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Ionotropic Nucleation of Calcium Carbonate by Molluscan Matrix[1]

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SYNOPSIS. The hydrophilic, sulfated fraction of the organic matrix found in molluscan shells appears to be involved in crystal nucleation. It is located primarily at the sites of initial nucleation. The hydrophilic fraction favors *in vitro* formation of calcified deposits, when it is fixed in place on the hydrophobic fraction. Calcium is bound by the hydrophilic fraction with high affinity and selectivity. Enzymatic desulfation reduces the calcium binding. However, the binding stoichiometry of one calcium for every two ester sulfates is not altered. The calcium binding induces local anion binding, which induces secondary calcium binding. This coordinated ion binding is known as ionotropy. The resultant local high concentration of ions is thought to bring about nucleation.

INTRODUCTION

The organic matrix of molluscan shells is thought to be an essential mediator of shell formation. Professor Wilbur, in whose honor this symposium is being held, has been the primary proponent of this view (Wilbur, 1976, 1984; Wilbur and Simkiss, 1979). He has suggested that the matrix may control crystal nucleation, polymorph selection, crystal orientation, crystal growth direction and/or crystal growth inhibition. The initial nucleation event probably regulates some or all of the other processes. Thus, the determination of how the matrix induces nucleation is an important element of any description of shell formation.

In order to induce nucleation, the matrix probably first binds calcium (Wilbur, 1976). The ethylenediaminetetraacetic acid (EDTA) soluble, hydrophilic fraction of the matrix selectively binds calcium (Crenshaw, 1972*a*). Since the EDTA soluble matrix is not destroyed by treating powdered shell with hypochlorite, it must be firmly associated with the mineral (Crenshaw, 1972*a*).

The EDTA soluble matrix appears to be located at the nucleation site. Gregoire (1972) described the phylogenetic pattern of pores in matrix prepared in either EDTA or hydrochloric acid. These pores are concentrated at the nucleation site in nacre (Crenshaw and Ristedt, 1975, 1976; Iwata, 1975). However, if shells are decalcified in solutions which fix glycoproteins, the nucleation sites contain an electron dense material that binds calcium and contains sulfur and acid mucopolysaccharide (Crenshaw and Ristedt, 1975, 1976; Iwata, 1975). The difference between these preparations should be whether or not the EDTA soluble, sulfated matrix remains fixed on the insoluble, hydrophobic fraction of the matrix. However, the histochemical mucopolysaccharide methods do not differentiate between glycoproteins and glycosaminoglycans. Sulfated mucopolysaccharide has also been found in the calcifying granules that form on glass cover slips inserted between the shell and the mantle (Wada, 1964, 1980) and at the initial site of shell regeneration (Abolins-Krogis, 1958; Saleuddin and Chan, 1969). Toluidine blue displaces calcium from the regeneration sites, further indicating that the mucopolysaccharide binds calcium (Abolins-Krogis, 1958). In addition, sulfur has been found by microprobe analysis to be concentrated at the rapidly growing shell edge and in the cover slip granules (Wada, 1980). Although the relative solubilities of these sulfated macromolecules have not been studied, they are all probably similar to the EDTA soluble, sulfated matrix.

One of the models explaining how the EDTA soluble matrix induces nucleation

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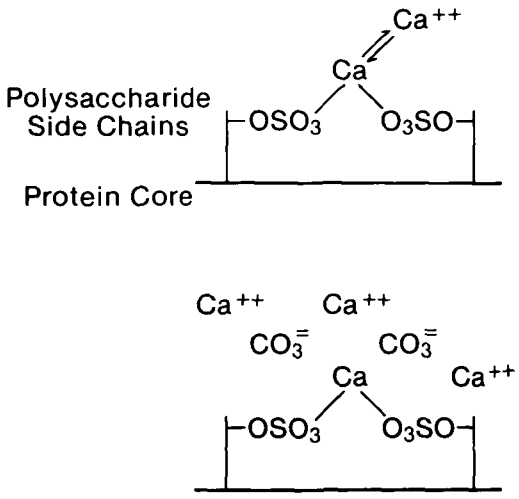

FIG. 1. Ionotropic binding. The top panel indicates the high affinity calcium binding. The lower panel indicates the carbonate and secondary calcium binding.

(Crenshaw and Ristedt, 1975; Crenshaw, 1982) is known as ionotropy (Thiele and Awad, 1969). The template model is the primary alternative to ionotropy (Weiner 1979, 1984; Weiner and Traub, 1983). The ionotropic model (Fig. 1) assumes that the calcium binding moiety is spatially fixed on a macromolecular scaffolding. The bound calcium would then attract a local concentration of those anions that form ion pairs with calcium. In solutions similar to seawater, the attracted anions would be predominantly carbonate (Crenshaw, 1982). The anions would attract a local secondary concentration of calcium ions as free ions and/or ion pairs. Nucleation results from this high local concentration of the precursor ions. Since this model does not require that the bound calcium fit a crystallographic lattice, the initial deposit would probably be amorphous. Amorphous deposits may be energetically favored in the initial events of biomineralization (Mann, 1983; Simkiss, 1984). Amorphous calcium carbonate has been found in a number of organisms, including some molluscs (Lowenstam, 1981).

Following an amorphous to crystalline phase transformation, crystal growth would ensue until it is stopped by additional matrix binding to the growth sites (Crenshaw, 1982). When it is free in solution, the EDTA soluble matrix can bind to growing crystals and inhibit crystal growth (Wheeler *et al.*, 1981; Sikes and Wheeler, 1983; Wheeler and Sikes, 1984). Thus, the matrix would act as a nucleator when fixed on a surface and as a crystal growth inhibitor when bound to crystal growth sites. Similarly, EDTA adsorbed on Formvar films (Walton *et al.*, 1967), chondroitin sulfate entrapped in polyacrylamide (de Jong *et al.*, 1980) and osteonectin bound to insolubilized collagen (Termine *et al.*, 1981) are nucleators, while all three inhibit crystal growth when in solution (Bowness and Lee, 1967; Harris *et al.*, 1969; Termine and Posner, 1970; Menanteau *et al.*, 1982).

RESULTS AND DISCUSSION

Ion binding studies

The original description of the EDTA soluble matrix demonstrated that it selectively binds calcium in the presence of large excesses of magnesium, sodium or potassium (Crenshaw, 1972*a*). Flow rate dialysis (Womack and Colowick, 1973; Crenshaw and Bawden, 1981) has been used to further investigate ion binding by the EDTA soluble matrix isolated from *Mercenaria mercenaria* shells. In the presence of 0.5 *M* sodium chloride, there are 0.67 μmoles of high affinity ($k = 1.3 \times 10^{-5}$ *M*) calcium binding sites per mg of matrix (Fig. 2A). This corresponds to approximately 100 tightly bound calcium ions per molecule, assuming a molecular weight of 160,000 (Crenshaw, 1972a; Wheeler *et al.*, 1981. However, see Samata and Krampitz, 1982; Weiner, 1983; Wheeler and Sikes, 1984). Reducing the salt concentration to 0.1 *M*, reveals a second class of 1.2 μmoles low affinity sites per mg ($k = 2.8 \times 10^{-4}$ *M*) (Fig. 2B). Since this binding is inhibited by sodium chloride, it is less selective than the high affinity binding and would not occur in extrapallial fluid.

The EDTA soluble matrix also binds phosphate, but only in the presence of calcium (Fig. 3). The nonlinear Scatchard plot indicates the presence of either cooperative binding or multiple types of sites.

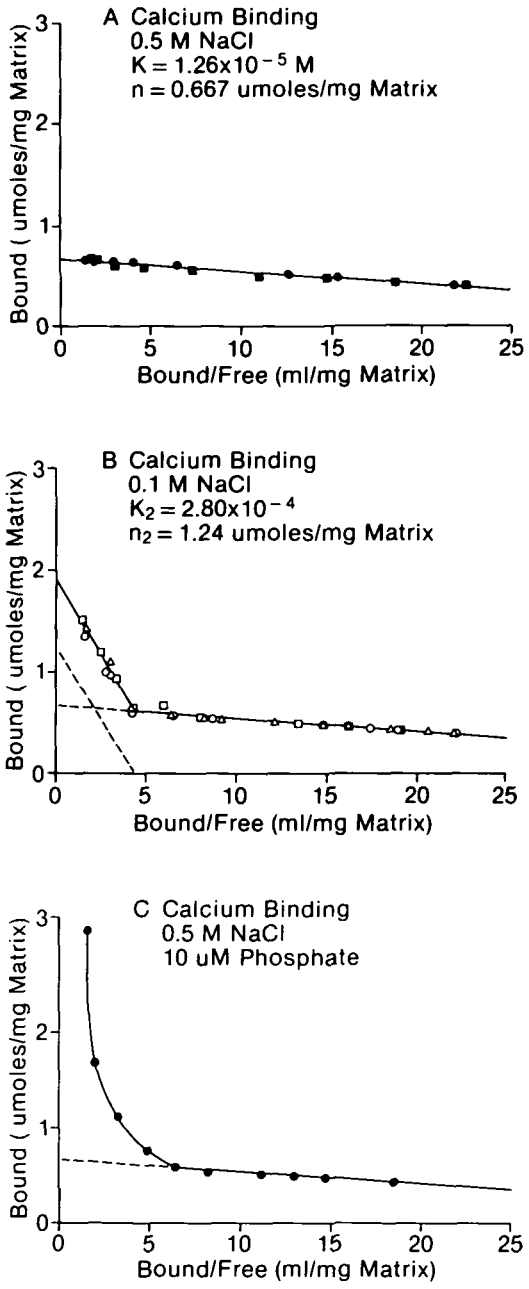

FIG. 2. Scatchard plots of calcium binding (50 m*M* Tris-Cl, pH 8). The differently shaped symbols indicate replicate runs. A. 0.5 *M* sodium chloride. The solid line is a fitted linear regression. B. 0.1 *M* sodium chloride. The high affinity line is reproduced from Fig. 3A. The low affinity line portion of the solid line is a linear regression and the low affinity dashed line has had the high affinity binding subtracted out. C. 0.5 *M* sodium chloride plus 10 μM phosphate. The high affinity line is reproduced from Figure 3A.

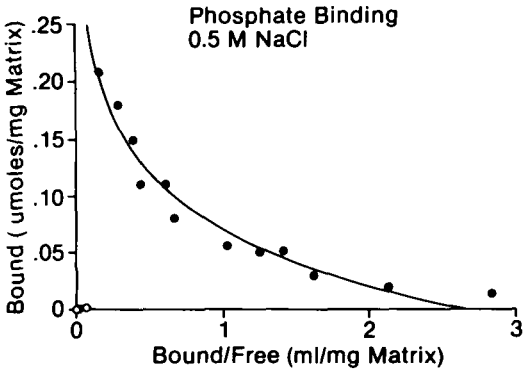

FIG. 3. Scatchard plot of phosphate binding (0.5 *M* sodium chloride, 50 m*M* Tris-Cl, pH 8). Solid circles represent calcium loaded matrix (0.63 μmoles calcium/mg matrix), open circles calcium free matrix.

Phosphate was used to demonstrate anion binding rather than carbonate to avoid exchange with the atmosphere.

Calcium binding is increased considerably by including 10 μM phosphate in the 0.5 *M* sodium chloride (Fig. 2C). This new low affinity binding is distinct from the low affinity binding found in the 0.1 *M* salt. It occurs in 0.5 *M* sodium chloride, involves considerably more calcium, has a much lower affinity and a nonlinear Scatchard plot. Thus, calcium binding by the EDTA soluble matrix induces anion binding, which induces secondary calcium binding as required by the model (Fig. 1).

Similarly, calcium binding to chondroitin sulfate in cartilage induces phosphate binding, which induces secondary calcium binding (Boyd and Neuman, 1951). In addition, phosphate increases calcium binding to the acidic phospholipids extracted from bone and calcifying tumors (Cotmore *et al.*, 1971; Anghileri, 1972). This ion binding appears to be the first step in acidic phospholipid induced hydroxyapatite formation from metastable solutions (Boskey and Posner, 1977).

Weiner (1979, 1983) has shown that the EDTA soluble matrix reacts with carbodiimides. Therefore, he argued that the aspartic acid residues in the EDTA soluble matrix contain free β-carboxyl groups and may be involved in calcium binding. The template model of nucleation requires that

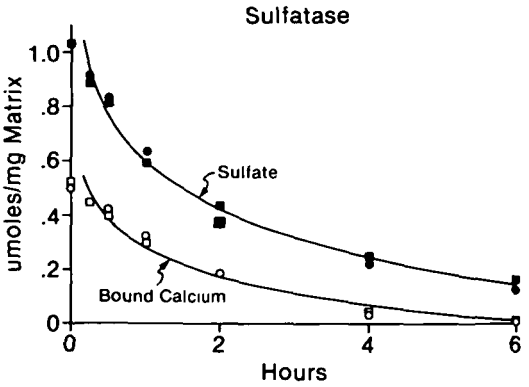

FIG. 4. Incubation with limpet digestive gland arylsulfatase. Digestive glands were homogenized in 1 *M* sodium chloride (Hatanaka *et al.*, 1975), the 30–60% ammonium sulfate fraction (Nishida-Fukuda and Egami, 1970) applied to a Sepharose-Concanavalin A affinity column and eluted with mannose. The final preparation contained 18 units per mg of protein, as measured with p-nitrophenol sulfate. It did not contain measurable proteolytic activity against casein or bovine serum albumin in the ninhydrin assay. The EDTA soluble matrix (1 mg/ml) was incubated with this sulfatase (10 μg/ml). At intervals, aliquots were desalted on a Biogel P-2 column and the total bound calcium and sulfate determined as previously described (Crenshaw, 1972*a*). Solid symbols represent remaining sulfate, open symbols bound calcium.

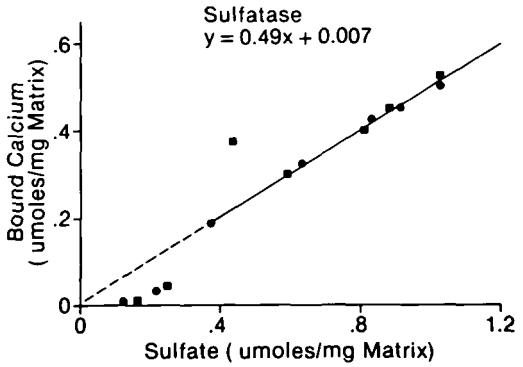

FIG. 5. Bound calcium to remaining sulfate ratios during desulfation. The line is a fitted linear regression omitting the aberrant point above the line and the points at less than 0.3 μmoles sulfate per mg matrix.

the calcium binding aspartic acids be arranged so as to match the crystallographic lattice (Weiner, 1979, 1984; Weiner and Traub, 1983). However, the EDTA soluble matrix contains enough amide nitrogen so that most of the aspartic acid is amidated and thus, unavailable for calcium binding (Crenshaw, 1972*a*). Further, the EDTA soluble matrix contains groups other than free carboxyls which may react with carbodiimides. Under some conditions, carbodiimides react with sulfates (Khorana, 1953), which make up 10 per cent of the EDTA soluble matrix. Carbodiimides may also crosslink proteins and EGTA (Walsh and Stevens, 1977). Similar crosslinking may have occurred between the EDTA soluble matrix and the EDTA that exists in the preparations (Weiner, 1979, 1983).

The calcium appears to be bound to the ester sulfates on the EDTA soluble matrix, rather than the aspartic acid residues. Incubation with limpet (*Diodora cayenensis*) digestive gland arylsulfatase reduces the amount of both ester sulfate and calcium bound to the EDTA soluble matrix (Fig. 4). No proteolysis could be detected with ninhydrin during the desulfation. One mole of calcium is bound for every two moles of sulfate remaining on the EDTA soluble matrix (Fig. 5). This stoichiometry is the same as that previously found with the intact EDTA soluble matrix (Crenshaw, 1972*a*) and a cartilage glycoprotein (Woodward and Davidson, 1968). The constant ratio over a large range of sulfate concentrations indicates that the sulfates are directly involved in the calcium binding, rather than indirectly through a conformational change. However, at very low levels of sulfate (<0.3 μmoles per mg matrix) much less calcium is bound than would be expected. The remaining sulfates may not be physically close enough to one another to allow two of them to interact in order to bind a single calcium atom. The elimination of calcium binding by desulfation and the failure of magnesium to inhibit calcium binding (Crenshaw, 1972*a*) indicate that the binding is not due to the presence of EDTA as a contaminant in the soluble matrix preparation (cf. Weiner, 1979, 1983; Wheeler and Sikes, 1984).

Nucleation studies

Macromolecules that induce nucleation *in vivo* should also favor *in vitro* crystal for-

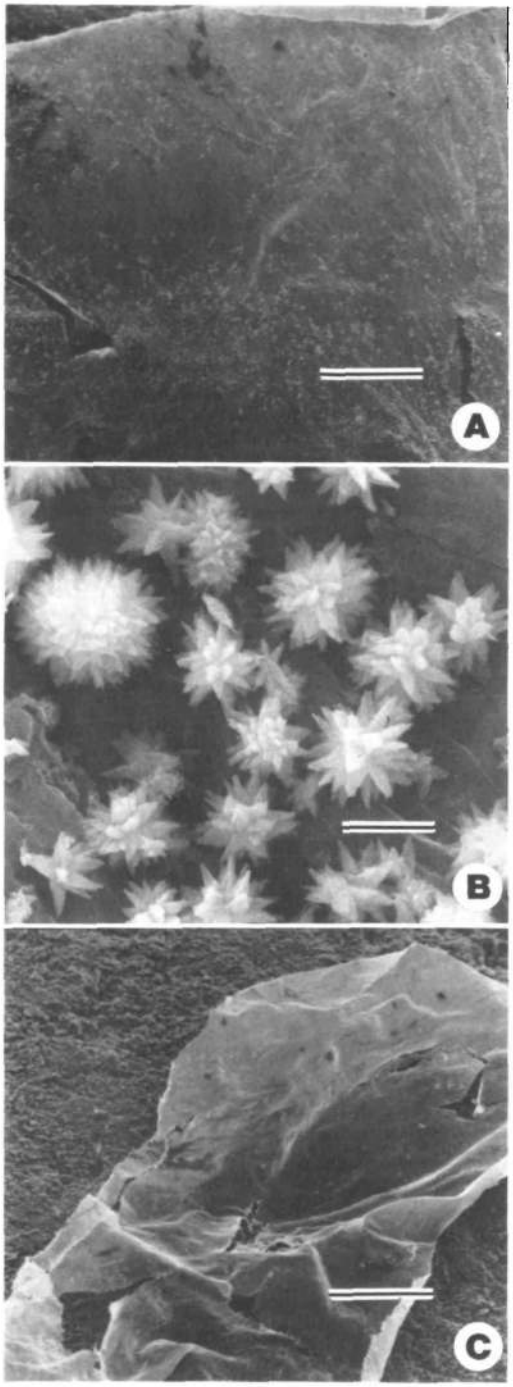

FIG. 6. Scanning electron micrographs of crystals deposited on *Nautilus* matrix preparations during 3.5 hr incubations in metastable solutions as described in the text. A. Complete matrix was prepared by partially decalcifying nacre in 0.5% cetylpyridinium chloride, 0.1 *M* EDTA (pH 8) for 15 min (Crenshaw and Ristedt, 1975). Matrix sheets were teased off from the side of the septum which had faced the mantle and briefly rinsed in distilled water. The sheets were transferred to solutions containing calcium and magnesium chlorides. The incubation was initiated by adding freshly prepared 0.1 *M* sodium bicarbonate to make the solution 5 m*M*. After 3.5 hr, the matrix was briefly rinsed in dilute ammonium hydroxide and then in acetone, mounted on a carbon stub, carbon coated, examined by energy dispersive analysis and then sputter coated with gold. The bar denotes 500 μm. B. Same matrix as in A. The bar denotes 2 μm. C. Matrix without the EDTA soluble fraction was prepared as in A, except that it was incubated in 0.5 *M* EDTA (pH 8) overnight after being teased off of the nacre (Crenshaw and Ristedt, 1975). The bar denotes 500 μm.

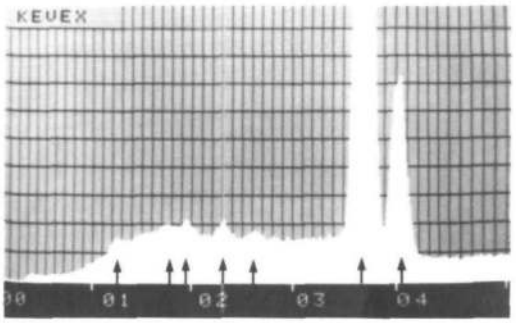

FIG. 7. Energy dispersive analysis of a crystal formed on the matrix. Calcium is represented by the two large peaks at 3.69 and 4.01 kV. Magnesium, sulfur and chloride are at 1.25, 2.31 and 2.62 kV respectively. The silicon peak at 1.74 kV is derived from the detector. The peak at 1.95 kV is a silicon escape peak from the 3.69 kV calcium emission.

mation. In studies of this type, the investigation of proper control molecules is crucial and often overlooked. Accordingly, *Nautilus pompilus* septal nacre matrix was prepared with the EDTA soluble fraction fixed in place or removed. These matrices were incubated in metastable solutions containing 10 m*M* calcium chloride, 5 m*M* sodium bicarbonate and 30 m*M* magnesium chloride (extrapallial fluid contains approximately 11, 5 and 60 m*M* respectively, Crenshaw, 1972*b*). After a 3.5 hr incubation, the complete matrix contained numerous 1–2 μm, spherulitic crystals (Fig. 6A, B). However, the matrix without the EDTA soluble fraction contained only a

few (Fig. 6C). Thus, the EDTA soluble matrix appears to favor crystal nucleation and growth, probably by ionotropy. This difference was not observed in the absence of magnesium, a calcium carbonate nucleation inhibitor (Pytkowicz, 1965). Therefore, the effect probably depends on the EDTA soluble matrix selectively binding calcium (Crenshaw, 1972*a*), thus escaping the inhibition due to magnesium.

Energy dispersive analysis confirmed that the crystals contain calcium (Fig. 7). Unfortunately, this method cannot detect carbonate. Small amounts of sulfur, magnesium and chloride were also present. The sulfur should be derived from the matrix and the other two ions from the incubation solution. In the absence of crystals, the matrix contained only a small amount of calcium. The remainder of the spectrum was similar to the one from the crystal.

Phosphoryn

The molluscan EDTA soluble matrix seems able to induce nucleation by ionotropy. In addition, macromolecules with similar properties have been found in other calcified tissues. A number of these have been discussed above. However, the best studied of the group is phosphoryn, the principle noncollagenous protein in mammallian dentin. Calcium binding by phosphoryn has been shown by nuclear magnetic resonance (NMR), infrared spectra and stoichiometric analysis to involve both phosphoserine and aspartic acid residues (Lee *et al.*, 1977; Cookson *et al.*, 1980; Lee and Veis, 1980). Enzymatic dephosphorylation significantly reduced the calcium binding (Lee and Veis, 1980; Zanetti *et al.*, 1981). Recent NMR studies indicate that calcium binding induces phosphate binding (Lee *et al.*, 1983). Secondary calcium binding has not been investigated.

Phosphoryn is bound to collagen (Veis *et al.*, 1981; Linde *et al.*, 1981) at the mineralization front (Weinstock and Leblond, 1973). In some solutions, phosphoryn aggregates and becomes insoluble (Lee *et al.*, 1977; Kuboki *et al.*, 1979; Cookson *et al.*, 1980; Lee and Veis, 1980). Under conditions where it would be expected to aggregate (Lee and Veis, 1980), phosphoryn induces hydroxyapatite formation (Nawrot *et al.*, 1976). Partial dephosphorylation eliminated the activity. However, nonaggregated phosphoryn binds to hydroxyapatite and, thus inhibits nucleation and growth (Termine *et al.*, 1980). Thus, phosphoryn appears to function in a manner very similar to that proposed for the molluscan EDTA soluble matrix.

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