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Biopolymer-mediated Ca and Mn accumulation and biomineralization

P. Westbroek, E.W. de Vrind-de Jong, P. van der Wal, A.H. Borman & J.P.M. de Vrind
Department of Biochemistry, University of Leiden, Wassenaarseweg 64, 2333 AL Leiden, The Netherlands

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Abstract

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Various metals are known to be accumulated by both intra- and extracellular biopolymers. Such accumulations may lead to the formation of biominerals, whereby the biopolymers may assume a regulatory role in crystallization processes. Two examples are discussed that are studied in our laboratory, calcium and manganese. *Emiliania huxleyi* is a unicellular marine alga that produces calcite discs – coccoliths – within an intracellular vacuole. Much attention has been given to the probable involvement of a complex acidic polysaccharide that is associated with the crystalline phase. *E. huxleyi* is the most productive lime-secreting species on earth and is likely to play an important role in the oceanic carbon cycle. Manganese-oxidizing bacteria occur associated with recent sedimentary Mn-deposits. Manganese oxidation in two species is briefly discussed. There are strong indications suggesting that extracellular biopolymers regulate the binding of reduced manganese and its subsequent oxidation. Biopolymer-mediated metal accumulation and biomineralization occur at a huge scale in the biosphere, and hence may have important geological implications. The processes may be utilized for the reclamation of heavy metals from natural and polluted environments, for the prevention of 'scaling' in industrial practice, and for the treatment of pathological (de)mineralizations in humans.

Introduction

In the biopolymer-dominated microcosm that is a living cell, metal ions behave in a different fashion than in the inorganic world: they become part of the biological organization (Fig. 1). Some are needed in bulk amounts (notably sodium, potassium, calcium, and magnesium), others (e.g. iron, manganese, copper, zinc, and molybdenum) can only function at trace concentrations, while metals such as cadmium, mercury, lead or uranium are generally considered to be non-functional and harmful even in very small amounts. The useful metals perform a wide array of vital functions. They may be bound to the active site of enzymes and play a key role in the catalytic cycle, or else be used for regulatory, osmotic, or structural purposes.

The maintenance of optimum intracellular concentrations of the useful elements and the removal of redundant or toxic metals is a major function of the cellular organization if it is to survive fluctua-

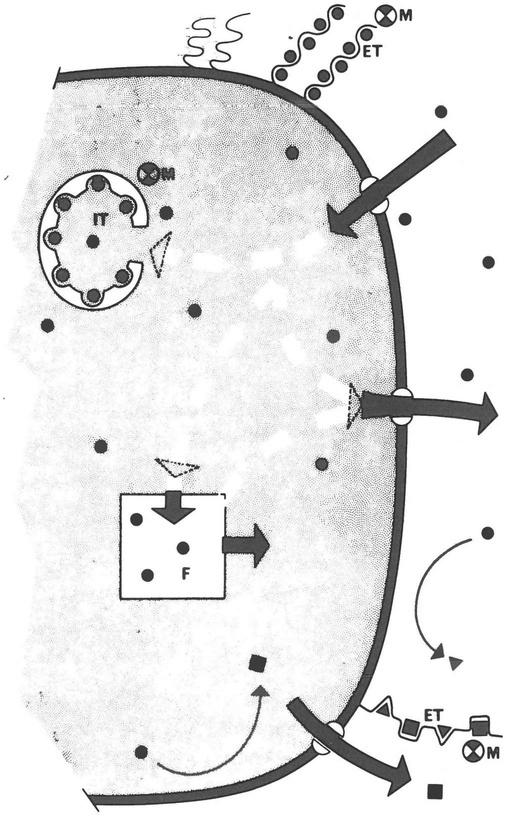

Fig. 1. Some interactions between a metal and a simple cell. F: compartment or site where the metal performs a function; IT: intracellular metal trap; ET: extracellular metal trap. ●→■: intracellular transformation of metal; ●→▲: extracellular transformation of metal; transformation includes alkylations, dealkylations, oxidations, reductions, etc. ⊗: possible site of metal accumulation; M: possible site of mineralization.

ting environmental conditions. The cell membrane, a non-polar lipid bilayer that separates the intracellular space from the environment, is a major barrier to metal ions. The latter can only pass into and out of the cell through highly selective openings provided by transport macromolecules that are embedded in the membrane. In addition, intra- as well as extracellular macromolecular 'metal traps' exist, capable of scavenging the unwanted elements. These may give rise to considerable local accumulations of metals, even when the latter occur at very low ambient concentrations. For reviews on this subject see, e.g., Kelly et al. (1979), Brown & Lester (1979), and Brierley (1982).

Cell wall fragments of the gram-positive bacteria *Bacillus subtilis* and *B. Licheniformis* bind substantial amounts of various metal ions at different extracellular macromolecules (teichoic acid, teichuronic acid, peptidoglycan); the binding of Au(I) results in the deposition of elementary gold (Beveridge & Murray 1980, Beveridge et al. 1982). In the cell envelopes of the gram-negative *Escherichia coli* K12 also the lipopolysaccharides, phospholipids, and proteins of the outer membrane are possible metal chelators (Beveridge & Koval 1981, Hoyle & Beveridge 1983). Extracellular polymers of *Klebsiella aerogenes* compound Cu, Ni, Mn, and Co. Extraction of these polymers reduces the capability of the cells to bind metals (Brown & Lester 1979). Substantial amounts of uranium (Shumate & Strandberg 1978), and mercury (Murray & Kidby 1975) are bound to the cell walls of *Streptomyces cerevisiae.* The most widespread example of metal accumulation in nature is the microbial transformation of iron and manganese resulting in the precipitation of insoluble oxides at the cell surface.

Under appropriate conditions, such accumulations may be conducive to the formation of biominerals. The great variety of known biominerals has been surveyed by Lowenstam & Weiner (1983). They include calcium, magnesium, silicon, strontium, barium, iron, and manganese salts and oxides. Moreover, deposits of elemental sulphur and ice are known. In some cases, the biominerals appear to be incidental by-products of the biological organization. But very often highly functional products are formed with an elaborate shape and structure. The original macromolecular metal traps may now acquire a matrix function and thus play a regulatory role in the crystallization process. Especially in the well-organized biominerals biopolymers are invariably found to occur in close association with the mineral phase. Thus, the cell appears to create specific microenvironments, where the crystallization processes are subjected to the subtle regulatory powers of biopolymers.

Calcification in the marine alga *Emiliania huxleyi*

General aspects

Emiliania huxleyi, a unicellular alga that belongs to the phylum Haptophyta, is dominant among the marine phytoplankton and may even be the species with the largest biomass on earth. Hence, it plays an important role in the marine food chain. The cell is covered with coccoliths (Figs. 2 and 3) – calcite plates with a very delicate structure. The coccoliths are produced intracellularly, and extruded after their completion. Because of its widespread occurrence in the oceans, *Emiliania huxleyi* is the most productive lime-secreting species on earth. Satellite imagery has shown the significance of blooms of this species as transitory but important events on a geographical scale (Holligan et al. 1983). The processes of coccolithophorid consumption by copepods and the subsequent faecal pellet transportation of algal debris to the ocean floor are subjects of oceanographic studies. Coccolithophorids themselves have specific biochemical features both in terms of their coccoliths and in their lipids and other macromolecules (De Jong et al. 1976, Westbroek et al. in press, Volkman et al. 1980). Such unique features can survive water column processes and provide a signature of algal blooms in underlying sediments (De Leeuw et al. 1980).

Since the production of the blooms is linked to light availability, water column temperatures and nutrient supply, the climatic record can be evaluated from these sediments. Huge deposits of limestone, consisting mainly of coccoliths, have indeed been accumulated in the geological past, and coccoliths are valued biostratigraphical markers. Some coccolith layers may form excellent oil reservoirs. It may be tentatively assumed that coccolith production may influence the oceanic pH and the CO_2 cycle of the oceans and the atmosphere, and hence may have a significant impact on climate. *Emiliania huxleyi* can be easily cultured in the laboratory, and hence it is an eminent model system for the study of biopolymer-mediated mineralization.

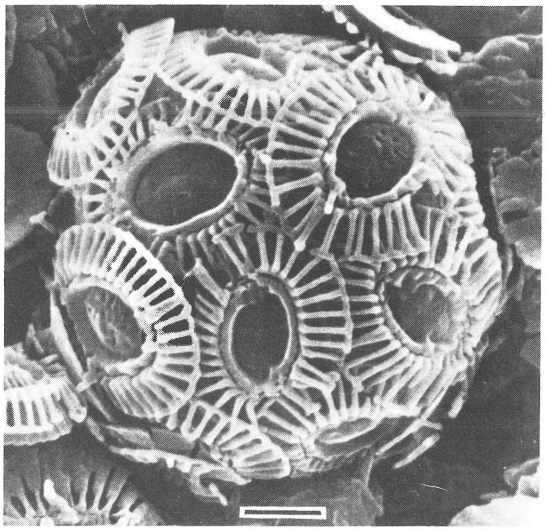

Fig. 2. Scanning electron micrograph of coccolith cover and loose coccoliths of *Emiliania huxleyi.* Bar = 1 μm.

Biochemical studies

Biochemical studies on this system have been carried out in our laboratory since 1970, in collaboration with the Department of Geology of this University. After the cultured cells of *Emiliania huxleyi* are harvested by centrifugation, the coccoliths can be easily purified by ultrasonication and centrifugation over a sucrose gradient. Dissolution

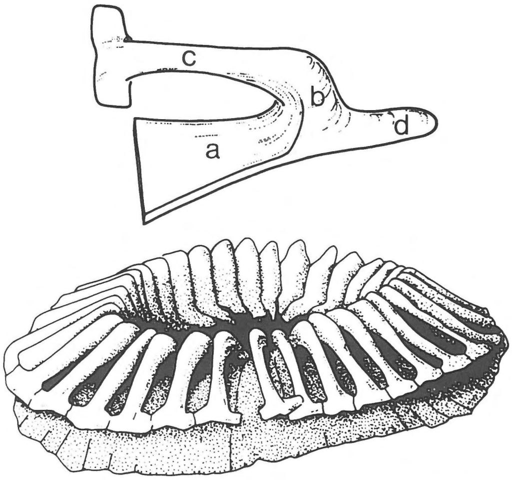

Fig. 3. Below: coccolith of *Emiliania huxleyi,* consists of a radial array of delicately sculptured segments of calcium carbonate. Above: one segment, consisting of (a) a flattened lower and distally directed element; (b) a vertical element with (c) a T-shaped distal directed end; and (d) a small medially-directed element.

of the crystalline phase releases an acidic polysaccharide which is very soluble in aqueous media. It should be kept in mind that this macromolecule is closely associated with the crystalline phase. It can only be liberated from the coccoliths by dissolution of the calcium carbonate, and not by mild chemical procedures that would normally remove adsorbed molecules but leave the crystallites intact.

Interestingly, acidic soluble macromolecules (proteins, glycoproteins, or proteoglycans) are generally found to be associated with calcified tissues (cf. Blomen 1982, Veis & Sabsay 1983, Weiner et al. 1983), and they are thought to play a regulatory role in the crystallization process. By analogy, we suppose that the coccolith polysaccharide has an important function in coccolith formation. It has several unique characteristics that sustain this assumption.

Polysaccharides are elaborate and often branched chains of sugar monomers; they are particularly rich in alcohol moieties, which may give these polymers a distinct hydrophylic character. Polysaccharides are produced by the cooperative action of several enzymes. Their structure is not immediately encoded in the DNA, and as a result there generally is some degree of variation in size and shape within a collection of polysaccharide molecules of a particular kind. As a rule, polysaccharides consist of a sequence of repeating units, each containing a limited number (one to four) of different monosaccharides.

The monosaccharide sequence in the macromolecule of *E. huxleyi* has been studied by Fichtinger-Schepman et al. (1981) (Department of Organic Chemistry, University of Utrecht). It is a polysaccharide of exceptional complexity, containing at least thirteen different monosaccharides. Many of the alcohol moieties are modified to accomodate methyl, carboxyl, or sulphate groups, thus providing the macromolecule with domains containing non-polar and acidic properties. Attached to a backbone of successive mannose moieties are complicated side chains with the methylated and carboxylated sugars concentrated at the periphery (Fichtinger-Schepman et al. 1981). It is likely that the biosynthesis of this polymer is a demanding operation for which an extensive array of enzymes is needed. Such a costly polymer can only be expected to have envolved if it has an important biological function. Its close association with the calcium carbonate crystals is indirect evidence for a role in the calcification process.

Physical-chemical studies in collaboration with Dr. C. Van Bloys Treslong (Department of Physical Chemistry) and Dr. C.W.A. Pleij (Department of Biochemistry, both this University) have shown that the molecules are relatively uniform in size as compared with other polysaccharides. The molecular weight varies between 56 000 (as determined with osmometry), and 96 000 (light scattering). In the analytical ultracentrifuge it behaves as a single monodisperse macromolecule. It has a rather rigid ellipsoid form in aqueous solution. The addition of Ca^{++}-ions has a profound effect on its conformation (manuscript in preparation). Equilibrium and flow-rate dialysis experiments have demonstrated that the polysaccharide preferentially binds Ca^{++}-ions in the presence of excess concentrations of Mg^{++}, or Na^{+}.

The *in vitro* crystallization of calcium carbonate from supersaturated aqueous solution is inhibited by very low concentrations of the polysaccharide (Borman et al. 1982). After the selective removal of its carboxyl groups, the macromolecule no longer influences the crystallization process, while removal of the sulphate ester has no effect. It is likely, therefore, that the polysaccharide envelops crystal nuclei, or is attached with its carboxyl moieties to multiple growth sites (lattice defects) on the crystal surface, thus inhibiting further growth. Other acidic polysaccharides, containing 2.5 times more carboxyl groups than the coccolith macromolecule (on weight basis), inhibited the calcium carbonate crystallization to the same, or even a much lesser extent. This suggests that the spatial distribution of the functional groups, and thus the conformation of the coccolith polysaccharide are specifically adapted to interaction with the crystalline phase.

In collaboration with Drs. O.L.M. Bijvoet, L.J.M.J. Blomen and D. Kok (Dept. of Endocrinology, University of Leiden) it was shown that the polysaccharide strongly inhibits the growth of calcium oxalate crystals, but stimulates their aggregation. Probably, the carboxyl groups are not concentrated on one side of the macromolecule, but are exposed in all directions, so that they can adhere to lattice defects of different crystals.

Indirect evidence has been found suggesting that the polysaccharide, when not in the free form, but bound to an inert insoluble support, will *stimulate* the *in vitro* crystallization of calcium carbonate.

Presently, studies on the biosynthesis of the polysaccharide are underway, and several precursors have been isolated. These are associated with a small protein. At an early stage of coccolith synthesis, the protein may serve to anchor the polysaccharide onto the surrounding membrane (manuscript in preparation).

Electron microscopial studies

Electron microscopical studies on coccolith synthesis of *Emiliania huxleyi* have been carried out in our laboratory by P. Van der Wal, in close association with W.T. Daems and collaborators (Laboratory of Electron Microscopy, University of Leiden). Research elsewhere (Wilbur & Watabe 1963, Klaveness 1972) has shown that the cellular component responsible for coccolith synthesis consists of a 'coccolith vacuole' (cv) surrounding the growing coccolith, and the 'reticular body' (rb), a system of anastomosing tubes. The lumina of these vesicles are connected (Fig. 4). Precise polysaccharide localization according to Thiery (1967) allowed the visualization of structural relations between these substances and the growing coccoliths (Van der Wal 1983). The calcifying system of *E. huxleyi* is strongly stained at four sites: (1) materials associated with the membranes of cv and rb; (2) polysaccharide threads anchored in the membranes and sticking into the vacuolar lumina; (3) material associated with the base of the coccolith (base plate); and (4) a thin skin surrounding the coccolith (Fig. 5).

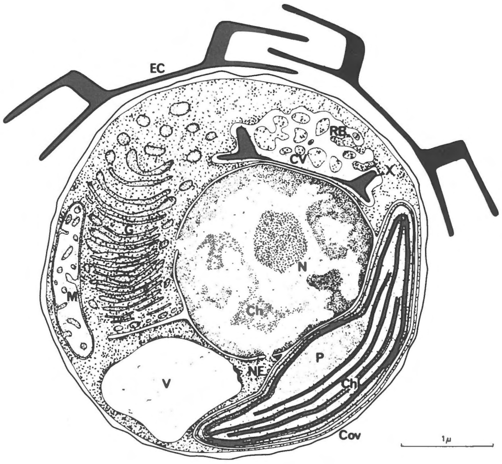

Fig. 4. Cross-section through calcifying cell of *Emiliania huxleyi*. Drawing based on micrographs of ultrathin sections of specimens fixed in glutaraldehyde and OsO_4. Within the coccolith vesicle (CV) there is an immature coccolith. Only two of the extracellular coccoliths (EC) constituting the coccosphere are shown. Abbreviations: Ch: chromatin; Chl: chloroplast; Cov: cover; CV: coccolith vesicle; ED: extracellular coccolith; G: Golgi complex; M: mitochondrion; N: nucleus; NE: nuclear envelope; RB: reticular body; V: cell vacuole; X: crystalline matter. From: Van der Wal et al. (1983), with permission of Springer-Verlag.

Studies with high-resolution electron microscopy (Parker et al. 1983) have shown that the parts of the coccoliths which are associated with the base plate form single crystals, while the latero-distal parts consist of a mosaic of fused microcrystals (Fig. 6). Thus, in the formation of a coccolith two different mechanisms appear to be operative.

Working hypothesis (Fig. 7)

Our present ideas on coccolith biosynthesis in *E. huxleyi* are summarized in the following model (Westbroek et al. in press). Three stages of coccolith synthesis are distinguished. (a) A new, small coccolith vacuole-reticular body complex is formed. $CaCO_3$-crystallization is inhibited inside the lumen by polysaccharide threads (p.t.) anchored onto the membrane. (b) The system grows and a base-plate is formed. As a result of lateral dilation of the coccolith vacuole a small space is created where the polysaccharide threads are absent. Along the rim of the base-plate on oriented calcite crystal can form. The (anchored) polysaccharide now stimulates the crystallization. (c) Further growth of the coccolith is generated by a dilation of the coccolith vacuole in certain well-defined directions. Radial extension leads to co-ordinated growth of the base-plate and the $CaCO_3$. The

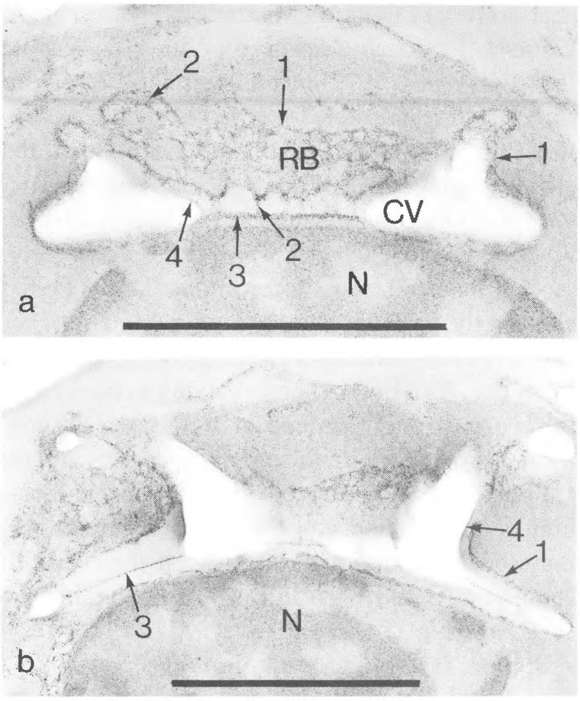

Fig. 5. Two stages of coccolith biosynthesis; electron micrographs of sections through cells and intracellular coccoliths of *Emiliania huxleyi.* Sections stained for polysaccharide. The coccolith in (a) is at an earlier stage of maturation than the one in (b). Polysaccharide is located at the following sites: (1) materials associated with the membranes of the coccolith vesicle (CV) and the reticular body (RB); (2) polysaccharide threads anchored in the membranes and sticking into the vacuolar lumina; (3) material associated with the base of the coccolith (base plate); and (4) a thin skin surrounding the coccolith. N: nucleus; CV: coccolith vesicle; RB: reticular body. Compare with Fig. 4. Calcium carbonate was dissolved during preparation. Bar: 1 μm. From: Van der Wal et al. (1983), with permission of Springer-Verlag.

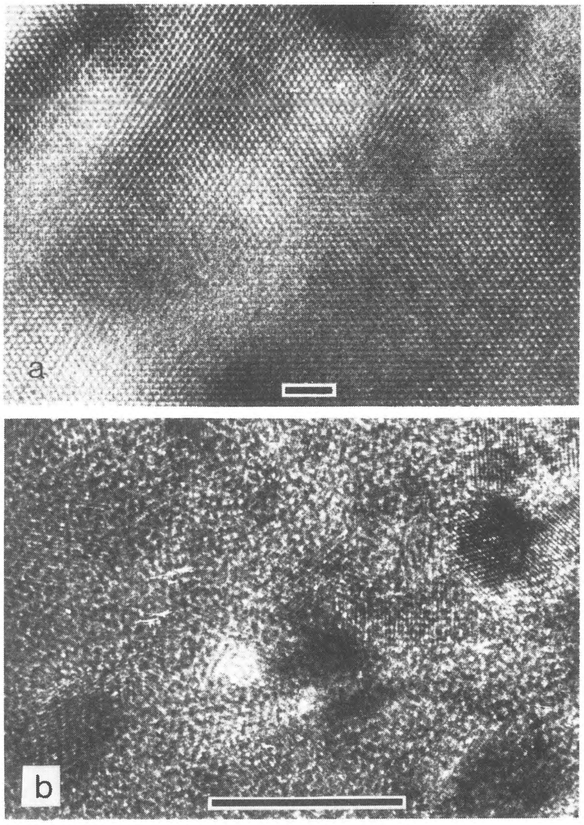

Fig. 6. High-resolution electron micrographs of (a) a lower, flattered element of a coccolith segment, and (b) an upper, T-shaped element. The lower element is a single crystal, while the upper element consists of a mosaic of fused microcrystals. Bar: (a) 20 Å; (b): 100 Å. Reproduced by kind permission of Dr. S.B. Parker, Oxford.

base-plate serves as a substratum for crystal growth and an ordered crystalline layer is formed. Dilation in disto-radial direction leads to a less well-ordered crystallization process, because no supporting substratum is available. The polysaccharide then adheres to the surface of the growing crystal, is detached from the membrane and forms a protective cover that inhibits further crystal growth. It is hypothesized that the co-ordinated dilation of the coccolith vacuole is brought about by the 'cytoskeleton', a fibrillar proteinaceous network that pervades all eukaryote cells and regulates intracellular movement.

At least three different functions are attributed to the polysaccharide in this hypothesis. While it is anchored to the membrane of a narrow vesicle it inhibits crystallization. Where the vesicle becomes dilated it induces crystal formation and growth. And when finally the polysaccharide is detached from the membrane it may cover the crystal surface and inhibit its further growth, in accordance with *in vitro* inhibition studies with free polysaccharide described above. In addition, the polysaccharide may act in inducing crystal agglomeration and in stabilizing the resulting agglomerates, as found in the upper (hammer shaped) elements.

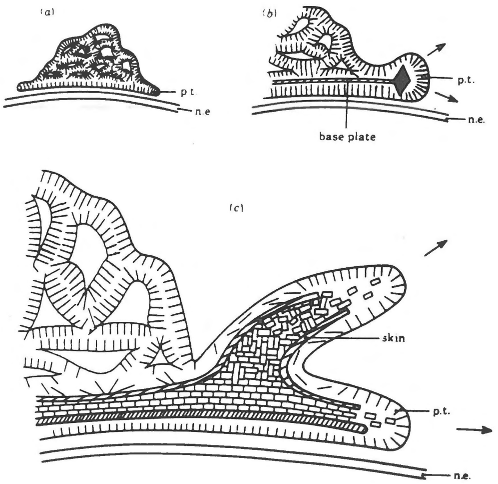

Fig. 7. Working hypothesis on the mechanism of coccolith formation. Only the coccolith vacuole, the reticular body and the nuclear envelope are shown. (a), (b), and (c) represent successive stages in coccolith synthesis. It is suggested that coccolith growth is held in check by the controlled expansion of the coccolith vesicle and by interaction with the associated acid polysaccharide. pt: polysaccharide threads (cf. Fig. 5 (2)); skin: polysaccharide cover of crystalline phase (cf. Fig. 5 (3)); ne: nuclear envelope. For detailed description see text. Reproduced from Westbroek et al. (1983).

Microbial manganese oxidation

General aspects

Manganese is a common crustal metal, amounting to about 0.1% of the total mass. Since it is a transition metal, it can have several valency states ranging from −2 to +7. Most common in nature, however, are the divalent and tetravalent forms. The divalent form prevales at low pH and under reducing conditions and is soluble in water. At high pH and under oxic circumstances the element exists as the +4 form, which readily forms highly insoluble oxides. It cannot be volatilized and lacks stable soluble forms in oxic waters. Therefore, the geochemical cycle of manganese in the outer earth is generally operating at a relatively slow pace and is largely dependent on hydrothermal activity and geological processes acting on solid materials. In restricted environments, however, manganese may cycle rapidly, due to its fast oxidation and reduction, and consequent conversion of insoluble to soluble forms and backwards. Moreover, it is an activator of several enzymes, and needed in photosynthesis. Consequently, biological transport systems are available for concentrating the element from the environment. A very important factor in the local cycling of manganese are the organisms which catalyze the transition between valency states. Manganese oxidizing and reducing microbes (including bacteria, yeasts, fungi and algae) are wide-spread and found in soils, fresh waters, and marine environments. They are often associated with manganese-rich sediments (e.g., manganese nodules) and thought to be involved in their formation. In the following we will concentrate on manganese oxidizing organisms.

Mechanisms of biological manganese oxidation

The mechanisms by which microorganisms oxidize the soluble divalent manganese to insoluble manganese oxides may be indirect or direct. Indirect oxidation results from increasing the Eh of the medium by O_2 production or excretion of oxidizing compounds, or by elevating the pH, e.g. by NH_3 production. Oxidation is considered to be direct when living organisms produce specific compounds (proteins, carbohydrates) which bind manganese, and enhance its oxidation in the bound form. The exact mechanisms of direct manganese oxidation and its function for the organism are still unclear. Since energy is liberated in the oxidation reaction (Ehrlich 1976, 1978b) it has been suggested that certain organisms use this reaction as an energy source for their metabolism (Ehrlich 1978a, Ali & Stokes 1972). However, autotrophic growth on manganese has never been unambiguously demonstrated. In several bacteria the involvement of enzymes in the oxidation reaction is likely (Ehrlich 1968, 1976; Douka 1977, 1980; Van Veen et al. 1978). In other cases cell surface polymers seem to be involved (Ghiorse & Hirsch 1979, Rosson & Nealson 1982). In our laboratory we recently started the study of manganese oxidation by spores of a marine *Bacillus sp.*, isolated from a near-shore sediment (Rosson & Nealson 1982) and by *Leptothrix discophorus,* a common sheath-forming organism from fresh-water environments.

Bacillus spores

Bacterial spores are dormant, stable germ cells with no metabolic activity. *Bacillus* SG.1 spores are composed of a spore germ, surrounded by a cortex consisting of peptidoglycan (a macromolecular complex of amino acids and sugar). The outermost layers are formed by the spore coats (which are composed of protein) and a structure resembling a so-called exosporium (consisting of protein, lipid and polysaccharide) (Tebo 1983). The spore is surrounded by a fibrillar material with an anionic character, presumably polysaccharide (Tebo 1983). When these spores are incubated in a medium containing Mn^{2+}, a dark brown precipitate of manganese oxide is accumulated on the spore surface (Fig. 8). *Bacillus* SG.1 can form spontaneous mutants which do not oxidize Mn^{2+}. A macromolecular compound is thought to be involved in the binding and oxidation of Mn. As a rule, macromolecules, especially proteins, are sensitive to heat: upon heating their conformation changes drastically. SG.1 spores gradually loose their manganese binding and oxidizing activity when the temperature of incubation is increased (Rosson & Nealson 1982). We presently investigate the possibility that one or more components of the spore coats, exosporium or extracellular material are involved in manganese oxidation. After extraction of a large percentage of spore coat protein and a small sugar fraction the residual spores only show minor activity. Whether this is due to solubilization or to denaturation of active components remains to be investigated.

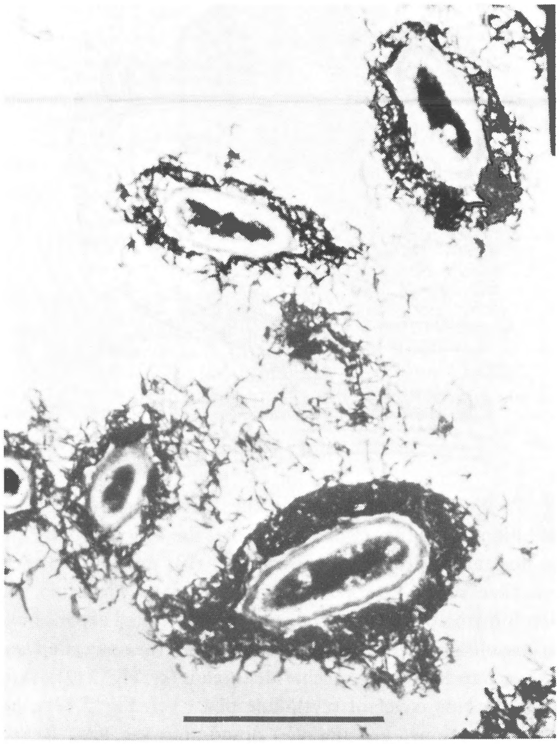

Fig. 8. Electron micrograph of section through spores of the marine bacterion *Bacillus sp.*, strain SG1. The spores are encrusted with manganese oxide. Bar: 1 μm. Photograph: Dr. B.M. Tebo.

Leptothrix discophorus

L. discophorus belongs to the so-called sheathed bacteria of the *Leptothrix-Sphaerotilus* group. The cells are enveloped in a long organic tube or sheath that may be attached at one end to a solid substratum. In habitats where reduced iron or manganese is present, the sheaths become encrusted with iron and manganese oxides. These cells may contribute to the formation of iron deposits associated with bogs. They are also frequently involved in the clogging of water-pipes with iron oxide. While iron oxidation is non-specific, the oxidation of manganese seems to be catalyzed by a protein excreted by the organism (Van Veen et al. 1978). This protein is partly associated with the sheaths, and partly released into the medium. Other components of the sheaths, or of a slime layer which under certain conditions may be produced by the organism, may also be involved in Mn oxidation. Even empty sheaths or sheaths of dead organisms may catalyze the oxidation of Mn^{++} (Van Veen et al. 1978). As with *Bacillus* spores, the Mn-oxidizing activity of *L. discophorus* is heat-sensitive (Van Veen et al. 1978). We intend to isolate the macromolecules involved in manganese oxidation by extracting isolated sheaths as described above.

Geological significance and technological applications

Biopolymer-mediated metal accumulation and biomineralization occur at an immense scale in Nature, and have profound geochemical effects. In the ocean, e.g., these processes cause depletion of surface waters with respect to metals (Broecker 1983, Whitfield & Watson 1983). Also, they are conducive to the formation of various sedimentary ores (Holland & Schidlowski 1982), play a crucial role in the formation of calcareous and silicious sediments, and may influence evaporite sedimentation and dissolution (Cornee 1983). Hence, the study of the underlying mechanisms is a matter of utmost geological interest.

If indeed the biota make use of these mechanisms at such a massive scale, the question arises whether they can be applied to the benefit of society. The present developments in biotechnology hold the promise that interesting biopolymers of whatever source may be produced at a technical scale, once the pertinent genetic information has been isolated, cloned into a suitable vector, and brought to expression. Alternatively, synthetic substances may be produced with properties similar to those of interesting biological compounds. In either case, the available repertoire of technologies related to the accumulation of metals and to the crystallization of sparingly soluble salts is likely to become extended, diversified, and refined.

What applications may eventually emerge from the study of biopolymer-mediated metal accumulation and biomineralization? We recently started the program on manganese-oxidizing bacteria with the aim to use this as a model system for the study of the biological accumulation of heavy metals that are important pollutants, or have a major function in society. Such natural mechanisms may then be optimized, by genetic engineering or otherwise, and be used for the reclamation of these metals from natural and polluted environments.

Biopolymer-mediated crystallization control may be important in the prevention of 'scaling', i.e., the clogging-up of tubing in water-desalinization plants, boreholes, etc., by deposits of sparingly soluble salts. Finally, this field may have interesting medical overtones: biopolymers involved in biomineralization or in the inhibition of crystallization in biological systems, or synthetic analogs thereof, may be used in the treatment of pathological mineralizations or demineralizations, such as arteriosclerosis, stone formation, caries and osteoporosis.

The field is in its infancy. Little is known with certainty regarding the mechanisms of biopolymer-mediated metal accumulation and biomineralization. Yet, the variety and sophistication of natural systems is overwhelming. The further exploration of this important subject needs the integrated effort of specialists in many different fields. There are geological, (micro)biological, organic, inorganic and physical chemical, crystallographic, biochemical, electron microscopical, genetic, and technological aspects, and expertise in all these fields is not available in any single research group. As the above description of our own work may demonstrate, appropriate information circuits are readily established. Whether any technology that may emerge from such studies will ever be successfully deployed in Society largely depends on the question whether the production will be feasible at an economically competitive price.

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