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Polyanion-mediated mineralization – a kinetic analysis of the calcium-carrier hypothesis in the phytoflagellate *Pleurochrysis carterae*

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Summary. Polyanions are postulated intermediates in biomineralization because they sequester large numbers of calcium ions and occur in high concentrations at mineralizing foci in distantly related organisms. In this study mineral ion and polyanion metabolism was examined in *Pleurochrysis carterae* to determine whether polyanions function as intermediate calcium-carriers during coccolith (mineralized scale) formation. In this organism mineralization occurs intracellularly in coccolith-forming saccules, and mature coccoliths are extruded through the plasma membrane into the coccosphere. The polyanions (acidic polysaccharides known as PS-1 and PS-2) are synthesized in medial Golgi cisternae and transported to the coccolith-forming saccule prior to the onset of mineral deposition; they also cover the mineral surface of mature coccoliths. Pulse-chase experiments with $^{45}Ca^{2+}$ and $^{14}CO_3^{2-}$ show the calcium uptake into the coccolith-forming saccule is much slower than carbonate uptake. The extended intracellular half-life of calcium ions destined for the coccosphere suggests that calcium is initially sequestered in more distal Golgi elements (perhaps in association with the polyanions) and enters the coccolith-forming saccule only after passage through the endomembrane system. This is consistent with previous cytochemical studies showing that the polyanions are complexed with calcium prior to mineral deposition. It has been suggested that polyanions may be degraded at the mineralization front in order to free calcium ions for precipitation with available carbonate or phosphate ions. However, this study demonstrates that the polyanions are not degraded; essentially all PS-1 and PS-2 are eventually secreted with the mineral phase into the coccosphere. The kinetics of mineral ion and polyanion secretion are consistent with a polyanion-mediated calcium transport; however, the manner in which calcium might be sequestered by and freed from the polyanions is still obscure.

Keywords: Calcium; Coccolithofore; Mineralization; Polyanion.

Abbreviations: PS-1/2/3 polysaccharide 1/2/3; EDTA ethylenediaminetetraacetic acid; TCA trichloroacetic acid.

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Introduction

Highly acidic macromolecules are postulated intermediates in biomineralization because they sequester large numbers of calcium ions and occur in high concentrations at mineralizing foci in distantly related organisms (Lee et al. 1977; Marsh 1989, 1994; Marsh and Sass 1983; Marsh et al. 1992). The mineral-associated polyanions are phosphoproteins in molluscan bivalve shells and vertebrate dentin but are acidic polysaccharides in a photosynthetic protist, the coccolithophore *Pleurochrysis carterae*. Although the animal and algal polyanions are chemically different, both are extremely electronegative, suggesting that they have a common function in protistan and metazoan mineralization.

P. carterae is a unicellular alga surrounded by a layer of mineralized scales called coccoliths (Fig. 1). The coccoliths are organic oval-shaped scales (base plates) with a rim of $CaCO_3$ crystals. An organic coat surrounding the crystals (Fig. 2) consists primarily of two acidic polysaccharides PS-1 and PS-2. The most abundant polyanion PS-2 has a repeating unit consisting of D-glucuronic, *meso*-tartaric, and glyoxylic acid residues (Fig. 3) (Marsh et al. 1992). The tartrate and glyoxylate residues are probably introduced in a post-polymerization process by oxidative cleavage of C2–C3 bonds in alternate residues of a nascent polyuronide (prePS-2). With four carboxyl groups per repeating unit, PS-2 sequesters more calcium than any polyanion yet described. The molecular weight of PS-2 is very heterogeneous; its degree of polymeriza-

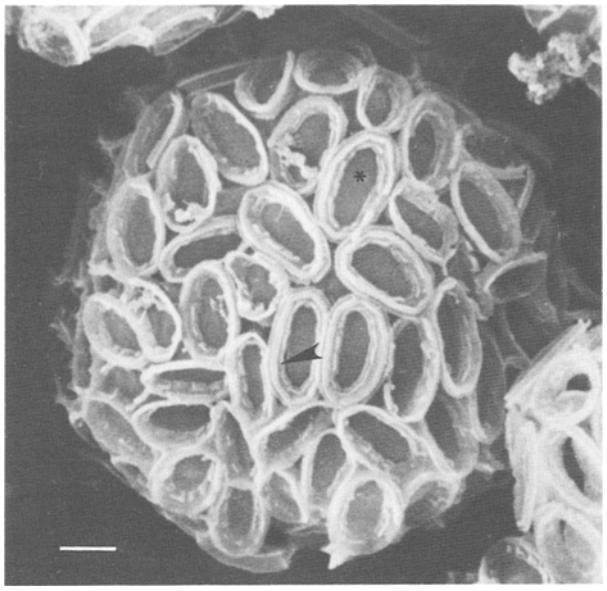

Fig. 1. Scanning electron micrograph of a *P. carterae* cell showing the mineralized scales of the coccosphere. These scales, called coccoliths, consist of organic oval-shaped bases (*) with rims of $CaCO_3$ crystals (arrowhead). An organic coat surrounding the crystals cannot be distinguished in scanning images. From Marsh (1994). Bar: 1.0 μm

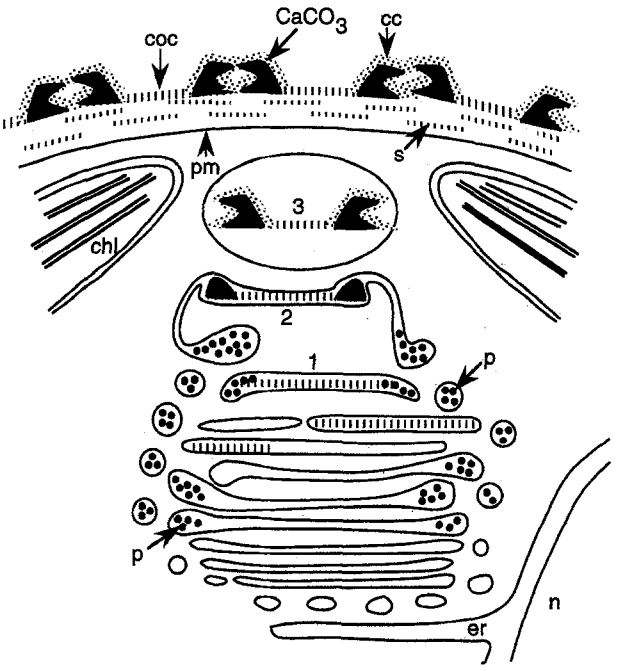

Fig. 2. Cross-sectional model of coccolith formation in the Golgi system of *P. carterae*. The coccolith-forming saccule is shown before (*1*), during (*2*), and after (*3*) mineral deposition. Ca-PS-1/PS-2 complexes are synthesized in medial Golgi cisternae. They are organized in discrete particles (*p*, large black dots) before and during mineral deposition (*1* and *2*) and in amorphous crystal coats (*cc*) after mineralization ceases (*3* and coccosphere). All structures above the plasma membrane (*pm*) are contained in the coccosphere. Hatch marks, coccolith bases; *coc* coccoliths, *s* unmineralized scales, *chl* chloroplast, *er* endoplasmic reticulum, *n* nucleus. From Marsh (1994)

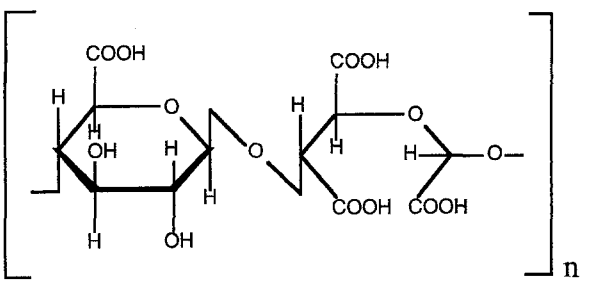

Fig. 3. Structure of PS-2

tion ranges from less than 10 to over 100 repeating units per molecule. The primary structure of PS-1 has not been determined; it is a polyuronide with a glucuronic/galacturonic acid ratio of 1 : 3 and contains small amounts of uncharged glycosyl residues. PS-3, a polyanion of low abundance (less than 2% of total polyanions) and high sulfur content, has also been isolated from coccolith preparations; however, its presence in the crystal coats has not been established.

PS-1 and PS-2 are synthesized in medial Golgi cisternae where they coaggregate with calcium ions to form 20 nm particles (Marsh 1994, van der Wal et al. 1983b). The particles are released in small vesicles which fuse with other vesicles containing the base of a future coccolith (Fig. 2) (van der Wal et al. 1983a). After fusion, the polyanion particles are localized on the rim of the base where mineral deposition will occur. $CaCO_3$ nucleation and crystal growth take place in the presence of the PS-1- and PS-2-containing particles. During the final phase of coccolith formation, the polyanion particles are reorganized into an amorphous organic coat which surrounds the mature crystals and remains with the mineral phase after the coccoliths are extruded from the cell into the coccosphere (Marsh 1994, Outka and Williams 1971). PS-3 has not been localized at the ultrastructural level; however, this study shows that it is not transported to the cell surface by the same pathway as PS-1 and PS-2.

The mineralizing function of the polyanions is unclear. Theoretically PS-1 and PS-2 may have a role in $CaCO_3$ nucleation in their particulate state and later a role in crystal growth inhibition in their amorphous form (Marsh 1994, Crenshaw 1982). The polyanion particles may also act as calcium carriers (Marsh

1994, van der Wal et al. 1983a, Dimuzio and Veis 1978); i.e., the calcium used to build coccolith crystals may accumulate at the mineralization front in association with the polyanions. The purpose of this study is to address the carrier hypothesis with a kinetic analysis of polyanion, calcium and carbonate ion transport from intracellular pools to the coccosphere.

Material and methods

Pulse-chase

Pleurochrysis carterae (synonyms *Hymenomonas carterae* and *Cricosphaera carterae*) clone CCMP 645 (formerly COCCO II) was obtained from the Bigelow Laboratory (West Boothbay Harbor, ME), and cultures were grown aseptically at 18 °C with a 12 h light/12 h dark photocycle in F/2 medium (Guillard 1975) prepared with synthetic sea water (Lyman and Fleming 1940) containing 10 mM Ca^{2+}, 27.6 mM SO_4^{2-}, and 2.3 mM HCO_3^-. Cultures (10^6 cells/ml) were pulse labeled at 18 °C in the light with $^{45}CaCl_2$ (50 µCi/ml) or $Na_2{}^{14}CO_3$ (20 µCi/ml) in growth medium minus bicarbonate plus 0.36 mM sodium carbonate and 10 mM Tris, pH 8.6, or with $Na_2{}^{35}SO_4$ (250 µCi/ml) in growth medium minus sulfate plus 10 mM Tris, pH 8.6. Labeled cells were pelleted 2 min at 250 **g** and resuspended at 15 or 18 °C in unlabeled growth medium plus 10 mM Tris, pH 8.6 (chase medium) at 10^5 cells/ml. All studies were repeated at least once and similar results were obtained in duplicate experiments.

Separation of intracellular and coccosphere ions

For separation of coccosphere calcium, carbonate, and polyanions from intracellular pools of the same molecules, 10^6 cells were pelleted as above from aliquots of labeled cultures, washed with 3 × 1 ml chase medium, and incubated 1 min in 0.5 ml of 0.1 M EDTA, 60 mM Tris, pH 8.6. The EDTA-soluble extract (collected at 250 **g** for 2 min) contained the extracellular mineral and polyanions. The cells were sonicated 30 s with an additional 0.5 ml aliquot of the same EDTA solution, and the intracellular calcium, carbonate and polyanions were recovered in the supernatant fluid after centrifugation of the sonicate for 10 min at 16,000 **g**.

Isolation and detection of polyanions

Labeled polyanions were isolated by addition of 50 µg of unlabeled PS-2 (Marsh et al. 1992), 0.1 ml 1 M $CaCl_2$, and 0.1 ml 3 M Tris, pH 8.8, to 0.25–0.5 ml aliquots of intracellular or coccosphere EDTA extract followed by centrifugation for 5 min at 16,000 **g**. The polyanion-containing pellet was vortexed with 250 µl of 5% TCA and centrifuged at 16,000 **g** for 5 min. Polyanions were precipitated from the supernatant fluid by addition of 25 µl 1 M $CaCl_2$ and 50 µl 3 M Tris, pH 8.8. After sedimentation as before, the pellet was redissolved in TCA and reprecipitated with alkaline calcium.

PS-1 and PS-2 were separated by addition of 10 µl 3 M Tris, pH 8.8, and 25 µl of 1 M $MgCl_2$ to either intracellular or coccosphere polyanions dissolved in 25 µl 0.1 M EDTA, pH 8.3, followed by sedimentation at 16,000 **g** for 5 min. The Mg-soluble and Mg-insoluble fractions contain PS-1 and PS-2, respectively. Labeled polyanions and PS-1- and PS-2-containing fractions were resolved on 12.5% polyacrylamide gels in the Laemmli (1970) system without sodium dodecyl sulfate. Bands were visualized by fluorography after treatment of gels with Enhance (DuPont NEN) as recommended by the manufacturer.

Isolation and detection of mineral ions

45-Calcium uptake was determined directly by liquid scintillation analysis of EDTA extracts after incubation of cultures with $^{45}CaCl_2$. After exposure of cultures to $Na_2{}^{14}CO_3$, ^{14}C-carbonate was isolated by acidification of EDTA extracts and collection of liberated $^{14}CO_2$ on filter paper saturated with NaOH. A 0.1 ml aliquot of the EDTA extract was placed in the bottom of a 1.5 ml microcentrifuge tube. The top of a gelatin capsule (size 00; Polysciences) was inverted and wedged in the tube above the extract. 0.2 ml of 4 N H_2SO_4 was added to the capsule, and the centrifuge tube was tightly capped. Affixed to the inside surface of the cap with a little silicon vacuum grease was a 6.5 mm circle of Whatman #3MM filter paper to which 17 µl of 4 N NaOH had been applied. After the acid had dissolved the gelatin capsule and entered the extract (15–30 min), the tubes were rotated for 1 h at an angle of about 70°. Then the filter pads plus caps were extracted over night in 4 ml of UniverSol (ICN) scintillation fluid containing 0.5 ml water.

Calcium-binding

Calcium-binding to unfractionated polyanions isolated from TCA extracts (Marsh et al. 1992) was measured in 0.15 M NaCl, 40 mM Tris, pH 8.6, using an ultrafiltration procedure (Marsh 1994). Uronic acid was measured by the phenyl phenol assay of Blumenkrantz and Asboe-Hansen (1973). The carboxyl content of the polyanion fraction is about 2.8 times the uronate level, since PS-2 with one uronate residue and three additional carboxyl groups per disaccharide unit and PS-1 (a polyuronide) represent about 75% and 25% of the polyanion fraction, respectively (Marsh et al. 1992).

Results

Analysis of isolation procedures

Mineral ions and polyanions associated with the coccosphere (but not with intracellular pools) were dissolved by a brief exposure to EDTA (Figs. 4 and 6). Subsequently, mineral ions and polyanions were extracted from intracellular pools by sonication in the presence of EDTA. Labeled polyanions were isolated in greater than 99% yield by extraction of cells or EDTA extracts with 5% TCA and precipitation with alkaline calcium in the presence of added unlabeled PS-2 (Fig. 5 a). The polyanions were separated into PS-1- and PS-2-containing fractions by precipitation with $MgCl_2$ (Fig. 5 b). The PS-1 (Mg-soluble) fraction also contained PS-3. The PS-2 (Mg-insoluble) fraction contained all PS-2 molecules and a trace of PS-1. Total carbonate was isolated by trapping carbon dioxide liberated from acidified extracts in filter paper saturated with a basic solution; a 101% recovery of carbonate was achieved from standard $Na_2{}^{14}CO_3$ solutions.

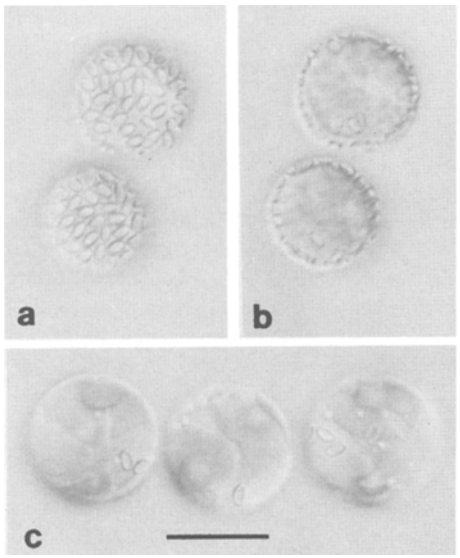

Fig. 4 a–c. Nomarski photomicrographs of *P. carterae* cells. **a** With the focal plane at the cell surface, the oval-shaped $CaCO_3$ rims of the extracellular coccoliths are apparent. **b** With the focal plane through the center of the cell, the coccosphere is seen in cross-section at the cell margin, and with careful inspection the mineral rims of internal coccoliths are discernible. **c** The presence of calcified rims on internal coccoliths and the absence of coccosphere mineral at the cell margins are apparent in photomicrographs of cells treated briefly with EDTA (Material and methods). Focal plane through cell interior. Coccosphere $CaCO_3$ has dissolved. Scanning electron images indicate that EDTA also dissociates the coccolith bases from the cell surface (not shown). Bar: 10 µm

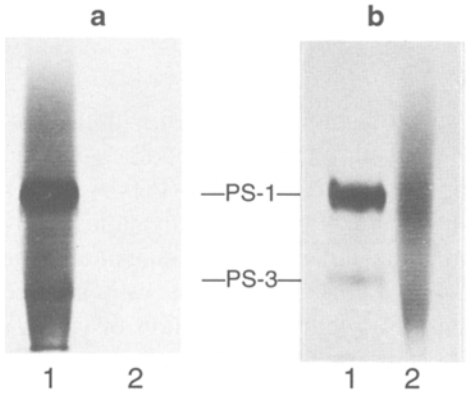

Fig. 5 a, b. Fluorography of polyanions resolved on 12.5% polyacrylamide gels. **a** Cells (10^6) were labeled with $^{14}CO_3^{2-}$ and then extracted successively with two 0.25 ml aliquots of 5% TCA. ^{14}C-labeled polyanions were precipitated from both extracts with alkaline calcium in the presence of added unlabeled PS-2. All ^{14}C-polyanions were recovered in the first TCA extract (*1*); none were recovered in the second extract (*2*). Polyanions were also quantitatively recovered from EDTA extracts treated with TCA (not shown). **b** ^{14}C-labeled polyanions were fractionated with magnesium ions (Material and methods). *1* Magnesium-soluble fraction; *2* magnesium-insoluble fraction, the multiple bands are different molecular weight species of PS-2

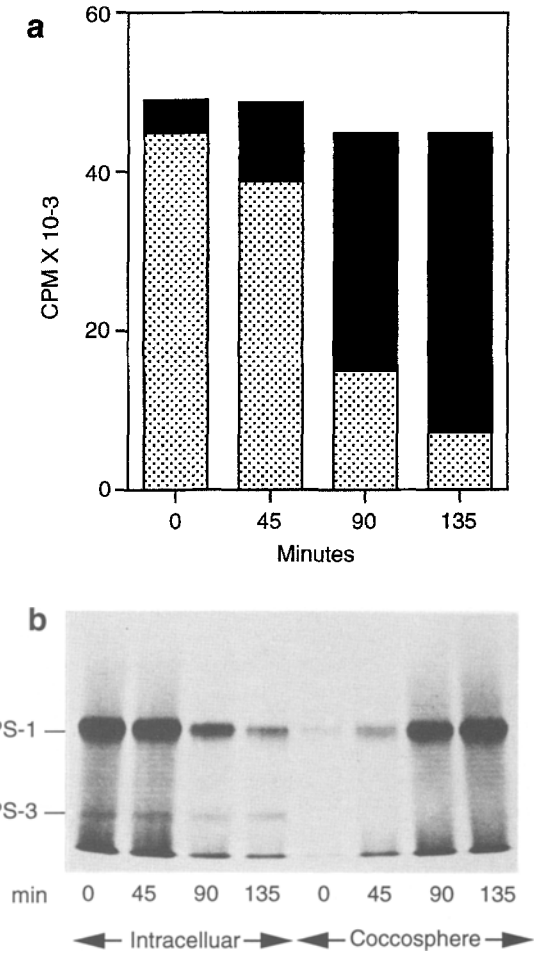

Fig. 6 a, b. Distribution of ^{14}C-polyanions in intracellular pools (stippled bars) and coccosphere (black bars) as a function of time after a 15 min pulse with $^{14}CO_3^{2-}$. Aliquots of intracellular and coccolith polyanion fractions were counted (**a**) and subjected to electrophoresis and fluorography (**b**). Note the uniform level of PS-3 in intracellular fractions, and the absence of PS-3 in coccosphere fractions. PS-1 and PS-3 bands are indicated to the left of the gel. The other bands are PS-2 molecules of variable lengths. Note that the distribution of PS-2 chain lengths is similar at t = 0 (intracellular) and t = 135 min (coccosphere)

Synthesis and secretion of polyanions

After a $^{14}CO_3^{2-}$ pulse, ^{14}C-labeled PS-1 and PS-2 were secreted into the coccosphere at similar rates with an intracellular half life of about 80 min at 18 °C (Figs. 6 and 11; Table 1). Secretion of the minor polyanion PS-3 after a $^{35}SO_4^{2-}$ pulse was much slower with a $t_{1/2}$ of over 4 h (Fig. 7). There was little if any polyanion degradation during mineral deposition. The labeled polyanion content (intracellular plus coccosphere) was essentially constant within experimental error throughout the chase period (Figs. 6 a and 7 a). The heterogeneity in ^{14}C-PS-2 molecular weight was

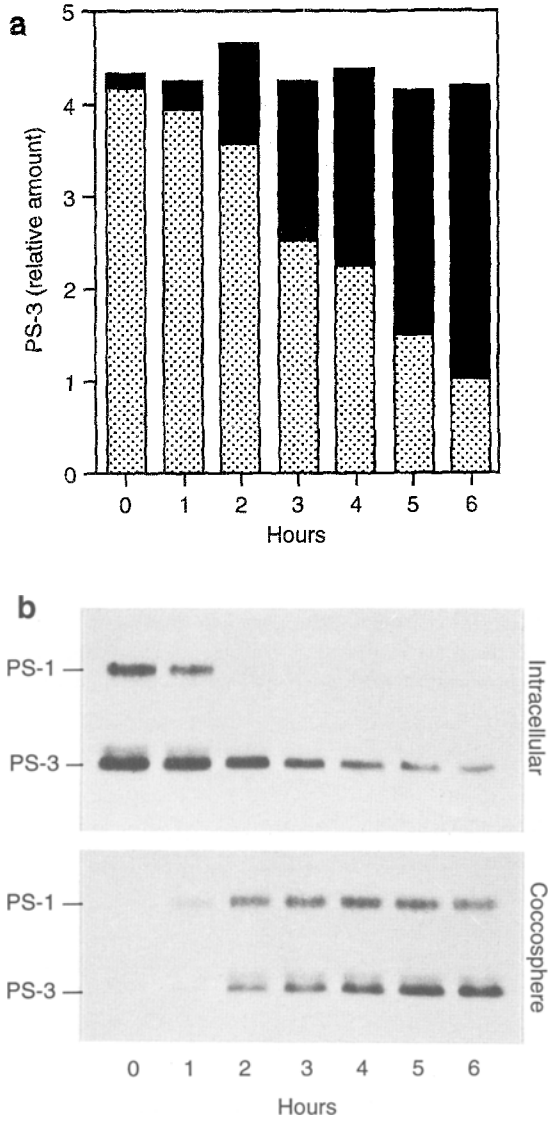

Fig. 7. a Distribution of labeled PS-3 in intracellular pools (stippled bars) and coccosphere (black bars) as a function of time after a 15 min pulse with $^{35}SO_4^{2-}$. **b** Relative amounts of PS-3 in each fraction were determined by electrophoresis, fluorography, and densitometry of intracellular and coccosphere polyanion fractions. PS-2 with no detectable sulfate does not appear on the gels. PS-3 is highly sulfated (Marsh et al. 1992). PS-1 contains only a trace of sulfate

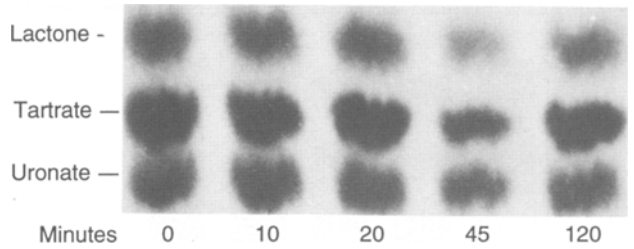

Fig. 8. Paper chromatography and fluorography of polyanion hydrolysates as a function of time after a 5 min pulse with $^{14}CO_3^{2-}$. Tartrate intensity relative to that of uronic acid (uronates plus lactones) was similar at all times. Polyanions were isolated from intact cells (cells with coccospheres) and hydrolyzed 3 h at 121 °C in 2 N trifluoroacetic acid. Hydrolysates were dried with a stream of nitrogen and chromatographed on Whatman 3MM paper in butanol : acetic acid : water (60 : 15 : 25). Bands were measured by densitometry

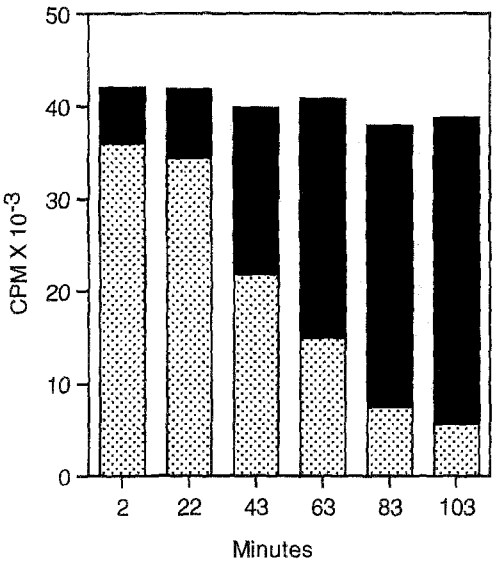

Fig. 9. Distribution of ^{45}Ca in intracellular pools (stippled bars) and coccosphere (black bars) as a function of time after a 15 min pulse with $^{45}CaCl_2$

similar at the beginning and end of the chase (Fig. 6 b) indicating that PS-2 is synthesized in multiple lengths and not as a single polymer which is subsequently depolymerized to a variable extent. The relative levels of ^{14}C-labeled tartaric and uronic acid residues in the polyanion fraction are similar at early and late intervals after a brief $^{14}CO_3^{2-}$ pulse (Fig. 8). Hence tartrate is introduced into PS-2 during or soon after polymerization.

Uptake and secretion of calcium and carbonate ions

Essentially all of the $^{45}Ca^{2+}$ and inorganic $^{14}CO_3^{2-}$ accumulated in a 15 min pulse remained associated with the cells throughout a 100 min chase (Figs. 9 and 10). This does not include labeled ions incorporated in rapidly exchanging pools; pools with half-lifes of less than five minutes are not detected in these experiments. Within 100 min most of the stably incorporated calcium and carbonate ions had been secreted into the coccosphere, with half-lifes of about 60 and 30 min, respectively.

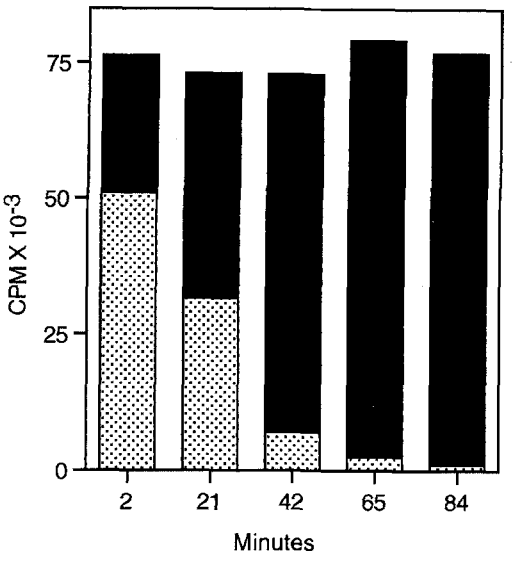

Fig. 10. Distribution of total ^{14}C-carbonate in intracellular pools (stippled bars) and coccosphere (black bars) as a function of time after a 15 min pulse with $^{14}CO_3^{2-}$

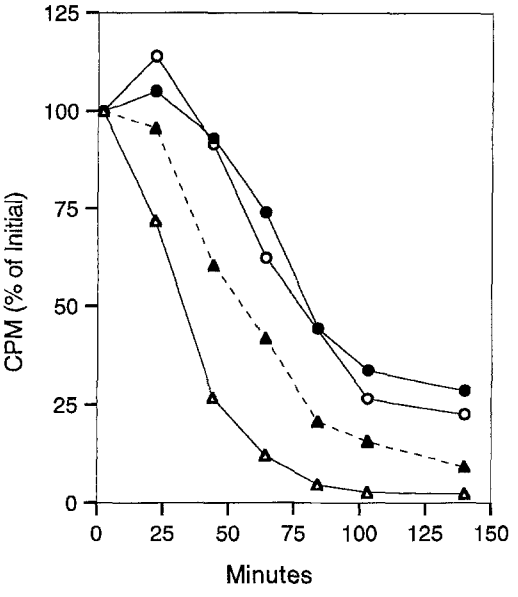

Fig. 11. Labeled intracellular mineral ions and polyanions as a function of time after a 15 min pulse with $^{45}CaCl_2$ (dashed line) or $^{14}CO_3^{2-}$ (solid lines) in duplicate cultures. The polyanions were separated into PS-1 (●) and PS-2 (○) containing fractions as shown in Fig. 5 b. Carbonate (△), calcium (▲)

Kinetics of Ca^{2+}, CO_3^{2-}, and polyanion secretion

Pulse-chase labeling of duplicate cultures with $^{45}Ca^{2+}$ and $^{14}CO_3^{2-}$ show that intracellular pools of mineral and polyanions are secreted at different rates (Fig. 11). At 18 °C carbonate, calcium, and PS-1/PS-2 are secreted with half-lifes of about 33, 58, and 78 min, respectively, and at 15 °C the half-lifes are approximately twice the level at 18 °C (Table 1). The measured polyanion half-lifes represent maximum values. In all pulse-chase studies, maximum incorporation of ^{14}C into the polyanion fraction was always observed 20 or 40 min after the end of the pulse period at 18 and 15 °C, respectively. The maximum level of ^{14}C incorporated was 3–10% above the level observed at the beginning of the chase period.

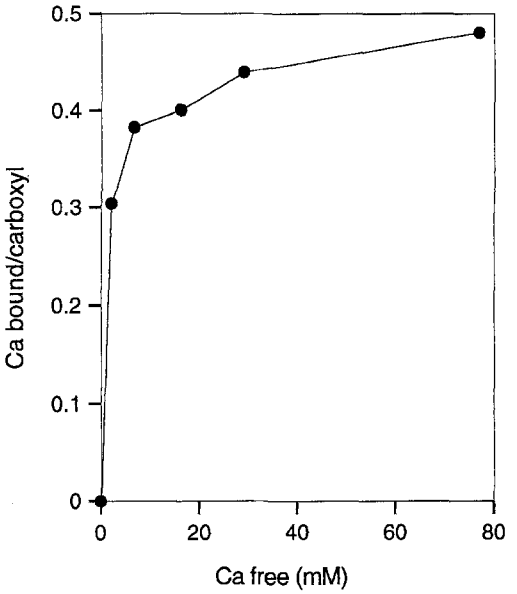

Fig. 12. Binding of calcium to polyanions in 0.15 M NaCl, 40 mM Tris, pH 8.6. Data are expressed as the number of calcium ions bound per polyanion carboxyl group. Polyanion concentration was 0.5 or 5 mg/ml

Table 1. Intracellular half-life of mineral ions and polyanions

Ion	$t_{1/2}$ (min)	
	15 °C	18 °C
Carbonate	75	33
Calcium	120	58
PS-1/PS-2	165	78
PS-3		252

Calcium-binding

Isolated coccoliths contain about 6.2 calcium ions in the mineral phase for each polyanion carboxyl group in the crystal coats (Marsh 1992). In vitro, the total polyanion fraction binds about 0.4 calcium ions per

carboxyl group at pH 8.3 and ionic strength 0.18 M in the presence of 10 mM calcium ions (Fig. 12).

Discussion

Polyanion secretory pathways

In pulse-chase studies, PS-1 and PS-2 are secreted into the coccosphere at a similar rate, suggesting that they follow a similar pathway through the Golgi system and calcifying vesicle to the cell surface. This is consistent with immunolocalization studies showing that these polyanions are intimately associated in elements of the Golgi apparatus, the coccolith-forming saccule, and the coccosphere (Marsh 1994). The minor polysaccharide PS-3 has not been immunolocalized, but this study shows that it is secreted into the coccosphere by a different pathway since its intracellular half-life is three times longer than that of PS-1 and PS-2. When and where, if ever, PS-3 becomes associated with the other polyanions and the mineral phase is unclear. It is reasonably well established that PS-1 and PS-2 have a mineralizing role in coccolith formation (van der Wal et al. 1983b, Marsh 1994), although the details of the process are unclear. Since PS-3 coaggregates with the calcium complexes of the other polyanions in vitro, it may have a mineralizing function requiring interaction with PS-1 and PS-2.

The mechanism by which the PS-1- and PS-2-containing particles are reorganized into an amorphous crystal coat is unknown, and the molecular entities to which the particles bind on the rim of the coccolith base before the onset of mineral deposition have not been identified. Given the potential for PS-3 interaction with PS-1 and PS-2, the former could function as docking elements for the polyanion particles on the rim of the coccolith base, or PS-3 could interact with the particles at a later time to effect a reorganization into crystal coats.

The significance of polyanion reorganization when mineralization ceases is unclear. The process is not accompanied by polyanion degradation or other detectable molecular modifications involving covalent bonds. None of the polyanions are turned over, and the molecular weights of intracellular and coccosphere polyanions are similar. The conversion of prePS-2 to PS-2 is not associated with polyanion reorganization. ^{14}C-tartrate residues appear in the polyanion fraction after a brief $^{14}CO_3^{2-}$ pulse, indicating that oxidation of Pre-PS-2 to PS-2 occurs during or shortly after polymerization.

Mineral ion secretory pathways

In pulse-chase studies, calcium was secreted into the coccosphere with an intracellular half-life greater than that of carbonate. Since the ions are cosecreted as $CaCO_3$ directly from the coccolith-forming saccule, it appears that Ca^{2+} and CO_3^{2-} are sequestered in the saccule by different pathways. Carbonate probably diffuses directly into the coccolith saccule as CO_2 (facilitated by rapid equilibration between carbonate species). Calcium may be sequestered in more distal Golgi elements and then transported to the coccolith-forming saccule by fusion of calcium-laden vesicles within the saccule membrane.

The intracellular half-life of calcium is about midway between that of carbonate and PS-1/PS-2. However, the measured polyanion half-life is high, since additional ^{14}C-PS-1/PS-2 is synthesized from ^{14}C-labeled organic precursors after the withdrawal of $Na_2{}^{14}CO_3$. Hence the actual rate of polyanion secretion may be closer to that of calcium secretion. This is consistent with ultrastructural and electron microprobe analyses demonstrating that the polyanion particles are complexed with calcium ions before they are localized at the mineralization front (van der Wal et al. 1983b).

However, it is uncertain whether all calcium is transported in polyanion complexes. The mature coccoliths contain about 6.2 calcium ions in the mineral phase for each polyanion carboxyl group in the crystal coats, but in vitro the polyanions sequester only about 0.4 calcium ions per carboxyl group. Thus it would appear that less than 10% of the coccolith calcium could have been transported by the coccolith polyanions. To account for the excess calcium, it was suggested that about 90% of the polyanions synthesized were never incorporated in the coccoliths but instead were degraded at the mineralization front to free calcium ions for precipitation with available carbonate ions (Marsh 1994, van der Wal et al. 1983b). However, this study demonstrates that the polyanions are not turned over. Essentially all PS-1, PS-2, and PS-3 synthesized by the cells is eventually secreted into the coccosphere. Clearly, if all calcium is carried to the coccolith-forming saccule in polyanion-containing vesicles, additional anions (either organic or inorganic) must be cotransported to maintain electrical neutrality.

Additional experimental approaches will be required to define more clearly the role of polyanions in coccolith mineralization. Localization of calcium pumps in the endomembrane system may help to elucidate the

route of calcium entry into the coccolith-forming saccule (Kwon and Gonzalez 1994); vesicles with the highest level of membrane-associated Ca^{2+}-transporting ATPase are likely to be the vesicles in which Ca^{2+} is initially sequestered.

Acknowledgements

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