Potent Natural Inhibitors of $CaCO_3$ Crystallization From Chalk Deposits[1]

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ABSTRACT. Fossilized calcareous structures in chalk collected from exposures of the Selma formation in Alabama yielded organic components comparable to the organic matrices of the calcium carbonate ($CaCO_3$) shells of sea urchin and oyster. The organic matrix of the chalk was composed of 0.05% soluble protein and 0.17% soluble carbohydrate per dry weight. The possible role of the organic matrix as a regulator of $CaCO_3$ formation was tested by use of two crystallization assays, the nucleation assay and constant composition assay. Results of the nucleation assay showed total inhibition of crystallization at 0.25 ug protein/ml and partial inhibition at 0.025 ug protein/ml. However, the Selma extract was not an inhibitor of the rate of crystal growth, once crystals had already formed, as shown by use of the constant composition assay. Analysis of the chalk extract by high performance liquid chromatography yielded distinct peaks in UV absorbance, indicating that it may contain more than one presumably protein component. In addition, the relatively high content of carbohydrates in the chalk extract suggests that some of the inhibitory activity may be due to the presence of these materials.

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INTRODUCTION

Calcified structures from biologically formed minerals normally contain a small amount of organic material called matrix. Possible ways that the organic matrix might participate in calcium carbonate ($CaCO_3$) deposition have been discussed extensively (e.g., Crenshaw 1982, Krampitz et al. 1983, Mann 1983, Greenfield et al. 1984, Weiner 1984). Following the discovery that the organic matrix from oyster shell contained a component that is a potent inhibitor of $CaCO_3$ formation (Wheeler et al. 1981), a number of studies have centered on the possible role of matrix as a regulator of crystallization that may limit and shape crystal growth (Sikes and Wheeler 1983, 1985, 1986a, Wheeler and Sikes 1984, 1985, Wilbur and Bernhardt 1984, Wheeler et al. 1986). These studies have focused mainly on molluscan shell matrix, particularly oyster shell matrix. However, a few other studies have examined the structure and function of matrices from other $CaCO_3$ biominerals. For example, de Jong et al. (1976a, 1976b, 1979, 1983) studied the matrix from coccoliths and reported that it is wholly polysaccharide in structure. This seemed unusual in that matrices from other biominerals are primarily proteinaceous (Weiner et al. 1983). Later, the coccolith matrix was shown to also have the capacity to inhibit $CaCO_3$ deposition (Borman et al. 1982, Sikes and Wheeler 1986b). In another study, Swift et al. (1986) examined the matrix from sea urchin tests and reported that it is a proteinaceous material that also has high levels of activity as an inhibitor of crystallization.

The purpose of the present study was to extend these observations to include matrix material from fossil deposits. Although there have been studies showing that matrices may be preserved in fossils (Weiner et al. 1976, de Jong et al. 1976a, Westbroek et al. 1983), there has been no evidence that fossil matrices retain their functions. Along these lines, we selected chalk samples from the Selma deposit in Alabama for study. The Selma chalk is comprised of deposits formed during the Cretaceous period of the Mesozoic some 65 to 135 million years ago. It contains the fossilized remains of many types of creatures including foraminiferans and large shells of the molluscan families Ostreidae and Anomiidae (Adams et al. 1926, Monroe 1941). Organic matrix from this chalk was extracted and characterized with respect to composition and possible function.

METHODS AND MATERIALS

EXTRACTION. In this study, a chalk sample was obtained from exposures of the Selma formation in Alabama. After the sample was crushed by hand, it was sifted on a screen (0.5 mm mesh) to remove organic debris-like fragments of leaves and twigs. The chalk was then ground to a fine powder in an electric mill. Ten grams of the powder were made into a slurry with distilled water in dialysis tubing (Spectrapor, 20,000 MW cutoff). Prior studies (e.g., Crenshaw 1982, Krampitz et al. 1983, Weiner et al. 1983, Wheeler et al. 1986) indicated that the components of matrix are retained within this tubing. The powder was dialyzed against 4 liters of 2% acetic acid for 24-48 hours, or until no chalk remained. Stirring was accomplished by attaching the tubing to a rotating cam that vertically displaced the tubing in the acid bath. The resulting extract was dialyzed two times against 4 liters of distilled water for four hours to remove the acetic acid and inorganic ions. The extract was neutralized (pH 7.50 ± 0.02) with 1N NaOH. Seperation of the soluble component from insoluble residue was accomplished by centrifugation at 3220 X g for 15 minutes with a Beckman Model J2-21 Centrifuge.

CHARACTERIZING THE EXTRACT. Protein concentration was determined by the Lowry assay (Lowry et.al. 1951). Proteinaceous matrices frequently are unreactive to the Bradford reagent (Bradford 1976); however, comparison of direct weighing of lyophilized oyster shell matrix to estimates of Lowry protein yielded a good correlation (Wheeler, pers. comm.). Carbohydrate was measured with the method developed by Dubois et. al. (1956). Standards ranged from 0-70 ug starch/ml in duplicate. To 1.0 ml of standard or sample, 1 ml of 5% phenol, and 5 ml of 96.5% sulfuric acid were added. The preparations were allowed to stand for 10 minutes before shaking and then placed in a water bath at 25-30°C for 10-20 minutes to let the solutions stabilize. Absorbance was read at 490 nm with 1-cm cells on a Perkin Elmer Model 160 spectrophotometer. Concentrations of protein and carbohydrate were calculated from their respective standard curves.

NUCLEATION ASSAY. Nucleation and growth of $CaCO_3$ crystals were traced in a curve reflecting pH. Constant pH is characteristic of the nucleation phase prior to crystal growth. Crystal growth produces a decrease in pH as CO_3^{2-} is removed from solution. A closed vessel containing 29.1 ml artificial sea water (0.500 M NaCl and 0.011 M KCl) was held in a water bath maintained at a constant temperature of 20.0 C (±0.1 C). The solution remained stable upon addition of 0.3 ml of 1.0 M $CaCl_2$ to produce a concentration of 10 mM Ca^{2+}. Addition of 0.6 ml of 0.5 M $NaHCO_3$ to yield 10 mM dissolved inorganic carbon (DIC) initiated the experiment. The solution was titrated to a pH of 8.30 (±0.02) with 1N NaOH, and then

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monitored until crystal growth ended. When the inhibitors were tested, they were added 10 minutes prior to the addition of $NaHCO_3$. The reaction was monitored by use of a pH electrode (Orion 901) and recorded by strip chart (Perkin Elmer, model 56).

CONSTANT COMPOSITION ASSAY. Calcium and carbonate ions can be replaced by automatic titration, as they are removed from solution as crystals form. This matches more closely real situations in which Ca^{2+} and CO_3^{2-} ions are supplied continuously. The pH also does not change very much during crystal growth. An assay based on this principle was performed as follows. The water-jacketed vessel was maintained at 20.0 C (±0.1 C). To 48.5 ml of artificial sea water, 0.5 ml of 1.0 M $CaCl_2$ and 1.0 ml of 0.5 M of $NaHCO_3$ were added to yield a solution of 10 mM Ca^{2+} and 10 mM DIC. The pH was adjusted to 8.34 (±0.02) by addition of 1N NaOH. In control curves without an inhibitor, pH began to drift downward after an initial period of about six minutes during which nucleation of $CaCO_3$ crystals occurred. However, pH was held constant at 8.30 (±0.04) by automatic titration of equal doses of 0.5 M $NaHCO_3$ and 0.5 M $CaCl_2$ from separate burettes. In experiments on inhibition of crystallization, the inhibitors were added when the pH had first reached 8.30. This allowed some crystals to form in the solution prior to introduction of the inhibitor, so that the effect of the inhibitor on rate of crystal formation instead of the effect on crystal nucleation was featured. A curve showing volume of the titrant versus time was generated. A computer-assisted titrimeter (Fisher Scientific), specially modified for simultaneous titration from two burettes, was used to perform these assays.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC). The protein component was separated into fractions with a liquid chromatograph (Varian 5560) equipped with a 15-cm, C-18 reversed phase column (Weiner 1982). A continuous gradient was generated that consisted of initial levels of 40% sodium acetate at a pH of 6.5 and 60% acetonitrile and final levels of 20% and 80%, respectively. The period of separation was five minutes with a flow rate of 0.2 ml/min. A filtered sample of chalk extract of 100 ul was manually injected via a Valco valve; the gradient program was begun simultaneously. Absorbance was measured by a UV detector set at 235 nm and was recorded on diskette (Chromlab Version 1.0 Chromatographic Data Collection and Data Reduction, Data Translation Inc.).

RESULTS

The extract consisted of a soluble component and an insoluble component. The insoluble material was not analyzed further in this study. The soluble component comprised 0.22% per dry weight of the chalk material, 0.05% soluble protein per dry weight, and 0.17% soluble carbohydrate per dry weight (Table 1). A comparison of the composition of the Selma extract to that of the matrices of shells of the sea urchin and oyster is also shown in Table 1. The data show a reversal of the relative concentrations of protein and carbohydrate in the organic matrix of the Selma extract in reference to the others.

The Selma chalk extract was a powerful inhibitor of crystal nucleation (Fig. 1). Nucleation was inhibited completely at 0.25 ug protein/ml. Partial inhibition was observed at 0.025 ug protein/ml, with an induction phase of 64.0 ± 17.0 (SD, N = 4) minutes. A lower concentration of 0.016 ug protein/ml also showed inhibition with an induction phase of 25.0 ± 0.7 (N = 3) minutes. Inhibition curves were compared with a control curve whose induction phase was 10.30 ± 1.46 (N = 10) minutes.

In the constant composition assay (Fig. 2), it was shown that hydroxyethylidine diphosphonate (HEDP) accelerated crystal growth above that of the control values. The effect of the Selma chalk extract on the rate of $CaCO_3$ deposition appeared to be similar to that of HEDP. The time axis in Figure 2 was set such that time zero represents the time at which crystals began to grow as evidenced by the onset of autotitration. In this way,

TABLE 1

The composition of various inhibitors given as mean percentage of total organic matter (SD < 10%).

Inhibitor	Protein (%)	Carbohydrate (%)	Molecular weight* 10^3 daltons
Selma chalk extract	24.7	75.3	30**
Sea urchin extract***	96.4	3.6	170
Oyster shell extract****	91.9	8.1	15 to 170
HEDP	—	—	0.208

*Determined by gel electrophoresis and gel filtration (Wheeler et. al. 1986).
**Wheeler (pers. comm.).
***Swift et. al. (1986).
****Wheeler et. al. (1981, 1986).

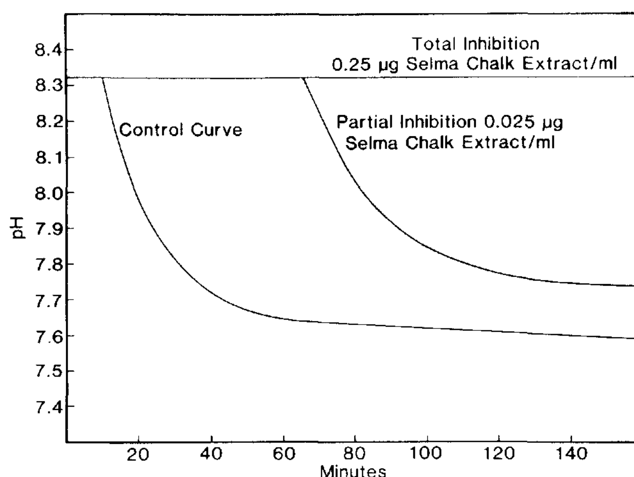

FIGURE 1. Recorder tracings of the nucleation assay showing the inhibition of $CaCO_3$ formation in the presence of Selma chalk extract.

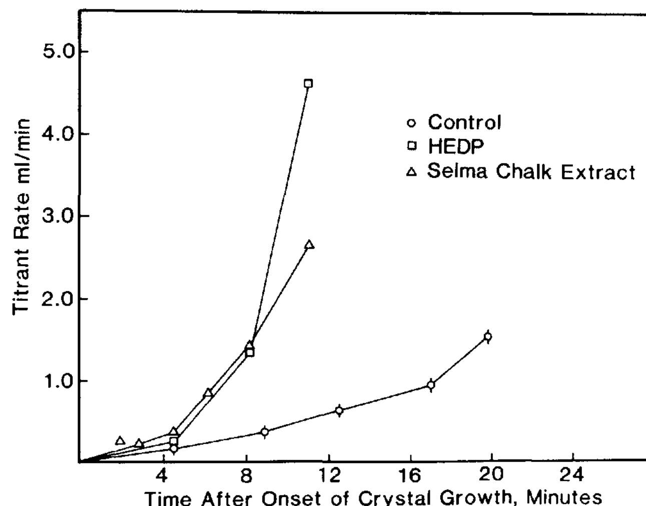

FIGURE 2. Rate of $CaCO_3$ crystal growth in the presence of Selma chalk extract (0.73 ug protein/ml) and HEDP (0.1 ug/ml) in the constant composition assay. In these assays, the concentration of inhibitor was chosen to yield a phase prior to crystal growth of about 20 minutes.

the effect of the inhibitors on the rate of crystallization could be compared directly.

Separation of the protein component of the chalk extract by HPLC resulted in two peaks (Fig. 3). Retention time characteristic of peak 1 was 4.37 ± 0.58 (N = 3) minutes and of peak 2 was 5.29 ± 0.49 (N = 3) minutes. The chromatogram indicates the presence of several fractions associated with the organic matrix from the Selma chalk.

DISCUSSION

The organic material from the Selma chalk, which is an inhibitor of $CaCO_3$ crystal nucleation, is among the most potent known materials of this type. On a weight basis, it compares favorably with HEDP as an inhibitor of nucleation. In assays similar to those employed in this study, HEDP was the most effective inhibitor of $CaCO_3$ nucleation. Total inhibition was observed at 0.06 ug/ml (Sikes and Wheeler 1985a), as compared to between 0.025 and 0.25 ug/ml for the Selma matrix (Fig. 1). This phosphonate, HEDP, is perhaps the most widely employed inhibitor of $CaCO_3$ crystallization throughout the world (Monsanto 1973), where it is added to water as a scale and corrosion inhibitor among many other uses (Drake et al. 1983). Discovery of the biopolymer crystallization inhibitors (Termine et al. 1976, Wheeler et al. 1981), of which the Selma chalk extract is an example, has resulted in the identification of equally effective and more environmentally sound water-treatment chemicals for control of crystallization on the surfaces of cooling towers, heat-exchangers, boilers, scrubbers, and similar devices (Sikes and Wheeler 1985, 1986b, Wheeler and Sikes 1985).

Interestingly, in spite of their utility as inhibitors of the formation of crystal nuclei, neither HEDP nor the Selma extract were effective at slowing the rate of crystal growth under the conditions of the constant composition assay. At the levels tested, both of these materials seemed to actually enhance $CaCO_3$ crystallization once it had begun. The explanation for this phenomenon is not obvious, although it may be that at a concentration of inhibitor that is too low to stop nucleation altogether, the crystals that form are distorted such that the surface available for crystal growth is increased (Nancollas 1974). This phenomenon requires further study.

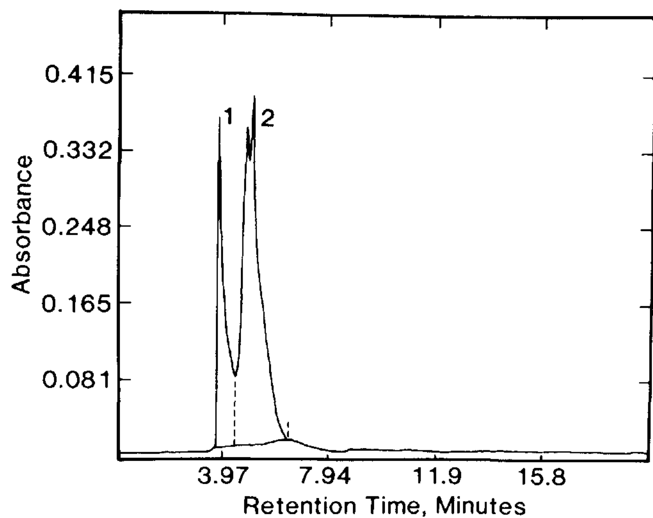

FIGURE 3. HPLC chromatogram with a C-18 reversed phase column and UV detector set at 235 nm. Peak 1 represented 30% or 2.86 ug of protein; Peak 2, 70% or 6.68 ug of protein.

In contrast, neither the organic matrix from oyster shell nor polyaspartate were observed to enhance the rate of $CaCO_3$ formation in the constant composition assay in another study (Sikes and Wheeler 1986c). In fact, these polypeptide inhibitors were observed to suppress strongly both crystal nucleation and crystal growth. It is possible that the Selma matrix is a qualitatively different material than the oyster shell matrix. The Selma matrix clearly contained more polysaccharides than the oyster matrix, which in purified form is almost wholly proteinaceous (Wheeler and Sikes 1986). Alternatively, it is possible that the mixture of components in the extract from the Selma chalk interacted with each other to produce either synergistic or antagonistic effects. For example, the high activity of the Selma matrix with regard to crystal nucleation may have resulted in part from synergism between the proteinaceous and polysaccharide components. Effects like this have been reported between different types of inhibitors (Krueger et al. 1974). Although antagonism between inhibitors that would produce an enhancement of crystal growth has not been reported, it will be interesting to isolate the components of the extract from Selma chalk and determine their individual and collective effects.

This process has been started with the HPLC technique. There are at least two distinct, UV-absorbing molecules, presumably proteinaceous components, from the matrix of the Selma chalk. The composition of the polysaccharide portion is being studied currently with the HPLC coupled to a refractive index detector.

The high polysaccharide content of the Selma chalk extract may be due to the presence of coccoliths that are known to have a polysaccharide matrix (de Jong et al. op. cit.), and that are commonly observed in chalk deposits. However, the Selma chalk is also characterized by an abundance of fossilized foraminiferans (Adams et al. 1926, Monroe 1941). The makeup of the organic matrix from the tests of forams has not been studied extensively, but there have been reports of both proteinaceous and polysaccharide components in foram matrix (Weiner et al. 1983). Consequently, it is also likely that some of the polysaccharides from the Selma chalk are attributable to this source. On the other hand, the matrix from molluscan shell, another component of the Selma chalk, has been observed routinely to be predominantly proteinaceous and not polysaccharide (op. cit.).

The relative amounts of protein and polysaccharide contained presently in the Selma chalk certainly do not reflect the amounts originally present when the deposit was formed during the Cretaceous. In fact, they represent some of the few organic remainders of the creatures of that period. Thus, it seems somewhat remarkable that these otherwise labile molecules are preserved over the years, as has been reported previously (de Jong et al. 1976a, Weiner et al. 1976, Westbroek et al. 1983), and also retain some of their function as shown in this study. The preservation of these molecules seems to indicate that they are protected by the crystal lattice from oxidation, and therefore are deployed in an intracrystalline fashion.

The actual location of organic matrix within biominerals has been discussed in relation to possible functions of matrix. On the one hand, electron micrographs often reveal a periodic secretion of layers of matrix between tablets of crystals (e.g., Bevelander and Nakahara 1969, 1980). This morphology usually has been interpreted as evidence for one organic layer acting as a template for initiation of crystallization, with the other perhaps acting to limit it. Another possibility that could occur, in addition to the periodic secretion of specific layers of matrix by organisms, is the continual secretion of matrix that becomes incorporated into crystals continuously. The existence of an intracrystalline matrix has been described by a number of authors (e.g., Watabe 1965, Crenshaw 1972, and Westbroek 1973) and, as discussed by Wheeler and Sikes (1984), might be expected for a class of matrix that controls crystal growth and structure. These regulatory proteins and polysaccharides would seem to be the most likely type of matrix to be recovered from calcareous fossils.

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