and is sometimes thought to live in the surface layers.

Shell and spines are formed of calcite. The whole animal is illustrated (top) together with a more detailed micrograph of the first chamber (bottom) showing both pores and spines. This group of organisms is frequently used to determine palaeotemperatures based on the different proportions of oxygen isotopes in the shell.

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Macromolecules in mollusc shells and their functions in biomineralization

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Mollusc shells are used as a model for studying 'organic-matrix-mediated' biomineralization, in which crystals are nucleated and grow in a pre-formed structural framework composed of proteins and polysaccharides. In particular, the possibility that the organic matrix functions as a template for crystal formation by epitaxial growth, is examined.

In general, individual organic matrix sheets are composed of a thin layer of β-chitin sandwiched between layers of proteins adopting the antiparallel β-sheet conformation. The protein polypeptide chains are oriented perpendicular to the chitin fibrils. The matrix surfaces contain acidic proteins and polysaccharides. X-ray and electron diffraction patterns of matrices and mineral crystals from the nacreous layers of a bivalve, a gastropod and the cephalopod, *Nautilus*, show that the chitin fibres and the protein polypeptide chains are aligned with the *a* and *b* aragonite crystallographic axes, respectively. This strongly suggests that the mineral formed epitaxially upon the matrix surface. However, as the degree of orientation of the organic constituents is much less than the mineral constituents, it is postulated that the site of nucleation of the mineral crystals comprises only a small part of the matrix structure and is itself composed of well oriented macromolecules, probably acidic proteins. Two acidic proteins, which may constitute part of the nucleation site for calcite in a bivalve, were identified by comparing all the acidic proteins in the calcite layer with those in the aragonite layer. The two proteins were present in the calcite layer only, and in addition were found to have an aspartic-acid-containing amino acid sequence, not present in any of the other soluble matrix proteins. The concept of a matrix composed of a structural core coated with acidic macromolecules, some of which constitute a nucleation site, may well be applicable to other mineralized tissues.

Introduction

Organisms are known to form more than 40 different minerals in over 30 different phyla. More than half of these minerals contain calcium and among the most common are the various calcium carbonates, which generally form the exoskeletons of invertebrates, and the calcium phosphates, found in vertebrate bones and teeth (Lowenstam 1981; Lowenstam & Weiner 1983). Mineralized tissues are usually formed by the initial elaboration of a structural organic framework composed of proteins and polysaccharides into which ions of the mineral phase permeate and crystallize. Many eukaryotes and possibly even some prokaryotes, have adopted this so-called 'organic-matrix-mediated' mineralization process (Lowenstam & Weiner 1983; Lowenstam 1981). The mineral crystals are characteristically aligned in a preferred direction and often adopt habits distinctly different from their inorganically-formed counterparts (Lowenstam 1981). The structural framework or 'organic matrix' thus appears to be intimately involved in the crystal formation process. In addition it provides advantageous mechanical

properties to the formed mineralized tissue (Wainwright *et al.* 1976). The organic matrix is composed of a complex, heterogeneous mixture of macromolecules. However, very little is known yet about the specific functions of the various components.

The mechanism for crystal formation most often proposed is that of epitaxial growth of the mineral phase upon an organic matrix template. These proposals, in the past, were based on what may be misleading morphological observations of an alignment of the *c*-axes of hydroxyapatite crystals with the collagen fibre axis in bone (Glimcher 1981). Similar morphologial observations, more reminiscent of epitaxial growth in the inorganic world, have been made on the growing surfaces of the inner shell layer of some molluscs (Wise 1970) (figure 1).

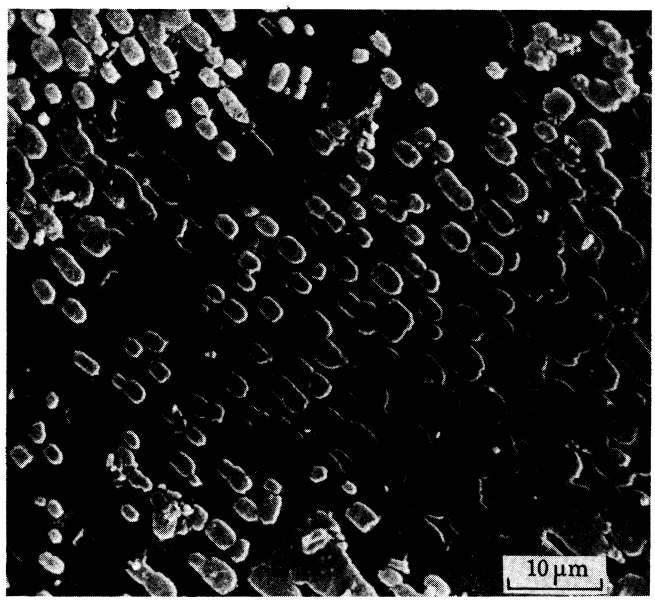

FIGURE 1. Scanning electron micrograph of the growth surface of the nacreous layer of the bivalve, *Neotrigonia margaritacea*. Note the preferred orientation of the newly-formed aragonite crystals and their merging together in the more mature areas to form a 'pavement-like' surface.

Here the newly formed crystals show a well defined preferred orientation, which suggests that controlled nucleation and growth took place upon the surfaces of the matrix sheets separating each mineral lamella (Nakahara 1979).

Epitaxy refers to the 'oriented overgrowth of one crystalline phase on the face of another' and arises when the 'atomic (lattice) arrangement in the substrate matches the lattice arrangement of the nucleating phase' (Posner & Betts 1981; p. 260). These concepts can be extended to include crystal nucleation on a non-crystalline template, which nevertheless has a regular structural repeat matching the crystalline lattice. To determine whether this applies to biological mineralization processes, we must examine structural relation at the molecular level and show, for example, that the crystallographic axes of the mineral overgrowth phase are aligned with the matrix macromolecular constituents of the substrate, and that the lattice dimensions of interacting surfaces of the two phases are matched. In this paper we will review evidence of this type, based primarily on studies of mollusc shells, which in our opinion tends to support the notion that molluscs form their shells by epitaxial growth.

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Mollusc organic matrix organization

Mollusc organic matrices are composed of many different macromolecules, only some of which dissolve after the mineral phase has been removed by chelation with ethylene-diamine-tetra-acetic acid (EDTA) at or close to neutral pH (Meenakshi *et al.* 1971). The major portion of this soluble fraction consists of proteins rich in acidic amino acids. (Weiner (1979), using the method of Hoare & Koshland (1967), showed that the aspartate and glutamate residues are predominantly present in their acid form and not as amides). Acidic sulphated

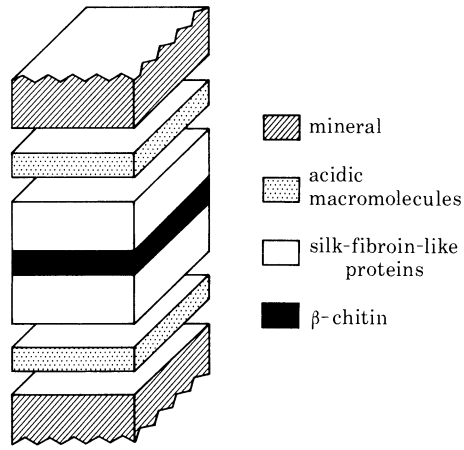

Figure 2. Schematic representation of a composite section of one individual matrix sheet bounded on both sides by mineral. The identification of each layer is discussed briefly in the text and in more detail in Weiner *et al.* (1983*a*). The total thickness of individual matrix sheets may vary between 30 nm and 300 nm (Watabe 1965).

polysaccharides are also present (Crenshaw 1972), possibly as part of proteoglycan complexes (Weiner *et al.* 1983*a*). The proteins of the insoluble fraction are quite different in composition, being generally hydrophobic (Crenshaw 1982) and rich in glycine and alanine (Meenakshi *et al.* 1971; Gregoire 1972). The polysaccharide chitin has also been found in the insoluble matrix fraction (Jeuniaux 1963) in its β-form (Weiner & Traub 1980). For recent reviews of the biochemistry of mollusc organic matrices, see Crenshaw (1982), Krampitz *et al.* (1983) and Weiner *et al.* (1983*a*).

Mollusc shells are composed of two or more shell layers, each of which has a distinctive microarchitecture. In some cases they also differ with respect to the polymorph of calcium carbonate present, namely aragonite or calcite (Boggild 1930). The inner shell layers of many molluscs have a nacreous microarchitecture, characterized by alternating layers of aragonite and organic matrix aligned parallel to the inner shell surface. The aragonite layers are also disrupted laterally by thin vertical matrix sheets, which in planar view subdivide the mineral layers into polygons (Watabe 1965; Gregoire 1972). The regular geometrical arrangement of the nacreous microarchitecture makes it particularly convenient for studying matrix–mineral relation.

Transmission electron microscope (t.e.m.) examinations of stained sections, cut through the organic matrices, reveal that matrix sheets may be composed of a number of different layers (Nakahara 1979). Figure 2 shows a schematic block diagram of a composite matrix section. (See also the discussion in Weiner *et al.* (1983*a*).)

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Nakahara (1983) identified the thin chitin core in gastropods, but not in bivalves. The electron-luscent layers on either side of the chitin core are thought to be composed of the glycine- and alanine-rich proteins of the insoluble fraction, which adopt the antiparallel β-sheet conformation and show X-ray diffraction patterns similar to silk-fibroin (Weiner & Traub 1980). The proposal that the chitin forms a thin layer sandwiched between two protein layers is consistent with the X-ray diffraction results, in that the two are clearly present as separate phases, though intimately related. The latter is inferred from the fact that the mean chitin fibril direction is perpendicular to the mean orientation of the protein polypeptide chains (Weiner & Traub 1980). This unusual plywood-like construction of the insoluble matrix probably contributes to its mechanical strength. A similar molecular organization is present in arthropod cuticle (Wainwright *et al.* 1976). The insoluble chitin–protein complex contains many pores or holes, the shapes and sizes of which are characteristic of the class of molluscs from which they originate (Gregoire *et al.* 1955). The surfaces of the insoluble complex are overlaid by an electron-dense layer (Nakahara 1983), which is thought to be composed primarily of the soluble acidic matrix components (Nakahara *et al.* 1982; Weiner *et al.* 1983*a*). The proteins of the soluble fraction adopt a random coil conformation in solution (Mann *et al.* 1983). Recent investigations of the conformations of proteins from this fraction made with Fourier transform infrared spectroscopy, suggest that some of these adopt the β-sheet conformation (Worms & Weiner 1983).

Not all organic matrices appear to contain the full complement of layers. For example, the matrix of the gastropod *Strombus gigas* is composed of only electron-dense layers (Bevelander & Nakahara 1980), which are almost entirely soluble after decalcification with EDTA (Weiner 1979). In the gastropod, *Turbo*, the horizontal interlamellar matrix contains the electron-luscent layers, whereas the vertical matrix layer does not (Nakahara 1979). It is not yet clear whether the chitin layer is present in all molluscs, although assays using chitinases show that chitin is present in many different molluscs, albeit in small amounts (Goffinet & Jeuniaux 1979).

Weiner *et al.* (1983*a*) have proposed that the insoluble chitin and silk-fibroin-like protein layers function primarily as a non-mineralizing structural framework, which contributes significantly to the mechanical properties of the shell. The fact that these layers are not essential for mineralization is demonstrated by their apparent absence in certain organic matrix layers. On the other hand, the prevalence of the acidic matrix components in the Mollusca, and in fact among many widely differing phyla, attests to their fundamental importance in mineralization (Weiner *et al.* 1983*a*).

Organic matrix–mineral spatial relations

A well defined spatial relation between the substrate and overgrowth phase is an essential property of all epitaxially formed crystals. This property is, however, difficult to examine in biological systems as the molecular structure of the organic components is easily perturbed by X-ray and especially electron beams. Furthermore, the relatively small amounts of matrix (0.01–5% by mass; Hare & Abelson (1965)) compared to mineral, necessitates the complete or partial demineralization of the specimen before analysis. In the course of this preparative procedure, the important acidic matrix constituents dissolve out of the specimen and their relation to the mineral phase cannot be analysed. In addition, the demineralization and dehydration procedures involved in sample preparation cause disorder in the remaining insoluble fraction to some unknown extent.

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Earlier X-ray diffraction studies (Weiner & Traub 1980, 1981) of some 10 different molluscan shell layers, revealed that in only one case, namely the septal nacreous layer of *Nautilus repertus*, could preferred orientation of the insoluble matrix constituents be detected in the plane of the specimen that interfaced with the aragonite mineral (the *ab* plane). It was found that the β-chitin fibrils and the protein polypeptide chains (which are mutually perpendicular to each other) were aligned with the *a* and *b* aragonite crystallographic axes, respectively. The unanswered question was whether *Nautilus* was typical in this aspect or not.

Recently, Weiner *et al.* (1983*b*) developed the methodology for using electron diffraction to study matrix–mineral relations in small areas (some 6 μm², compared to about 10000 μm² for X-ray diffraction). The areas studied correspond approximately to the cross sectional area of a single mantle epithelium cell. Using partially decalcified samples cooled to −100 °C to reduce radiation damage, we obtained oriented organic matrix diffraction patterns within the plane of the specimens from all four of the nacreous layers studied. (We examined the nacreous layers of the bivalves *Pinctada margaratifera* and *Neotrigonia margaritacea*; the gastropod, *Tectus dentatus* and the cephalopod, *Nautilus repertus*.) Clearly, the approximately 1000-fold reduction in area analysed was instrumental in revealing the presence of preferred orientation of the matrix macromolecules. In addition, for the bivalve *Pinctada* and the gastropod *Tectus*, electron diffraction photographs were obtained that included both mineral and matrix reflexions from the same small specimen region. For both, the matrix–mineral spatial relations proved to be the same as that previously found in *Nautilus* by X-ray diffraction. So these observations strongly support the notion that aragonite crystal growth in nacreous layers occurs epitaxially upon a matrix template.

The gastropod *Tectus* represents a particularly interesting case. X-ray diffraction patterns for the matrix (in the plane of the specimen) and the aragonite (*ab* plane) were randomly oriented, whereas the electron diffraction patterns of the matrix showed orientation of the macromolecules in a preferred direction and single crystal order for the aragonite. By using larger selective area diffraction apertures, it could be shown that the matrix is composed of a mosaic of relatively ordered areas and that the aragonite crystal associated with each area is aligned with the local matrix orientation.

Such an arrangement of matrix and mineral is inconsistent with theories of shell formation involving fields, gradients or currents over large areas, such as has been proposed, for example, by Digby (1968). We do emphasize, however, that we examined only the insoluble matrix components and not the soluble ones, which are *in situ* adjacent to the mineral layer itself.

The electron diffraction study shows that the mineral is much better oriented than either the protein or the chitin structures. Although specimen damage during preparation and analysis may well be responsible for part of this difference, we believe that these different degrees of order reflect an integral property of the shell layer. Explanations of the role of the organic matrix in mineral formation will, therefore, need to account for these differences. This is discussed in the next section.

The nucleation site

Little is known about the nucleation site, and hence much of the following discussion is of a speculative nature. We propose that the nucleation site is composed of an acidic protein or proteins, adopting the β-sheet conformation. These proteins are in a localized area on the matrix surface. The nucleation site constitutes only a small part of the total matrix protein β-sheet

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structure formed by each individual mantle epithelium cell, but it is aligned in some specific manner with the other matrix macromolecules. The nucleation site proteins bind a number of calcium ions at specific points so that their two-dimensional distribution on the matrix surface matches an appropriate plane in the mineral crystal lattice. When this occurs, and when in addition the local concentrations of calcium and carbonate ions are sufficient, crystal nucleation will take place.

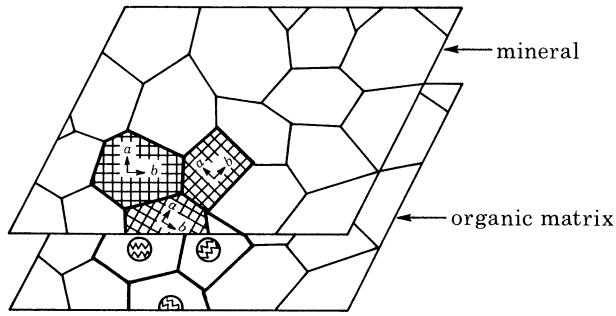

FIGURE 3. Schematic diagram illustrating the mosaic nature of the matrix and mineral sheets as deduced from the electron diffraction study of the nacreous layer of the gastropod, *Tectus dentatus* (Weiner *et al.* 1983*b*). The aragonite crystallographic axes are aligned with the polypeptide chains of the corresponding nucleation site (circles) β-sheet proteins present on the organic matrix surface.

We postulate localized nucleation sites within well-ordered and aligned macromolecular assemblies, to account for the X-ray and electron diffraction observations that show the disparity in degree of orientation between the organic and mineral phases. We envisage that in nacreous layers, where the aragonite crystals are aligned over large areas (over 1000 μm²), the nucleating-site β-sheet regions formed by each epithelium cell would also be well aligned with each other over large areas. However, the β-sheet regions outside the nucleating site have a range of different orientations and hence the diffraction patterns of the matrix are less well aligned than those of the mineral. In gastropods, the nucleation sites formed by different cells are evidently not aligned with each other and so the associated minerals grow to form a mosaic pattern (figure 3). In *Nautilus* nacreous layer, nucleation sites formed by different cells would be less well aligned with each other, as compared to the two bivalves examined, and so the resultant mineral crystals are also less well aligned. However, within the portion of matrix formed by individual cells, all the various β-sheet regions have orientations quite close to that of the nucleation sites. Hence in *Nautilus*, the X-ray diffraction patterns of the organic matrix show the same overall degree of orientation as those of the mineral.

The sheets of aragonite lamellae that form the bulk of the nacreous layer, when examined in the scanning electron microscope, are seen to be disrupted laterally by vertical organic matrix layers. The result is that each lamella is composed of a 'pavement' of polygonal-shaped mineral tablets (Gregoire 1967) (figure 1). When the aragonite is removed, the same polygonal pattern can be seen on the interlamellar matrix surfaces, which are termed 'crystal imprints' (Gregoire 1967). It is not known whether each 'crystal imprint' corresponds to the portion of matrix formed by an individual mantle epithelium cell. Crenshaw & Ristedt (1975) have demonstrated, by using *Nautilus*, that the centres of 'crystal imprints' can be differentially stained by calcium red (as well as other stains) only after the shell fragments were fixed in such a way as to stabilize the soluble acidic matrix components. This experiment amply demonstrates that the chemistry of the matrix surface is not uniform. Crenshaw & Ristedt (1975) conclude that the centres of

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these polygons contain acidic sulphated polysaccharides or sulphated proteins, or both, which are capable of binding calcium. There is no conclusive proof that aragonite nucleation occurs at the polygon centres, but the results are consistent with the general concepts we propose above, with regard to the presence of a discrete localized nucleation site.

An indirect approach to identifying some of the possible matrix macromolecules involved in crystal nucleation was recently reported by Weiner (1983). The experimental strategy

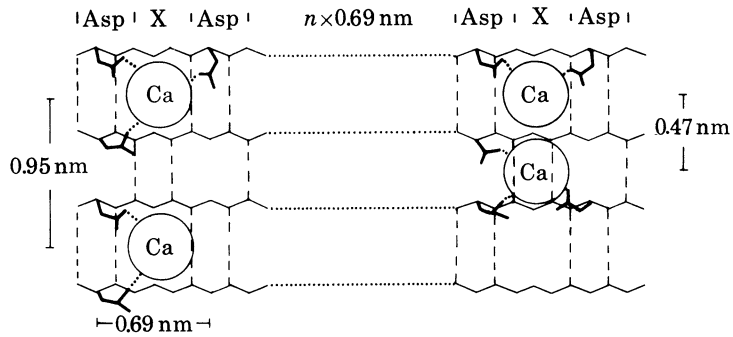

FIGURE 4. Schematic illustration of possible modes of calcium binding to the surface of a β-sheet containing aspartic acid residues. The calcium ions are liganded to 2 or 3 aspartic acid residues. The distances shown correspond to those measured by X-ray diffraction of the insoluble matrix protein fraction, which adopts the antiparallel β-sheet conformation (Weiner & Traub 1980).

adopted was to compare the assemblage of EDTA-soluble macromolecules present in the calcite layer with those of the aragonite layer from the same shell, on the assumption that macromolecules present in both shell layers perform common functions and hence cannot be responsible for the mineralogical difference, whereas those that are present in only one layer may possibly be involved in mineral nucleation. Approximately half of the 40 or so soluble matrix constitutents isolated from each of the outer prismatic and nacreous layers of the bivalve *Mytilus californianus*, by using a combination of ion-exchange and high performance liquid chromatography (Weiner 1982), were located in only one of the shell layers. Two of the proteins that were present only in the prismatic calcite layer contain a common, possibly repeating, amino acid sequence in which aspartic acid is separated by the dipeptide, Pro-Thr. The two proteins were purified to homogeneity and their apparent molecular masses were determined. So proteins of this type can be regarded as potential candidates for the nucleation site. By using this methodology antibodies can now be raised against 'candidate' nucleating proteins, which will enable us to determine, for example, whether or not a particular protein is indeed localized at the 'crystal imprint' centres.

A nucleation site constructed primarily of portions of acidic proteins adopting the β-sheet conformation has the advantage that it can form a two-dimensional surface capable of binding calcium ions in a finely regulated manner and under direct genetic control (as opposed to, for example, acidic polysaccharides). Figure 4 is a schematic illustration of part of such a site, which emphasizes that calcium binding through carboxyl groups requires the cooperativity of at least 2 or 3 ligands (Kretsinger & Nelson 1976).

Note that an amino acid sequence in which aspartic acid is separated by a single amino acid could constitute part of each calcium binding site. Significantly, amino acid sequences of this type are commonly found in the EDTA-soluble proteins of both aragonite and calcite layers of mollusc shells (Weiner & Hood 1975; Weiner 1983).

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Epitaxy in organic-matrix-mediated processes from other phyla?

No comparable information with regard to matrix–mineral spatial relations at the molecular level, exists for phyla other than molluscs. Even for the molluscs, our information is limited to just one of eight microarchitectural arrangements by which shells are formed (Boggild 1930).

In the absence of such information, one can indirectly assess the situation by comparing the complement of matrix constituents present in different mineralized tissues. Weiner *et al.* (1983*a*) report that acidic proteins are common constituents of all matrices for which information is available, whereas the insoluble more hydrophobic constituents, when present, differ considerably with respect to their biochemical properties. These observations support the notion that acidic proteins play important roles in mineralization, be it epitaxial or not.

For vertebrate mineralizing systems a vast amount of detailed information is available on the organic matrix, much of which emphasizes the importance of the soluble acidic components (or the so called non-collagenous components of bone and dentine). Many of the current proposals for the functions of these molecules, in the mineralization of bone and dentine, envisage them binding to a specific area on the surface of the collagen fibril and in turn binding calcium ions in such a way as to match an appropriate Ca–Ca spacing of the mineral lattice of hydroxyapatite (Veis *et al.* 1981; Glimcher 1981; Termine *et al.* 1981). The concepts proposed are in essence, very similar to those presented here for the organization and function of mollusc shell macromolecules in mineralization.

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Discussion

S. B. Parker (*Inorganic Chemistry Laboratory, Oxford, U.K.*). (1) From the presence of proline in the repeating amino acid sequence for calcite formation, one assumes that a β-sheet is not present. Does Dr Weiner have any idea of the structure of the organic matrix in this case?

(2) There is no reason why the calcite should align along its unit cell axis; the [111] zone consists of calcium ions 0.499 nm apart in an equilateral triangular arrangement, sitting proud of carbonate ions, which form a plane beneath them. This would, in theory, be a perfect situation for epitaxial growth of calcite from an organic template. In our work, we rarely, in fact, find the [111] zone, but often find the [421] zone, which has related properties.

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S. Weiner. (1) The two proteins from the calcite-prismatic layer of *Mytilus californianus*, which contain the -Asp-Pro-Thr-Asp- sequence, have proline contents of approximately 4 mole %. This amino acid sequence is, therefore, not predominant. We do not have any information on the conformation of these proteins *in vivo*.

(2) No direct information on the spatial relations between the matrix constituents and the mineral lattice is available for calcite shell layers. Without this, it seems premature to predict optimal arrangments as we do not understand the theory of mineral–protein interactions.

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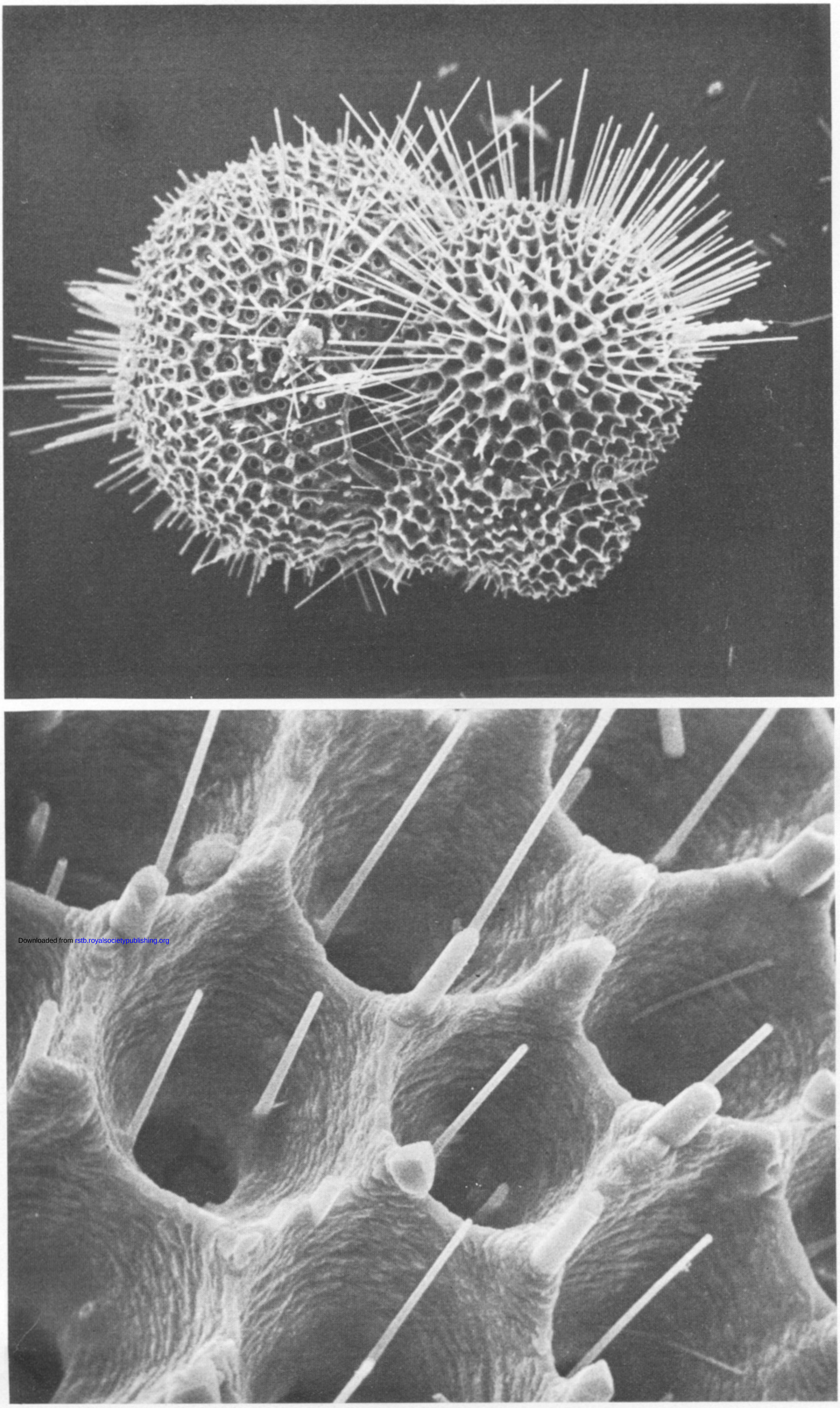

CALCIUM CARBONATES

Globigerinoides sacculifer; Globigerina is found in all tropical to subtropical oceans, and is sometimes thought to live in the surface layers.

Shell and spines are formed of calcite. The whole animal is illustrated (top) together with a more detailed micrograph of the first chamber (bottom) showing both pores and spines. This group of organisms is frequently used to determine palaeotemperatures based on the different proportions of oxygen isotopes in the shell.

The photographs for frontispieces I–IV were kindly provided by The Natural History Museum, London.

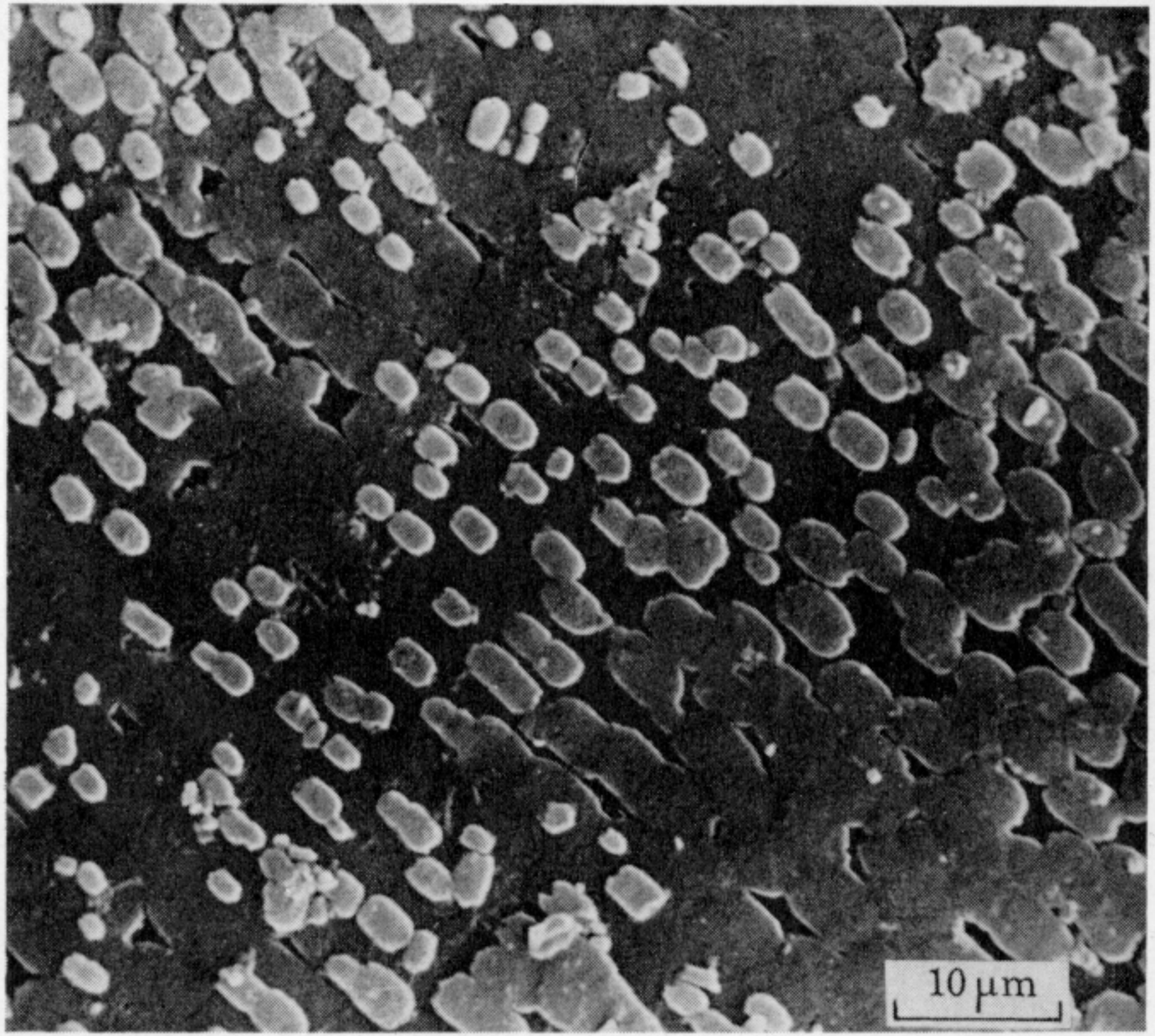

FIGURE 1. Scanning electron micrograph of the growth surface of the nacreous layer of the bivalve, *Neotrigonia margaritacea*. Note the preferred orientation of the newly-formed aragonite crystals and their merging together in the more mature areas to form a 'pavement-like' surface.

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