Golgi-associated calcification in the coccolithophorid phytoflagellate Hymenomonas carterae

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Golgi-associated calcification in the coccolithophorid phytoflagellate *Hymenomonas carterae*

by

Daniel Charles Williams

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of The Requirements for the Degree of DOCTOR OF PHILOSOPHY

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INTRODUCTION

Coccoliths have been known for many years to geologists and paleontologists as a prominent form of calcium carbonate microcrystal in certain types of chalk and marine bottom ooze. The biological concern in these crystalline "concretions" is derived from the fact that they encompass the bodies of certain unicellular, predominantly marine algae. More recently the interest of cell biologists and physiologists has been aroused by observations that the coccoliths are formed intracellularly, thus providing a cellular system for studying biological calcification.

The coccolithophorid algae, as a group, present a spectrum of complexity with reference to biological calcification ranging from the relatively simple case in which calcite rhombohedrons are crystallized outside the cell plasmalemma but within a cell "skin," to a more complex situation, as represented by Hymenomonas, in which the highly contoured, morphologically distinct calcite elements which make up the coccolith are formed, and arranged in a precise and orderly manner within the cell's Golgi system.

Morphological and biochemical information suggests that coccolith formation is typical of most biological mineralization systems with respect to the use of an organic matrix as a cellular means of controlling inorganic crystallization. The role of the matrix in coccolith formation would seem to be exceedingly clear as compared to many other mineralizing systems since matrix formation and calcification are confined to the membrane-bound

---

1 The terms alga and phytoflagellate will be used in a descriptive sense and are not meant to imply botanical vs. zoological affinities for the coccolithophorid organisms.
Golgi cisternae, and the continuum of events is not broken by secretion of the unmineralized matrix into an extracellular environment as it seems to be, for instance, in most vertebrate systems.

During the course of this work it has become apparent that along with its application to the study of biological mineralization, coccolithogenesis also serves as a model system for cellular secretion and provides excellent corroborative evidence for the cytomembrane interchange hypothesis. Morphological observations additionally suggest a possible role of site specific localization of biological messages on membranes as a means of cellular control over macromolecular events.

This work includes new information on the structure of the coccolith of *Hymenomonas* and on the sequence of intracellular events associated with its formation. Chemical modification of the cellular environment was used, and its effect on population dynamics, coccolith kinetics, and fine structural modifications of the coccolithogenic machinery was examined.
LITERATURE REVIEW

It is generally agreed that Ehrenberg (30) was the first to observe coccoliths (which he referred to as morpholiths or crystalloids) (10, 22, 138). He however considered them to be inorganic crystalline forms peculiar to chalk (138). T. H. Huxley, in 1858, reported observing "very curious rounded bodies,...looking, at first sight, somewhat like single cells of the plant Protococcus" in deep sea deposits. He called these "rounded bodies," which were rapidly and completely dissolved by dilute acids, "coccoliths" (44). H. C. Sorby (138) observed coccoliths in chalk and suggested that they were not of "crystalline or concretionary origin," and in addition, that they were not separate individuals, but rather portions of larger cells. At about the same time G. C. Wallich observed coccoliths from North Atlantic soundings both in the free state, and "as adjuncts to minute spherical cells, upon the outer surface of which they were adherent in such a manner as to leave no doubt of that being their normal position" (152, 153). He termed the spherical cells "coccospheres," and later observed them as free-floating organisms in tropical seas (154).

Various suggestions were made as to the nature and taxonomic relationships both of coccoliths and coccospheres. Coccoliths have been considered to be inorganic crystalloids (30), parts of various types of organisms (44, 138, 155), and also as independent organisms themselves (e.g. 18, 53). Coccospheres have been variously regarded as secondary aggregates of coccoliths (44), rudimentary or reproductive phases in the life cycle of other calcareous organisms (154), or independent organisms (e.g. 138). Wallich (155), while holding the view that coccospheres were associated with
Foraminiferan development, suggested that an alternate view might be that they had algal affinities. He also appears to be the first to apply generic and specific identification to them, however, he failed to clearly indicate supergeneric affinities in his descriptions.

The first clear proposal of algal affinities for the coccospheres seems to be by the naturalists of the Challenger expedition (147). However, it was not until 1902 that Lohmann correctly associated his family Coccolithophoridae with the Chrysomonad (Chrysophycean) group of phytoflagellates (74).

An extensive, and very diverse body of literature has developed during the twentieth century regarding coccoliths and the coccolithophorid algae. This literature has been discussed in varying degrees of detail in the reviews of Lohmann (74), Schiller (132), Deflandre (22), Black (10), Noel (101), Paasche (115), and others. Loeblich and Tappan have compiled a very current and comprehensive index to this literature (69-73). This review will restrict itself primarily with that portion of the literature directly concerned with coccolith formation (coccolithogenesis), a subject that has been discussed in the recent reviews of Wilbur and Watabe (162), Paasche (115), and Pautard (120).

Terminology

It is particularly important to emphasize at the onset of this discussion that the formation of coccoliths does not involve the haphazard crystallization of calcium carbonate. Rather, the crystallization process, and as a result the shape of the coccolith, must be under close cellular control. The coccoliths of each cell or genetically related population of
cells are morphologically unique. A complex terminology for describing and classifying fossil and modern coccolithophorids has been developed on the basis of coccolith morphology, as discussed by Halldal and Markali (40) and summarized by Braarud, Deflandre, Halldal, and Kamptner (13). In a very general sense coccoliths can be divided into two broad groups, the holococcoliths, which are formed by a large number of regularly packed calcite crystals of simple hexagonal-prismatic or rhombohedral shape, and the heterococcoliths, such as the cricolith of *H. carterae*, in which the basic calcite rhombohedral shape has been modified to such an extent that the crystal faces become partially or wholly obliterated.

Recent laboratory investigations have shown that this system of speciation based on coccolith morphology is misleading since within the life cycle of the same coccolithophorid two (or more?) stages may exist which possess distinctly different types of coccoliths (e.g. the motile phase of *Coccolithus pelagicus* has holococcoliths, while the non-motile phase bears heterococcoliths), and in some species it is common to see two types of coccoliths on the same cell (94, 115). Additionally, cells which possess no coccoliths may be common in the life cycles of many coccolithophorids (62, 63, 115).

The life cycle of the organism currently under investigation, *Hymenomonas carterae* (and other apparently related organisms) has been the subject of several new studies (62, 63, 66, 118). Leadbeater (62, 63) has recently reviewed the taxonomic and life cycle histories of these organisms. Two major, morphologically distinct stages have been established, a coccolithophorid (*Hymenomonas*) stage, and a non coccolith-bearing vegetative or benthic (*Apistonema* or *Pleurochrysis*) stage. At least in the
Hymenomonas-Apistonema system, the coccolithophorid stage seems to be diploid, and the benthic stage haploid, with the two stages linked through meiosis (meiospores) and syngamy (gametes) (62, 129, 143). In both the Hymenomonas-Apistonema and Hymenomonas-Pleurochrysis systems, non-coccolith-bearing "swarmer" stages are also known (62, 63).

Protoplast structure appears to be similar in each of these stages, with the major morphological difference being the type of cell cover and presence or absence of external appendages, i.e. flagella or haptomema (62, 63, 118). Lefort (66) has very recently reported an additional stage in the life cycle of Hymenomonas, an "Ochrosphaera" stage. He has proposed, on the basis of his observations of cultured material, that the coccolithophorid Ochrosphaera verrucosa, which bears heterococcoliths that are distinct from the cricoliths of Hymenomonas, is actually an additional stage in the life cycle of H. carterae. Thus, the life cycle of H. carterae may consist of at least three major stages, one naked and two bearing mutually distinct coccoliths, as well as a number of transition phases. Control of coccolith formation in Hymenomonas must therefore involve not only the ability to determine coccolith morphology, but also the ability to turn coccolith formation on and off and to determine which of two or more coccolith types is to be formed at a particular time.

To avoid confusion as a result of the complex taxonomic and life cycle data associated with the Hymenomonas group of coccolithophorids, in this paper coccolith bearing stages of organisms shown to possess cricoliths will be referred to as Hymenomonas, plus a species designation where appropriate. This includes Syracosphaera carterae and species of Cricosphaera (62, 63, 87). Organisms designated Ochrosphaera, Apistonema, or Pleuro-
Chrysis by authors will retain the appropriate designation in this paper, with the understanding that they may represent stages in the life cycle of a hymenomonad. An additional terminological complexity is introduced by the use of different isolates or strains of the same species for laboratory investigations. While strains of the same species are presumably genetically similar, Paasche (115) has pointed out that under certain conditions, strain differences may be significant. Therefore, while identification will generally be limited to genus or genus-species designation, when appropriate, strain differences will be noted.

Coccolith Formation

Intracellular coccoliths had been observed very early by Dixon (27) and Lohmann (74), and the intracellular origin of coccoliths has been proposed by several authors as a result of their observations on collected material (19, 27, 65, 77). Laboratory investigations with cultured material using the light microscope (67, 108, 119), and more recently with the electron microscope (86, 87, 107, 123, 161), have clearly demonstrated that coccoliths are formed either partially or wholly in the intracellular milieu.

Physiological and nutritional studies

Physiological and nutritional studies on coccolith formation have been mainly limited to two genera, Hymenomonas and Coccolithus. The use of controlled laboratory conditions to elucidate the physiological parameters associated with coccolith formation has provided significant information regarding this form of biological mineralization. For the sake of discus-
sion this work can conveniently be divided among four categories - light, temperature, chemical effectors, and age.

**Light** The association between light, photosynthesis, and coccolith formation has been extensively investigated by several workers. In short-term experiments, light has been shown by Crenshaw (21) to be a requirement for coccolith formation in clones of *C. huxleyi* and *H. carterae*. Paasche (110), in another clone of *C. huxleyi*, found that the rate of coccolith formation in light was seven to ten times that measured when the cultures were placed in the dark. While most work seems to suggest that periodic exposure to light is necessary for coccolith formation (115, 162), Isenberg *et al.* (46, 52) suggest that under conditions of organic supplementation to the growth medium, *H. carterae* can grow and form coccoliths in the dark. Their observations however lack adequate controls and fail to differentiate between growth and coccolithogenesis occurring immediately after exposure to light and that occurring after long periods of dark incubation.

Paasche, in 1962 (108), noted a quantitative association between carbon-14 uptake in photosynthesis and coccolith formation in *C. huxleyi*, with the ratio of uptake in the two processes being about one. Subsequently, several workers suggested that this might result from the interaction of bicarbonate ions which could serve as a common source of inorganic carbon for both photosynthesis and coccolithogenesis (21, 109, 141). Paasche (110) summarized this proposed reaction as follows:

\[
2 \text{HCO}_3^- = \text{CO}_2 + \text{CO}_3^= + \text{H}_2\text{O}
\]

**photosynthesis** Coccoliths

Recent work has failed to support this hypothesis (110, 115, 116, 117). In particular, comparative studies using the motile (holococcolithophorid) and
non-motile (heterococcolithophorid) phases of C. pelagicus have indicated that the amount of carbon (as C-14) incorporated into holococcoliths amounted to less than 2% of that used in photosynthesis, while that incorporated into heterococcoliths was several times more than that assimilated photosynthetically (116). Thus, the one-to-one relationship between photosynthetic and coccolith carbon may be no more than coincidental. In addition, Paasche and Klaveness (115, 117) suggest that naked clones (not bearing coccoliths) can probably use bicarbonate as a source of photosynthetic carbon in the absence of coccolith formation.

Studies measuring the uptake of carbon-14 in coccolithogenesis and photosynthesis as a function of light intensity at various wave-lengths indicated that the action spectra for the two processes were similar, with absorption peaks at about 440 nm and 670 nm (112). Blue light, however, appeared to be relatively more efficient in coccolith formation than in photosynthetic assimilation. Paasche suggests that two photochemical reactions, one mediated by chloroplast pigments and the other mediated by some pigment absorbing specifically in the blue part of the spectrum may be involved in the light reactions associated with coccolith formation. Within the context of this hypothesis, it is interesting to note that Olson, Jennings, and Allen (105) have identified an extrachloroplastic pigment body in Hymenomonas whose maximal absorption is in the blue range. Any possible relationship between this body and coccolith formation, however, remains obscure.
While most physiological data show a relationship between light and coccolith formation, the nature of this relationship is unclear. Coccolith formation may be associated with photosynthesis through carbon assimilation, photophosphorylation, or some more complex manner. In addition, it appears that a light reaction other than photosynthesis may be involved in coccolith formation.

**Temperature**  
As one might expect, temperature changes affect growth and coccolith formation. In general, the optimal temperature for cell division and coccolithogenesis is in the range of 18°C to 22°C (49, 113, 161, 162).

Coccolith dimensions are also apparently affected by temperature changes. Watabe and Wilbur (157, 162) maintained cultures of *C. huxleyi* at constant temperatures over the range of 7°C to 27°C and examined coccoliths from these cultures in the electron microscope to determine their dimensions. There was no significant difference in over-all coccolith length or width at temperatures between 7°C and 18°C; however, at temperatures above 18°C both length and width decreased. Temperature also apparently affected the dimensions of the individual calcified elements making up the coccolith. These authors cite the work of McIntyre and Be (94) with natural populations of *C. huxleyi* as paralleling their work on laboratory clones. They also note that the number of cells forming morphologically abnormal coccoliths at 7°C and 27°C was two- to three-fold greater than at 18°C and 24°C, and that under conditions of nitrogen deficiency, temperature also affects coccolith crystalline structure (161).
**Chemical effectors**  The effect of the chemical environment on coccolith formation has been dealt with by several authors (21, 46, 47, 49, 51, 52, 110, 161). The use of defined nutritional conditions, and the controlled experimental modification of these conditions, has proven to be a valuable tool in studying coccolith formation. Investigations concerned with the effect of the chemical environment on coccolith formation can be divided into two categories, (1) those which employ quantitative modifications of nutrients, and (2) and those which employ specific inhibitors.

As one might expect, the role of calcium and carbonates in the chemical environment has received particular attention by investigators interested in coccolith formation. The concentration of these ions is important not only in the formation of coccoliths, but also in the maintenance of coccolith structure once it is exposed to the extracellular environment where coccolith CaCO$_3$ is in equilibrium with ionic calcium and the carbon dioxide-carbonate equilibrium system (21, 110, 115, 145).

Paasche (110), using carbon-14 uptake as a criterion, found the calcium saturation level for coccolith formation in *C. huxleyi* to be about 0.24 g/liter (2 x 10$^{-3}$M). At 1.5% of this concentration the rate of calcification was reduced to 1 to 2% of the saturation level, while photosynthetic uptake of C-14 was only slightly affected. Crenshaw (21), working with a different strain of *C. huxleyi*, found that the organisms failed to form coccoliths at 10$^{-3}$M calcium and reached calcium saturation at about 10$^{-2}$M. He also indicated that little growth takes place at calcium concentrations less than 0.75 x 10$^{-2}$M. Isenberg et al. (49) indicate that a calcium concentration of 10$^{-2}$M is optimal for growth of *Hymenomonas*, and coccolithogenesis is initiated at 10$^{-5}$M. In contrast to Crenshaw's work with
C. huxleyi, Isenberg et al. (49) state that growth of Hymenomonas occurs in calcium concentrations as low as $10^{-6}$ M.

In general, coccolith formation seems to be dependent on an adequate supply of inorganic carbon in the chemical environment. However, the exact nature of this dependency is affected by a number of factors including pH, light intensity (see section on light), and presence of organic carbon sources (21, 49, 52, 110). While both $\text{CO}_2$ and bicarbonate are apparently used as carbon sources for photosynthetic assimilation by C. huxleyi (110), bicarbonate seems to be the major carbon source for coccolith formation (21, 110).

The effect of pH on coccolith formation is probably associated with the pH dependent $\text{CO}_2$-carbonate equilibrium system. According to Paasche (110), the pH optimum for coccolith formation in C. huxleyi occurs between pH 7.5 and 8.5. The rate of coccolith formation drops somewhat at more alkaline pH, and is reduced drastically at acid pH (i.e. at pH = 6.3 coccolith carbon amounted to only about 3% of the control at pH = 7.5 - 8.0). Acid inhibition of coccolith formation may be due to inhibitory effects of molecular $\text{CO}_2$, or to the inability of cells to maintain a sufficiently high pH to allow precipitation of calcium carbonate.

The ability of organic compounds to stimulate growth and coccolith formation appears to differ between genera, and even within strains of the same species (115, 125). Isenberg and his colleagues have studied the effect of numerous nitrogenous and non-nitrogenous organic compounds (amino acids, imino acids, simple carboxylic acids, and their derivatives) on growth and coccolith formation of Hymenomonas (46, 52). Their data suggests that some of these compounds could differentially inhibit or stimulate
growth (cellular nitrogen) and/or coccolith formation. They additionally indicate the concomitant presence of supplementary carbonate in the test media can alter the effect of organic supplementation. Crenshaw (21) indicated that, at least for the case of lactate, *Hymenomonas* failed to utilize the organic component until carbonate and bicarbonate had been depleted from the media.

Several investigators have examined the effect of inorganic ions other than calcium and carbonate on coccolith formation. Indirect evidence suggests that salinity, within the growth range of an organism, does not seem to affect the ability of *Hymenomonas* to form coccoliths (11). Isenberg and his co-workers (28, 46, 48, 49, 50) have examined the effect of divalent cations in *Hymenomonas*. Mg$^{++}$ or Ba$^{++}$ apparently play no role in coccolithogenesis (49). The Mg$^{++}$/Ca$^{++}$ ratio may, however, affect the ability of organism to utilize some organic nutrients and therefore may indirectly affect coccolith formation (52). Neither Mg$^{++}$ or Ba$^{++}$ was able to substitute for Ca$^{++}$ as a growth requirement. Sr$^{++}$ on the other hand could substitute for Ca$^{++}$ for growth, though no effect on growth or coccolithogenesis could be detected when both Ca$^{++}$ and Sr$^{++}$ (in concentrations between 10$^{-6}$ M and 10$^{-2}$ M) were present in the growth media (46, 49).

Only nitrate among the non-carbonaceous anions has been found to have a significant effect on coccolith formation. Wilbur and Watabe (161) noted that a reduction in the nitrate concentration of their growth media from 150 mg/liter to 16.7 mg/liter could induce a non-coccolith forming strain of *C. huxleyi* to form coccoliths. In addition, as previously mentioned, nitrogen deficiency in conjunction with temperature variations resulted in
crystallographic changes in coccoliths of normal and nitrate induced calcifying strains of *C. huxleyi*.

The effects of several metabolic inhibitors on coccolith formation have been examined. Paasche (110) examined the effects of hydrocyanic acid, 2, 4-dinitrophenol (DNP), hydroxylamine, acetazolamide (2-acetylamino-1,3,4-thialdiazole-5-sulfonamide), and 3-(p-chlorophenyl) -1, 1-dimethyl urea (CMU) on photosynthesis and coccolith formation in *C. huxleyi*. Hydrocyanic acid affects both respiration and photosynthesis in algae, although the relative extent of inhibition may vary in different organisms. DNP acts primarily in uncoupling oxidative phosphorylation, with light-dependent phosphorylation being somewhat less sensitive. Hydroxylamine is an inhibitor of photosynthesis in some algae. Hydrocyanic acid, DNP, and hydroxylamine all inhibit both photosynthesis and coccolith formation, with photosynthesis being inhibited slightly less than coccolithogenesis. Acetazolamide, known to affect some calcification systems (20, 37, 41, 93), is an inhibitor of carbonic anhydrase, the enzyme which catalyzes the interconversion of molecular CO$_2$ and carbonic acid. It inhibits both photosynthesis and coccolith formation to an equal extent. CMU, an inhibitor of the Hill reaction (photolysis of H$_2$O and evolution of O$_2$), affects coccolith formation to a lesser extent than photosynthesis. Paasche suggests that this information supports his hypothesis that coccolith formation is linked to photosynthesis and is thus light-dependent, through a requirement of energy derived from photophosphorylation, which is not CMU sensitive, rather than mutually sensitive carbon uptake which would be CMU sensitive. He accounts for the effects of hydrocyanic acid and DNP by suggesting that
respiratory energy may also be required during some stage in coccolith formation.

The effects of DCMU, 1-(3,4-dichloro) phenyl-3,3-dimethylurea, a compound closely related to CMU, and acetazolamide on coccolith formation in *Hymenomonas* have also been evaluated. Crenshaw (21) found that DCMU affected photosynthesis and coccolith formation to an almost identical degree. Isenberg, Lavine, and Weissfellner (51) related the effect of Diamox (sodium acetazolamide) on coccolith formation to growth (cellular nitrogen) rather than photosynthesis. Their data indicate that coccolith formation could be completely inhibited at concentrations which reduce growth by only about one-third. The significance of the differing inhibitor data obtained with *C. huxleyi* and *H. carterae* is not clear since it is unlikely that these organisms differ markedly in the mechanism by which they form coccoliths. They may rather reflect differences in experimental techniques used by the investigators (see Discussion).

**Age** Isenberg and his colleagues have made extensive references to the importance of culture "age" in relation to coccolith formation. They contend that coccolith formation as a cellular function is a characteristic of cultures in very late logarithmic or stationary growth phase. They were unable to microscopically detect coccoliths on cells in actively growing cultures and were unable to chemically detect calcium exchange between the chemical environment, cell, and coccolith fractions until "non-proliferative" conditions existed in the culture (46, 47, 52). These results however are not substantiated by the work of other investigators (11, 21, 107, 110, 113, 129, 145), and in some cases culture aging led to the accumulation of a non-coccolith forming phase of the life cycle (11, 129).
Structural studies

A number of investigations have emphasized the importance of the electron microscope to structural studies of coccoliths (e.g. 9, 10, 14, 40, 94). The minute size of coccoliths (approx. 0.25 to 15μ) restricts work on these elements at the light microscope level, particularly in the more complex forms. Thus, while early investigators were able to differentiate between grossly different coccoliths, and note the birefringent nature of the calcite crystals, detailed structural studies awaited the advent of transmission and scanning electron microscopy.

At the electron microscope level of resolution, a coccolith can morphologically be divided into three types of structural components. CaCO₃ crystals characterize all coccoliths (9, 115), and a subtending scale-like base is often observed (21, 62, 63, 87, 90, 106, 107, 118, 119). In addition, a number of workers have reported that the calcified elements of the heterococcoliths of Hymenomonas and Coccolithus are ensheathed by organic material (14, 21, 63, 87, 90, 107, 163, 164).

The term "coccolith" has been used in a restricted sense to refer only to the complex of calcified elements (this may or may not include the organic sheath around them) (21, 87, 90, 118) or, in a broader sense, to refer to the integrated unit consisting of the complex of calcified elements ensheathed in their organic matrix, and the subtending organic scale-like base (107, 122, 123, 163, 164). For reasons discussed by Outka and Williams (107) the use of the term "coccolith" in the broader sense seems preferable.

The base of the coccolith appears to be morphologically homologous to the unmineralized scales produced by other members of the haptophyceae (17,
Brown and his co-workers (17) have identified the chemical nature of the scales of *Pleurochrysis* (*Hymenomonas*) scherffelii as being primarily polysaccharide, including cellulose and a pectin-like moiety, along with 3 to 9% protein. It seems likely that the coccolith base is chemically similar to these scales (35).

The organic sheath, or matrix, surrounding the rim elements is closely appressed to the crystalline surfaces, so that its form exactly mimics that of the crystalline elements, even after experimental removal of the mineral components of the coccolith (14, 21).

The structure and composition of the mineral parts of coccoliths have been the subject of several recent reviews (9, 23-26, 101). Crystallographic analysis has indicated that, in general, the CaCO₃ crystals that make up the coccoliths are in the form of calcite (9, 45, 68, 156). An exception to this has been noted for *C. huxleyi* when grown under conditions of nitrogen deficiency (161). Sr⁺⁺ and PO₄⁻⁻, if present in coccoliths, are at a very low level (68, 161).

Black (9) in discussing control of crystal growth notes that crystallographically, the simplest coccoliths, such as the crystalloliths of the motile phase of *C. pelagicus*, are formed of calcite elements no different than those that can be precipitated in a test tube, while in the more elaborate coccoliths, such as the placoliths of *C. huxleyi*, crystal morphology is suppressed and the molding of the crystalline units is completely controlled by the organism. He noted, however, that even in the simplest of coccoliths, where crystal structure may not be closely controlled, the placement of microcrystals within the coccolith structure is under cellular control. Outka and Williams (107) have additionally pointed out that
Hymenomonas, in forming its dimorphic cricoliths\(^1\) is able to control the crystallization of morphologically distinct calcite elements within the same coccolith.

Manton and Leedale (87) suggested that the organic sheath, arbitrarily referred to as a matrix (14, 21), surrounding the calcite elements may play an important role in determining coccolith morphology. Outka and Williams (107) support this contention, and, on the basis of developmental data suggest that the organic sheath represents a matrix for calcification in the \textit{strict sense} as defined by Eastoe (29). Thus it is this matrix that allows the cell to define and limit the crystallization process.

The first electron microscopic studies of coccolith formation appear to be those of Manton and Leedale (86) and Wilbur and Watabe (161). Manton and Leedale (86), studying holococcolith formation in the motile phase of \textit{C. pelagicus} were unable to demonstrate intracellular calcification or attachment of the coccolith to Golgi derived organic scales, as had been previously suggested by Parke and Adams (119). Rather, they suggested that calcite microcrystals are formed between the plasmalemma and an external "skin" from soluble materials secreted into this region.

Wilbur and Watabe (161) described heterococcolith formation in \textit{C. huxleyi} as occurring intracellularly in a homogeneous non-granular material which they refer to as an organic matrix region. This region was observed to be generally located between the nucleus proximally and a "reticular body" distally. Calcification was described as proceeding from many cen-

\(^{1}\)Cricoliths are formed from two morphologically distinct, elaborate mineral elements which alternate about the periphery of the coccolith base, thus forming a calcified rim on the organic scale.
ners near the future coccolith base. In the early stages of calcification a thin "membrane" was seen connecting the centers of calcification. They proposed that the form of the coccolith is determined prior to calcification by conformation to the shape of the matrix region. They additionally indicated that the crystalline base plate of the coccolith "may form on or within a thin sheet of material" which would therefore influence the pattern of coccolith development.

Crenshaw (21) described intracellular coccoliths in *Hymenomonas*, identified the organic sheath about the crystalline elements, and correctly associated the coccolith base, to which the interlocking calcite crystals were attached, with haptophycean scales. He, however, included no structural information regarding the formation of coccoliths.

Isenberg and his co-workers have conducted an extensive series of investigations into the structural basis of coccolith formation in *H. carterae*. Based on their ultrastructural observations on normal and experimentally modified cells, they proposed a theoretical pathway for coccolithophorid mineral deposition (45). These authors hypothesized that the Golgi apparatus gives rise to a WW (reticular) body and a mineral reservoir which in turn give rise to an intracellular coccolith precursor body (ICP) through the mediation of fibrous elements (fibers). The ICP then forms a coccolith, and a remaining portion which is either reincorporated into a new ICP or degenerates into a fat body. Cytochemical evidence cited by these authors (28, 45) indicated that sulfated polysaccharides are involved in the mineralization process. In addition, they extracted, isolated, and characterized a mineral binding fraction (fraction F-1) from isolated coccoliths. This fraction contained both proteins and carbohydrates, and,
interestingly, amino acid analysis indicated the presence of hydroxyproline, an amino acid normally associated with collagen. They proposed that the F-1 fraction constituted the organic matrix of the coccolith, and equated it, without morphological evidence, to the "fibers" that they observed ultrastructurally (45).

More recently several authors have criticized aspects of the ultrastructural scheme on which the Isenberg model was based. The fibers, which Isenberg and his co-workers considered the calcification matrix are now known to be plate-like scales and unlikely candidates for the functions that they ascribed to them (87, 107, 115, 123). The intracellular coccolith precursor body has been equated to an autophagic vacuole on morphological and developmental grounds by Outka and Williams (107), and on cytochemical evidence by Pienaar (1971, personal communication). The ICP would therefore appear to be involved more in catabolic rather than anabolic activities. Thus the Isenberg scheme for coccolith formation, as well a scheme proposed by Pienaar (123), which also employed an ICP body, have been discredited.

A more direct association of the Golgi apparatus in coccolith formation has been proposed by Outka and Williams (106, 107, 163, 164), Manton and Leedale (87), and Manton and Peterfi (90). In comparative studies of coccolith formation involving *C. pelagicus* (non-motile stage, *H. carterae* and *H. roseola*, Manton and her co-workers concluded that coccoliths arose in a manner analogous to the non-calcified scales of other haptophyceae that had been previously studied (78-86, 88, 89, 91). They concluded that calcification began as a marginal deposit on a preformed organic scale and hypothesized an organic matrix was important in determining coccolith morphology.
Outka and Williams (107, 163, 164), in a more extensive morphological investigation into coccolithogenesis in *H. carterae*, have proposed that coccolith formation occurs sequentially within a highly polarized Golgi apparatus. They suggested that the first stages in the step-wise assembly involve the accumulation of electron-dense granular bodies (coccolithosomes) and the apparently independent assembly of highly structured scale-like bases in cisternae of the proximal, less mature Golgi region. Subsequently, coccolithosomes appear in the base-containing vesicle where they apparently contribute material to the formation of the sheath-like matrix of the coccolith rim. Matrix formation is initiated at the periphery of the scale-like base, with Golgi cisternal membrane closely associated with the developing matrix. Calcification of the rim elements then occurs in areas predelineated by matrix material. After the completion of coccolith formation in the intracisternal milieu, the coccoliths are placed extracellularly. The observations of Leadbeater (63) and Pienaar (1971, personal communication) support various aspects of this scheme.

If one can assume that the structural homology between the coccolith base and the non-mineralized haptophycean scale can be extended to morphogenetic homology, as seems to be the case (17, 35, 87), then the morphogenetic work of Manton and her co-workers (e.g. 64, 78-91) with many algal species, Brown and his co-workers (17, 35) with *Pleurochrysis*, and Pienaar (123) with *Hymenomonas* becomes pertinent to this review. Over a period of many years, Manton and her associates have shown that extracellular structures associated with many types of algae are formed in the Golgi apparatus, and in particular, that the organic body scales of the haptophytes *Chrysochromulina* (64, 80, 81, 88) and *Pyrmnesium* (79, 85) are Golgi derived.
The polysaccharide nature of haptophycean scales has been indicated by the work of Green and Jennings on *Chrysochromulina* (38), and Brown and his co-workers on *Pleurochrysis* (17). Based on their ultrastructural observations, Brown *et al.* (17) proposed a hypothetical model for scale formation. They suggested that the Golgi apparatus is responsible for synthesis and assembly of scales. Glucose polymerization to form cellulose chains occurs in central dialation of immature Golgi cisternae. The completed cellulose fibers, in a series of steps, then form the structured portion of the scale. In the distal (mature) Golgi region, the scale-containing cisternae become inflated as a result of fusion of vesicles derived from the periplastidal cisternae containing non-cellulosic polysaccharide constituents of the scale. The scale is then released to the exterior of the cell by an exocytotic process. Pienaar (123) has independently proposed an alternate, though perhaps not mutually exclusive, Golgi-associated model for scale formation in *Hymenomonas* in which the scale radiations are laid down on a series of tubules.

**The coccolithogenic system in relation to other eucaryotic systems**

Coccolith formation is an excellent protistan model for biological mineralization. While physiological studies tend to emphasize its plant affinities, structural work suggests a close relationship to animal mineralization systems (28, 45-47, 107, 164). In addition, Outka and Williams (107, 164) have cited the applicability of coccolithogenic studies to certain other areas of cell biology. Coccoliths, though complex structures, are in one sense, no more than Golgi-derived secretory products. Thus, the study of coccolithogenesis becomes the study of Golgi secretion. Another
interesting aspect of coccolithogenesis is the means by which the cell controls the formation of the complex coccolith structure. As pointed out by these authors, compared to self-assembly systems, such as phage, coccolith formation must require additional and/or different kinds of ordering and control mechanisms.

While the literature in any one of these three areas—Golgi function, mineralization, or cellular control—is far too large to exhaustively review here, it would seem desirable at least to cite examples in order to put coccolith formation in perspective as a model system for biological research. To this end, I have selected a few references from each of these areas to present in the following sections.

While the Golgi apparatus is most certainly a multifunctional organelle, it is perhaps best known for its role in cell secretion. It seems to be involved in the synthesis and packaging of a large variety of cellular products, as illustrated in the cytochemical studies of Siekevitz and Palade (134, 135) on pancreatic protein secretion and Peterson and Leblond (121) and Neutra and Leblond (98) on glycoprotein secretion. Beams and Kessel (5) and Favard (32) have cited numerous similar studies in their recent reviews of Golgi structure and function.

Many Golgi products are destined to form cell walls or coats of some sort (e.g. 6, 42, 76, 96, 104, 127, 166) and are thus analogous to the "secretion" of "coccolith coats." It is interesting to note that Brown et al. (17) in his important work on the scale layers of Pleurochrysis provided direct proof relating cellulose synthesis and secretion to Golgi function and thus align it with other polysaccharide secretory systems (5).
An additional similarity between coccolithogenesis and other secretory systems can be inferred from the recent work of Brown and Franke (15, 16). They have suggested, as a possibility, that microtubular arrays associated with the Golgi apparatus in *Pleurochrysis* may be involved with scale secretion, as has been noted for other secretory systems (61, 100, 126, 136, 165).

Polarization of the coccolithophorid Golgi apparatus (17, 107, 163) is similar to the situation observed in numerous other cell types (5, 32). Recent morphological, cytochemical, and biochemical information has suggested that cytomembranes are in a dynamic state of interchange (e.g. 31, 33, 34, 54, 57, 92, 134, 135, 159), with the polarity of the Golgi apparatus playing an important role in membrane transitions (e.g. 32, 39). As pointed out by Williams and Outka (164), the coccolithophorid system may offer distinct advantages for studying the phenomenon of membrane interchange, since during the sequential formation of coccoliths, the various stages of coccolith development serve as "signposts" for identifying specific regions of the Golgi. This would seem particularly desirable for *in vitro* studies.

The association of the Golgi apparatus to biological mineralization is by no way unique to the coccolithophorids. Golgi involvement in mineralization has been observed in protistan (133, 142), invertebrate (1, 144), and vertebrate (2, 7, 8, 36, 158) systems. In none of these cases, however, except perhaps the autoradiographic study of Weinstock and Leblond (158), is the morphological evidence of Golgi participation as clear as in the coccolithophorid system. Botanical systems have been less extensively
studied, and, while organic control of inorganic deposition is apparent, the source or mechanism of control is not well documented (4).

The chemical studies of Isenberg and his co-workers (45) on their F-1 fraction of coccoliths are interesting, albeit somewhat questionable as to exactly what the F-1 fraction represents. If it is indeed the calcification matrix, then its glycoprotein nature is similar to that of other calcifying matrices (124, 131). The presence of hydroxyproline in this fraction is additionally interesting since it is considered to be a characteristic of collagenous material (131), and while collagen is an important constituent of many mineralizing systems, its presence in unicellular organisms has not been established (103).

With reference to the organic matrix-inorganic crystal relationship, coccolith formation has an interesting position relative to other systems. Travis, in her recent study of calcified tissues (149), ranks a crustacean system, where the matrix effects little morphological or crystallographic control, as a rather primitative type of mineralization. As compared to this system, as well as the "more advanced" systems studied by Dr. Travis, the coccolithophorid system, both crystallographically (9, 156) and morphologically (9, 107), would seem to be highly evolved.

The nature of cellular control over the assembly of three-dimensional macromolecular structures has been the subject of much study in recent years (e.g. 3, 55, 56, 60, 137, 146, 167). Self assembly systems in which the structural units contain all of the information necessary for their assemblage have been particularly useful models for biological investigation (3, 55, 60, 102). The formation and assembly of coccolith-scale precursors on the other hand would seem to require significant information in
addition to that provided by the structural subunits, i.e., "instructional" information as opposed to "structural" information, and an important involvement of cisternal membranes has been suggested by Manton and Ettl (82) and Outka and Williams (107), and implied by the earlier work of Manton (78, 80) and Wilbur and Watabe (161, see Discussion). Thus, while much biological effort seems to be expended in producing structures whose assembly requires a minimum of instructional information (4, 43, 59, 60, 130, 150), the members of the Coccolithophorideae seem to be investing a great deal of "instructional energy" into the formation of coccoliths (9, 107).

A question arises when one considers the amount of energy spent on coccolith formation. Why? What is the function of the coccolith that requires it to possess such complex form? The idea that coccoliths may function as elements in a cytoskeletal armor would seem to be the most obvious and apparently the earliest suggestion of function (97, 147). More recent ideas about coccolith function include a role in protection against high light intensity (14), photosynthetic bicarbonate utilization (21, 141), and disposal of excess cellular CO₂ (45, 46). Little convincing evidence however has been presented to support any one of them as the function of coccoliths. Whatever function, or functions, are eventually ascribed to coccoliths, it will be necessary to show not only that the coccolith contributes to, or is involved in the function, but also that the ascribed function is economically equal to the energy expenditure necessary to produce coccoliths.
MATERIALS AND METHODS

Standardization of Culture and Sampling Conditions

*Hymenomonas* (Syracosphera, Cricosphera) *carterae*, Plymouth Culture Collection No. 181 was obtained in unialgal culture from Dr. L. Provasoli (Haskins Laboratory, New York, N.Y.), treated with antibiotics (see Appendix A) for about 30 days, and maintained on a culture medium modified from that of Isenberg *et al.* (51), (Appendix A). When additions to the standard growth medium were required for experimental purposes, except for low molarity Ca$^{++}$ (i.e. less than $10^{-2}$M) or reduced salt concentrations, these additions were added to the sterilized medium, and the medium was resterilized by filtration through Millipore membrane filters with a pore size of 0.22 μ.

The standard conditions of culture are as follows: 100 to 150 ml of culture medium per 500 ml stainless steel-capped Delong culture flask were incubated in a Precision Scientific controlled-temperature incubator at 17°C to 19°C with an incident light intensity of about 600 ft-c from 14-watt cool-white fluorescent tubes delivered in a repeating photocycle of 16 hours light and eight hours dark. Unless otherwise noted, initial exposure to experimental conditions took place between the second and fourth hours of the light phase, thus reflecting an interphase rather than division phase of growth. Inocula varied with different experiments, but usually resulted in an initial concentration of organisms in the medium of between $5 \times 10^3$ and $6 \times 10^4$ organisms/ml. As discussed in Results and Observations, except in cases involving prolonged growth or kinetic studies, experimental cultures and inoculae were maintained on a 48 hour transfer schedule, and har-
vested for experimental studies at concentrations of less than $3 \times 10^6$ cells/ml. Deviation from these standard conditions will be discussed in this section under the appropriate headings.

Growth determinations for all experiments were made in triplicate by direct cell counts employing microscopic observation and haemocytometer chambers (A. O. Spencer). Aliquots from cultures were counted either live, or fixed with Lugols iodine (74). Culture aliquots taken for counts during the dark phase of growth were obtained in total darkness.

General Structure

Actively growing cells cultured under standard conditions were examined by fluorescent, phase contrast, polarization, dark field, and Nomarski differential interference contrast microscopy using a Zeiss photomicroscope. Original photographs were taken on Kodak High Speed Ektachrome (daylight) or Plus-X film using electronic flash. Electron microscopic observations were carried out with either a modified RCA EMU 3-f or Hitachi HU-11E-1. Original micrographs were taken on either Kodak Electron Image Plates or Dupont Cronar Ortho S Litho sheet film.

Sectioned material standardly was prepared according to the schedule appearing in Appendix B. Coccoliths and scales examined in whole-mounts were obtained from cultures of varying age by differential centrifugation. Cells were removed from 50 ml of culture medium by centrifugation at about 250 G for five minutes. Coccoliths were then centrifuged from the supernatant at 1000 G for 15 minutes, resuspended and rinsed in either dilute NaOH

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1 Growth is defined by these conditions as the increase in cell number per unit volume of culture medium.
(pH 10) or 0.1 M (NH₄)₂CO₃ (pH 10), and placed on parlodion-carbon coated grids. Unshadowed preparations were examined in the electron microscope without further treatment. Other preparations were shadowed with platinum metal at angles varying from 15° to 35°. Carbon replicas were prepared according to the procedure outlined by Black (10).

Comparison of Processing Procedures for Ultrastructural Observation of Forming Coccoliths

The following staining solutions were examined with respect to their effect on sectioned material and compared to unstained sections: 0.5 to 2% aqueous uranyl acetate, 1% uranyl acetate in 50% ethanol, 5 to 20% uranyl acetate in absolute methanol, 0.2 to 0.3% lead citrate prepared according to Venable and Coggeshall (151), and sequential uranyl acetate-lead citrate staining with the various combinations. The sectioning flotation fluids compared with respect to their effect on crystallized CaCO₃ in the sections included distilled water, dilute sodium hydroxide (pH 10-11), and an 0.1 M sodium cacodylate-0.01 M CaCl₂ buffer solution. In addition to fixation in the standard glutaraldehyde-osmium tetroxide sequence (Appendix B), cells were fixed 2.5 to 4.5 hours in the "standard" glutaraldehyde solution without osmium post fixation, or for a comparable time in the "standard" osmium solution without previous glutaraldehyde fixation.

Effects of Decalcifying Procedures on Components of the Coccolithogenic System

Decalcification procedures were carried out at three stages during a fixation-dehydration sequence modified from that described in Appendix B and compared to standardly processed cells. The details of the decalcification procedures are described under the corresponding heading of the
Results and Observations section and summarized in Table 1. Additions of
decalcifying agents to fixation and rinse solutions were accompanied by an
equimolar reduction in the sucrose content. Following fixation and decal-
cification, cells were dehydrated in a graded series of ethanol. The
remaining preparative steps are those listed in Appendix B with the
cacodylate-CaCl$_2$ flotion medium being used.

Decalcification of living material (i.e. in vivo decalcification) was
carried out by suspending cells for eight to 12 hours in at least two
changes of a medium similar to the growth medium (Appendix A) except that
it contained no added CaCl$_2$ and had a pH of about 5.5. The cells were then
subsequently suspended in a Ca$^{++}$-deficient growth media, pH 7.8, for a
period of one to 12 hours. Exceptions to this general scheme of in vivo
decalcification will be noted under the appropriate headings in this sec-
tion.

Kinetics of Coccolith Formation

The rate of coccolith formation was determined by counting the number
of coccoliths appearing on the surface of cells at time intervals following
resuspension of in vivo decalcified cells in the appropriate medium under
the specified conditions. Counts were made using Nomarski differential
interference contrast optics at a magnification of about 1250 diameters
with a plan oil immersion objective stopped down to a numerical aperture of
about 0.8. Accurate counts of surface coccoliths per cell could be made by
focusing carefully through individual cells carrying between one and 40 to
50 coccoliths. Reasonable approximations (10 coccoliths/"ave. cell")
could be made on cells carrying between 50 to 80 coccoliths, though in gen-
eral, counts of greater than 40 coccoliths per cell were considered "too numerous to count." The number of cells counted to obtain averages varied with the number of coccoliths per cell, however, in general, between 25 and 100 cells were counted when the number of coccoliths per cell was less than 15, and between ten and 25 cells were counted when the number of coccoliths per cell was greater than 15. The material for coccolith counts was examined either live, or fixed by a 1:100 dilution with the "standard" glutaraldehyde solution (Appendix B).

**Effect of Light (Dark) and Heterotrophic Nutrition on Coccolithogenesis**

_In vivo_ decalcified cells were incubated during the normal eight hour dark period in one ml of calcium deficient (i.e. no Ca$^{++}$ added) growth media in 50 ml stainless steel-capped Erlenmeyer flasks. After this initial eight hour dark incubation, 15 ml of normal growth media ($\text{Ca}^{++} = 0.01 \text{ M})$ was added to these cultures. Complete darkness was maintained during this operation. One set of flasks was then incubated under standard conditions in the light, and a second set was incubated in the continued absence of light. The rate of coccolith formation was determined by fixing the contents of light and dark incubated flasks at specified intervals with the standard glutaraldehyde solution and counting the number of coccoliths per cell as previously described. A second experiment was carried out under parallel conditions, except that lactate was omitted from all solutions, and the final incubation media was supplemented with $10^{-3}$ M Na$_2$CO$_3$. Cell counts were made at the beginning and termination of the experimental incubation period to determine if cell division took place.
Effect of Ca^{++} Concentration on Growth and Coccolithogenesis

*In vivo* decalcified cells were inoculated into growth media with CaCl_2 added to concentrations of 10^{-2} M (control value), 10^{-3} M, 10^{-4} M, 10^{-5} M, 10^{-6} M, and "0" M (i.e. no added Ca^{++}), with no concomitant increase in other salt concentrations to compensate for osmotic or other effects of decreased CaCl_2 concentration. The culture condition used for all experiments deviated from those previously described in Standardization of Culture and Sampling Conditions in that 16 x 125 mm screw cap culture tubes filled to a volume of five ml and slanted at an angle of about 15° from the horizontal were used rather than DeLong flasks. Cell and coccolith counts were made at intervals for kinetic and growth studies. Cells were fixed at 0 hour (uninoculated *in vivo* decalcified cells), 1 hour, 12 hour, 24 hour, and 48 hour intervals for fine structural analysis. All glassware was specially processed for this experiment, including soaking it in three changes of 0.1 M Na_4EDTA solution over a period of 24 hours.

The Effect of Sr^{++} on Growth and Coccolithogenesis and Comparison with High Molarity (0.1 M) Ca^{++} and Mg^{++}

Undecalcified cells (for growth experiments) or *in vivo* decalcified cells (for kinetic experiments) were inoculated into standard growth media with added SrCl_2·6H_2O at concentrations of 10^{-1} M, 10^{-2} M, 10^{-3} M, 10^{-4} M, 10^{-5} M, and "0" M (i.e. standard growth medium only) with no concomitant decrease in other salt concentrations to compensate for the increased SrCl_2 concentration. The culture conditions for all experiments differed from those previously described in that 16 x 125 mm screw cap culture tubes containing five ml of medium or 25 x 150 mm screw cap culture tubes containing
15 ml of medium, slated at about 15° from the horizontal, were used rather than DeLong culture flasks. Cell and coccolith counts were made at intervals for kinetic and growth studies. Cells were fixed at 0 hours (inoculum control), 1 hour, 4 hours, 12 hours, 24 hours, 48 hours, and 31 days for fine structural analysis. Cells fixed at 24 and 48 hours were not decalcified prior to exposure to Sr++ concentrations. The cells examined after 31 days were transferred to fresh media at 48-hour intervals during that period.

The comparative effect on growth and the kinetics of coccolith formation produced by the addition of 0.1 M SrCl$_2$·6H$_2$O, 0.1 M MgCl$_2$·6H$_2$O, or 0.1 M CaCl$_2$ to the standard culture media was examined. Undecalcified cells were used for the growth experiment. In vivo decalcified cells used for the kinetics of coccolith formation (Figure 65) differed from those standardly used in that they had been incubated in the Ca$^{++}$-deficient medium for five days previous to inoculation into the experimental medium. Culture conditions deviated from those previously described in Standardization of Culture and Sampling Conditions in that 16 x 125 mm screw cap culture tubes containing five ml of culture media slanted at about 15° from the horizontal were employed.

*H. carterae* grown in a medium containing $10^{-2}$ M SrCl$_2$·6H$_2$O, but no CaCl$_2$, has been maintained in culture for over four years, and, while growth studies using the technique employed are hampered by the tendency of these cells to clump in culture, there can be no doubt about their ability to grow under calcium deficient conditions. In addition to the solutions standardly employed in preparing cells for ultrastructural analysis (Appendix B), fixation and washing solutions in which equimolar SrCl$_2$·6H$_2$O was substituted for the $10^{-2}$ M CaCl$_2$ were used.
Effects of Acetazolamide on Growth and Coccolithogenesis

Acetazolamide, 2-acetylamino-1,3,4-thialdiazole-5-sulfonamide (K & K Laboratories), between concentrations of $10^{-5}$ M and $4 \times 10^{-2}$ M was investigated with respect to its effect on growth and coccolith formation. Growth experiments both with in vivo decalcified and undecalcified cells were performed with acetazolamide concentrations of "0" M (standard growth medium), $10^{-5}$ M, $10^{-4}$ M, $10^{-3}$ M, and $10^{-2}$ M, both in the standard growth medium and in a lactate deficient growth medium supplemented with $10^{-3}$ M $\text{Na}_2\text{CO}_3$. The culture conditions were either those "standard conditions" described in Standardization of Culture and Sampling Conditions, or modified by the use of 16 x 125 mm screw cap tubes instead of DeLong flasks. Cells not showing growth in acetazolamide media after five days were transferred to the normal culture medium by a 100:1 dilution, incubated for three days, and examined to determine if growth had been permanently impaired.

The rates of coccolith formation by cells suspended in acetazolamide concentrations of "0" M, $10^{-5}$ M, $10^{-4}$ M, $10^{-3}$ M, $10^{-2}$ M, and $4 \times 10^{-2}$ M, both in normal and lactate deficient-$\text{Na}_2\text{CO}_3$ supplemented media, were examined. The conditions of culture are the same as those described in the previous paragraph for growth studies. In the experiment described in Figure 75, redecalcification of cells was accomplished by concentrating experimental cells by centrifugation and washing them twice in the low pH-$\text{Ca}^{++}$ deficient medium and then resuspending them in fresh acetazolamide test media.

Ultrastructural studies were conducted on in vivo decalcified cells fixed at 4 hour, 12 hour, 24 hour, and 48 hour intervals after the initial
exposure of the cells to the test media. In addition, undecalcified cells were fixed at 12 hour and 24 hour intervals after exposure to acetazolamide concentrations of $10^{-2}$ M and $10^{-3}$ M in the standard growth medium. Cells were processed by the standard methods described in Appendix B.

Effect of Colchicine on Growth and Coccolithogenesis

Colchicine (General Biochemicals Corp.), at concentrations of $10^{-2}$ M (0.4%) and $10^{-3}$ M (0.04%) in the standard growth media was investigated with respect to its effect on growth, motility, and coccolithogenesis in both *in vivo* decalcified and undecalcified cells. The conditions of culture deviated from those described in Standardization of Culture and Sampling Conditions in that 16 x 125 mm screw cap tubes filled to a volume of five ml and slanted at an angle of $15^\circ$ from the horizontal were used rather than DeLong flasks.

Ultrastructural studies were conducted on cells fixed at 1 hour, 12 hours, and 48 hours after the exposure of cells to the test media. Cells were processed by the standard methods described in Appendix B. Cell motility was evaluated at the light microscope level at the same time that living cells were being observed for kinetic studies.
RESULTS AND OBSERVATIONS

Standardization of Culture and Sampling Conditions

Because of the complex hymenomonad life cycle, and the proposed effect of aging on coccolithogenesis (52), it is necessary to examine the growth pattern under the specified conditions of culture in order to establish standardized sampling procedures for experimentation. A growth curve for *H. carterae* is presented in Figure 1. Under standard conditions, the cultures exhibit division periodicity, with most of the cell division occurring during the eight-hour dark period. In the experiment illustrated in Figure 1, essentially all cells, during the first four division cycles, divided during this period. In similar experiments, the percentage of cells dividing between the eight to 12 hour period initiated by the onset of darkness ranged from 85 to 100%. During the fifth division cycle in Figure 1, initiated at a cell population of about $2.3 \times 10^5$ cells/ml, a decrease in cell counts and tendency for cells to clump and stick to the sides of the culture vessel was observed. After this decrease, small (ca. 6μ diameter) swarmers (meiospores?) were observed (arrow). It is presumed that a phase change was initiated in the culture at this time. Subsequent counts revealed that the population as a whole no longer exhibited division periodicity, and that in addition to "normal" motile cells, clumps of non-motile cells were present. Phase change, such as that illustrated in Figure 1, was not consistently initiated at any particular population density, though the $2.3 \times 10^5$ cell/ml level in this experiment tends to be minimal, and cultures of motile cells could be maintained indefinitely in an active phase of growth (0.85 to 1 division/day) at population densities as great
as $5 \times 10^5$ cells/ml on a 48-hour transfer schedule with no evidence of phase change.

On the basis of this information, all experiments were performed on cells subcultured at 48-hour intervals for a minimum of three transfer periods, with a maximum density of $2.5 \times 10^3$ cell/ml. Unless otherwise noted, initial exposure to experimental conditions took place between the second and fourth hours of the light period, thus reflecting the vegetative rather than division phase of the growth cycle. Under these conditions, cultures contained predominantly motile cells with diameters of 12 to 15 μ. Cells were completely covered by coccoliths at all population densities observed.

General Structure

The general morphology of *H. carterae* has been reviewed recently by several authors both at the light and electron microscope levels of resolution (62, 63, 90, 107, 123). Figures 2 through 4 are Nomarski differential interference contrast light micrographs illustrating cellular organization. Figure 2 is a surface view of a cell in which the coccoliths appear as elliptical rings with major and minor diameters of about 2 μ and 1.5 μ, respectively. The relationship between the two flagella (f) and the haptonema (h) is illustrated in Figure 3. The structure of these two appendages has been discussed in some detail by Manton and Peterfi (90) and Leadbeater (62). Figure 4 shows an optical section through the same organism illustrated in Figure 2. The flagella and peripherally located coccoliths are evident. Internally, the two lateral chloroplasts with bulging pyrenoids and posterior vacuolar region are prominent. The central region
contains additional vacuolar elements and the nucleus. The antero-central region is occupied by the Golgi apparatus and the intracellular parts of the kinetic apparatus. In the extreme anterior region, near the point of flagellar insertion, two intracellular coccoliths (arrows) can be observed.

Figures 5 through 8 afford several views of coccolith-scale structure in whole-mount preparations at the electron microscope level of resolution. Figure 5 is an unshadowed preparation of a coccolith in which the apparent density (i.e. dark areas) can be attributed to crystallized CaCO₃ (calcite). The ring of calcified elements seen in this figure corresponds to the elliptical structures seen on the surface of the organism in Figure 2. Two distinct kinds of calcified elements occur - larger elements, designated A, and smaller elements, designated B - which alternate about the ring.

The scale-like base of the coccolith is morphologically biphasic (87, 107, 122). Figures 6 and 7 are metal shadowed preparations which illustrate, respectively, the upper (distal) and lower (proximal) surface of the subtending base (b). The upper surface of the base often appears either unstructured, or shows numerous meandering whorls of ridges, as illustrated in Figure 6. Numerous spoke-like ridges radiating from a central region toward the periphery can be seen on the lower surface of the base (Figure 7). Figure 8 illustrates the two kinds of unmineralized scales produced by H. carterae. The larger scale appears to be distributed rather evenly about the surface of the organism between the plasmalemma and the coccolith layer, while the distribution of the smaller scale seems to be limited to the haptonemal region.

A degree of determinable preparative artifact is evident in Figures 5 through 8. In Figures 5 through 7, the calcified elements of the coccolith
are at least partially collapsed onto the underlying surface. This is more noticeable in Figures 5 and 6 than in Figure 7. In Figure 7, however, the metal shadow on the undersurface of the base (arrow) indicates a partial collapse of the base into the hollow formed as the coccolith rests upside-down on its "raised" calcified elements (see sectioned material Figures 9 and 10). The scales illustrated in Figure 8 also possess a degree of artifact. Based on thin section observations (86; Figures 10, 17, 18, and 88) the scale rim is an artifact of drying, and the pattern of the scales is probably a composite view of the upper (whorled) and lower (spoked) surfaces.

Figures 9 and 10 are sections through mature, extracellular coccoliths in a plane perpendicular to the surface view illustrated in Figures 5 through 7. In Figure 9, the section passes through the base (b), with the dense calcified elements (A and B) at its periphery. In Figure 10, decalcification reveals another component of the coccolith, the sheath of matrix material which surrounds the calcified elements and mimics their form, but is not removed by decalcification treatment. As will be illustrated later, the electron density of this material is primarily associated with lead staining. The relationship between the "calcified" elements and the sub-tending coccolith base is probably best illustrated in the decalcified section. A prominent rim (r) is located at the periphery of the base. The B elements have a notch (left side) on their lower surface which "fits" this rim, and the A elements have a corresponding groove (right side) on their lower surface. In addition, the highly contoured hook region (h) on the upper surface of A elements is evident.
For the purpose of subsequent discussion, both the A and B elements will be arbitrarily divided into two regions as illustrated in Figure 10. The A elements will be divided into upper (uA) and lower (lA) regions, and the B elements into inner (iB) and outer (oB) regions, with the intersecting axes of the elements (see Figure 10) defining the division plane.

Figures 11 through 14 are metal shadowed carbon replicas of isolated coccoliths. The structural detail of the coccolith is enhanced by the removal of the electron opaque calcite. In Figures 11 and 12, the coccolith is sitting on its base, and the upper (distal) surface of the coccolith is exposed (as Figure 6). The hook region of the A element (h), the very contoured nature of the inner portion of the B element (iB), and the compact packing of the elements are illustrated. In one area of Figure 12, the dense outline of the outer portion of the B elements (oB) is evident. Its length is relatively larger than the corresponding measure of the inner portion of the same element (0.37 μ vs 0.23 μ) and smaller than the length of the upper portions of the adjacent A elements (uA, 0.42 μ). In Figures 13 and 14, the coccoliths are lying on the hooks of the A elements, with the lower surface of the base exposed (as Figure 7). Three "rings" can be observed at the periphery of the coccolith. The inner-most ring is formed by the lower surface of the A elements (lA), and is "covered" by the coccolith base. The dense central ring is formed by the outer portion of the B elements (oB), and the outer-most ring is formed by the upper portion of the A element (uA). The basal portion of the A element appears to be smaller in width than the upper portion of this element (about 3.4 μ vs 4.2 μ).
Granularity of the lead stained matrix can be observed in calcified material that has been sectioned sufficiently thin. Figure 15 illustrates such a case. The matrix granularity is however more easily observed in decalcified material, as in Figure 16. The size of the granules is variable, but averages about 7 nm.

The general aspects of coccolith morphogenesis are illustrated in Figures 17 through 25, and diagramatically summarized in Figure 26. In general they recapitulate the information presented previously by Outka and Williams (107), though new observations relating to early base and coccolithosome formation, asynchronous "matrix formation-calcification" of elements in a forming coccolith, and timing associated with scale-coccolith formation will be presented. Figures 17 and 18 illustrate sections through the Golgi region of *H. carterae* in decalcified material. The Golgi apparatus is highly polarized with respect to coccolith formation. The lower, proximal region of the Golgi contains no observable coccolith precursors, though cisternae containing central dilations (cd, Figure 18), proposed by Brown et al. (17) to be sites of scale (=base) monomer polymerization are evident. The central region contains coccolithosomes (c) and bases or scales (b). It is often difficult to distinguish between bases and scales in cross section. Figure 17 includes a section through a cisternae containing a stage (eb) in scale-base formation analagous to the early two-layered fibrillar stage described by Brown et al. in *Pleurochrysis* (17).

The remaining assembly stages of coccolithogenesis occur in the distal Golgi region. Four clearly defined intracellular coccoliths are evident in both Figures 17 and 18. The coccoliths labeled w represent early stages in coccolith assembly in which the matrix is incompletely formed. In Fig-
ure 17, the association of coccolithosomes with the forming matrix is evident. This coccolith (w. Figure 17) is somewhat displaced laterally from the cisternal stack; however, the proximo-distal polarity of the Golgi is preserved by a long extension of the cisterna into the stacked region of the Golgi. Lateral displacement of the coccolith bearing portion of cisterna is often observed in this region, and similar cisternal "extensions" are frequently noted. The apparent fusion of cisterna containing the mature coccoliths, x and y, has also been observed in other sections, though more frequently a one-coccolith-to-one-cisterna ratio seems to hold. In Figure 17, the cisterna containing mature coccoliths contain no apparent coccolithosomes. In Figure 18, however, numerous small "residual" coccolithosomes (rc) are evident. As compared to the coccolithosomes in the cisterna containing the forming coccolith (w) the residual coccolithosomes have a "used-up" appearance.

The relationship between coccolith and the large scales on the cell surface is illustrated in Figure 17. Two or more layers of scales (S) lie beneath the single layer of coccoliths (C). Between the scale-coccolith layers and the cell surface is a flocculent material which Manton and Leedale (87) called "columnar material" (cl), and Crenshaw (21) suggested may be involved in holding the coccoliths onto the cell surface.

The description of coccolith formation by Outka and Williams (107) left open to question whether coccolithosomes and bases were formed within the same cisterna or in different cisternae which later fused. Based on Brown's et al. scheme of scale formation (17), the cisterna labeled eb in Figures 19 through 21 contain early stages in scale (base) formation. In
addition, coccolithosomes (c) appear to be present in peripheral pockets of these same cisternae.

Figures 22 through 25 allow more detailed observations of the assembly stages associated with coccolith formation. In Figure 22, three stages in coccolith formation are evident (w, x, y) as well as part of a fourth (z). The earliest stage (w) is very early in matrix formation. Coccolithosomes are associated with the base periphery, and the base periphery is associated with (left side) or connected to (right side) the cisternal membranes. In the more mature stage of coccolith formation illustrated in this figure (x), the matrix has already been formed, and calcification is evident. In the A element on the right side, however, several regions can be observed (small arrows) in which the completed matrix has outlined the shape of the coccolith, but calcification has not yet occurred. Coccolithosomes are present in large numbers in peripheral "pockets" (right side) of both stages w and x. The next stage (y) appears to be relatively mature, with calcification being nearly complete. Figures 23 and 24 are serial sections through the same cisterna. Matrix formation is incomplete and calcification not evident in the section through the coccolith shown in Figure 23. In Figure 24 a serial section through this coccolith reveals partially formed elements on both right and left sides, and in addition, on the left side, a grazing section through a B element in which matrix formation is complete, and calcification has at least begun. Thus a degree of asynchrony exists in coccolith assembly, with states ranging from incomplete matrix formation to calcification occurring in the same coccolith. Figure 25 illustrates the simultaneous presence of body scales (s) and coccoliths (C) associated with the Golgi apparatus.
Associations between cisternal membranes and forming rim (A and B) elements of coccoliths are illustrated in Figures 17 and 18 (w); 22, 23, 24 (large arrows). The most consistent association is between the base periphery and the adjacent cisternal membranes, either by an obvious direct contact (e.g. Figures 17, w; 18, w; 22, x) or connected by fine "threads" of material (e.g. right-hand side in Figures 22, w and 23). Additional points of contact become evident as the matrix develops the shape of the rim element (large arrows, Figures 22 through 24). Similar membrane associations are not generally observed in vesicles containing relatively mature coccoliths (e.g. Figures 17, x, y, z; Figure 18 y, z; Figures 22 through 24).

Figure 26 is an interpretive drawing of coccolith morphogenesis based on observations. All stages in coccolith formation which have been observed are associated within or very close to the Golgi apparatus. Participation of the perinuclear, periplastidal, or endoplasmic reticular membranes in coccolith formation, while implied, has not actually been observed at the electron microscopic level of resolution. The proximal, "forming face" of the Golgi is bordered basally by the nucleus and vacuolar elements. Laterally the Golgi cisternae are bordered by nucleus, rough endoplasmic reticulum, mitochondria, and the cell's two cup-shaped plastids. Distally, the "mature" face of the Golgi is associated with plasmalemma in the region of flagellar insertion.

The proximal region of the Golgi contains no recognizable coccolith precursors, though the cisternal dilations thought to be involved in early scale (base) formation are present. The central Golgi region is associated with base and coccolithosome formation. Coccolithosomes apparently are
formed, and accumulate in the peripheral regions of the same cisternae in which the bases are formed. In the next stage observed in coccolith assembly (A1), the coccolithosomes are associated with the base periphery and adjacent cisternal membranes. In the subsequent stage (A2), coccolithosomes are clustered about the forming matrix. A close association between cisternal membranes and regions of the forming matrix is evident in this stage. In stage A3 (right) the matrix has formed an outline of the shape of the elements which make up the calcified rim of the coccolith. In A3 left, calcification has begun in the space delimited by the matrix. Membrane associations are often still evident during the early stages of calcification. Stage A4 consists of a mature, fully formed, completely calcified, intracellular coccolith enclosed within the cisternal membranes. Specific associations between cisternal membranes and the coccolith membrane are generally not observed at this time. The final stage of coccolithogenesis consists of the extrusion of the coccolith to the cell exterior, presumably by fusion of the cisternal and plasmic membranes, and the appropriate placement of the coccolith on the cell surface.

Comparison of Processing Procedures for Ultrastructural Observation of Forming Coccoliths

Figures 27 through 32 illustrate the effects of staining and sectioning flotation media on the various components of the coccolithogenic system. Figures 27 and 28 are unstained preparations which were cut and floated on either distilled water (Figure 27) or a cacodylate-CaCl_2 solution at pH 8.0 (Figure 28). The electron lucent areas in Figure 27 (d) represent spaces occupied by CaCO_3 before flotation. Calcium carbonate in Figure 28 (Ca) on
the other hand is fairly well preserved, with only small areas of dissolution evident. Dilute solutions of NaOH (pH 10-11) proved to be intermediate with respect to their ability to maintain crystallized CaCO$_3$ in the section. The type of embedding media used had no evident effect on this decalcification process.

Though contrast is low, bases (b), matrix (m), and coccolithosomes (arrows) can all be identified in unstained sections. However, in Figures 27 and 28, as well as the uranyl acetate stained sections shown in Figures 29 and 30, the granularity associated with the coccolithosomes or matrix is relatively indistinct as compared to that seen in lead stained material (e.g. Figures 15, 16, 31). The granular substructure of the coccolithosomes and matrix is similar, and in lead stained material the substructural granules have an average diameter of about 7 nm. The section seen in Figure 32 was floated on distilled water, then sequentially stained in uranyl acetate and lead citrate, thus revealing the matrix material which remains after the bulk of the crystallized CaCO$_3$ has been removed.

Figures 33 and 34 illustrate the effect of fixative on coccolith components. The appearance of the base (b), coccolithosomes (arrows), and matrix (m) remains the same whether fixed in glutaraldehyde alone (Figure 33), osmium tetroxide alone (Figure 34), or sequentially in glutaraldehyde then osmium (e.g. Figures 15, 22, 35).

Effects of Decalcification Procedures on the Components of the Coccolithogenic System

The effect of decalcifying procedures (low pH-Ca$^{++}$ deficiency or Na$_2$EDTA chelation) during fixation and dehydration was examined at the fine structural level. The results of these experiments are summarized in
Table 1. Three of the four controls (Table 1-4, 11, 12; Figures 35 and 48) did not deviate from the observations previously described (General structure). However, in the fourth control (Table 1-3) in which Ca$^{++}$ was omitted from the fixation and rinse solution, CaCO$_3$ crystals were not apparent and matrices appeared degraded (Figures 38 and 39). In addition, external coccoliths and scales were not evident and only remnants of the columnar material remained (Figure 52). The coccolith base appeared normal in both control and experimental material, while CaCO$_3$ was absent in all but the three control preparations.

Decalcification during glutaraldehyde fixation either by low pH-Ca$^{++}$ deficiency (1-1, Figure 37) or chelation (1-2, Figure 36) resulted in complete to partial degradation of both coccolithosomes and matrices. Compared to the Ca$^{++}$-deficient control (1-3), matrix degradation appeared more complete, with only small remnants of matrix material being evident at the periphery of the bases (dm, Figures 36 and 37).

The remaining decalcification procedures (Table 1-5-10) were carried out after standard glutaraldehyde fixation and post-osmication. Matrices were either unrecognizable (1-5, Figures 40 and 41) or degraded (1-6, Figure 42) by chelation at low pH. Coccolithosomes were absent or severely degraded in aqueous solution (1-5) but appeared relatively normal under the same circumstances in a 30% ethanolic solution (1-6). External coccoliths and scales were not present on the cell surface in either case, and only remnants of columnar material were observed (e.g. Figure 53).

Chelation at a higher pH (1-7, Figure 43), or low pH alone, both in aqueous (1-8, Figure 44) and ethanolic (1-9, Figures 45 and 46) solutions resulted in the preservation of normal looking coccolithosomes and formed
Table 1. Effect of decalcification during fixation and dehydration

<table>
<thead>
<tr>
<th>No.</th>
<th>Treatment</th>
<th>Pretreatment procedures</th>
<th>Post treatment procedures</th>
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<tbody>
<tr>
<td>1</td>
<td>Ca(^{++})-deficient glutarald. soln. pH 5.0, 30 min.</td>
<td>Std. glutarald. soln., 10 hr.</td>
<td>Rinse Os-postfix dehydrate embed Ca(^{++})-omitted from all solutions</td>
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<td>2</td>
<td>0.01 M Na(_2)EDTA glutarald. soln. pH 7.4, 30 min.</td>
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<tr>
<td>3</td>
<td>Ca(^{++})-deficient glutarald. soln. pH 7.4, 30 min.</td>
<td>&quot;</td>
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<tr>
<td>4</td>
<td>Std. glutarald. soln. pH 7.4, 30 min.</td>
<td>&quot;</td>
<td>Std. rinse, Os-postfix, dehydrate and embed</td>
</tr>
<tr>
<td>5</td>
<td>0.01 M Na(_2)EDTA pH 5.0, 10 min.</td>
<td>Std. glutarald. soln. rinse Os-postfix</td>
<td>Std. dehydrate embed</td>
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<tr>
<td>6</td>
<td>0.01 M Na(_2)EDTA 30% ethanol pH 5.0, 10 min.</td>
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<tr>
<td>7</td>
<td>0.01 M Na(_2)EDTA 0.1 M cacodylate pH 7.4, 10 min.</td>
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<tr>
<td>8</td>
<td>0.1 M NaAc pH 5.0, 10 min.</td>
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<tr>
<td>9</td>
<td>0.1 M NaAc 30% ethanol pH 5.0, 10 min.</td>
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<tr>
<td>10</td>
<td>0.02 M UAc 30% ethanol pH 4.5, 10 min.</td>
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<tr>
<td>11</td>
<td>0.1 M cacodylate pH 7.4, 10 min.</td>
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<tr>
<td>12</td>
<td>30% ethanol pH 7.4, 10 min.</td>
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\(\text{a}^+\) = present in relatively unaltered form.

\(\text{b}\) = Control.

\(\text{d}\) = partially degraded but recognizable.

\(\text{=}\) = absent or unrecognizable.
<table>
<thead>
<tr>
<th>Extracell. coccoliths &amp; scales</th>
<th>Matrix (intracell.)</th>
<th>Coccolithosomes (intracell.)</th>
<th>Base (intracell.)</th>
<th>CaCO₃ crystals</th>
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matrices which mimicked the shape of the decalcified peripheral elements. In the aqueous solutions (1-7 and 1-8), external coccoliths were often absent, though having a "formed" matrix when present (similar to Figure 50). In the ethanolic solution, external coccoliths were present, though the matrices appeared degraded, and the amount of columnar material reduced (1-9, Figure 51). Decalcification with uranyl acetate in an ethanolic solution at a pH of 4.5 (1-10) preserved the integrity of the intracellular coccolithogenic system (Figure 47) and, in addition, cells treated in this manner retained their extracellular armature of coccoliths and scales (Figure 50).

Decalcification of living cells was accomplished by means similar to those employed by Paasche (110), as described in the corresponding section of Materials and Methods. The extracellular envelope of coccoliths and scales in the organisms remained in position on the cell surface, however, crystallized CaCO\textsubscript{3} was not evident, and the matrices appear to have collapsed (Figure 49). There was no evidence of intracellular effects on the coccolithogenic system, and normally calcified coccoliths were observed (C, Figure 49).

Kinetics of Coccolith Formation

The rate of coccolith formation was determined under the standard conditions of culture by resuspending \textit{in vivo} decalcified cells in fresh culture medium and then counting the number of coccoliths on the surface of individual cells at intervals. As illustrated in Figure 54, after an initial lag period of one to two hours, coccoliths were produced at an average rate of 10 to 11 coccoliths/cell/hour. Figures 54A-D illustrate the
appearance of organisms at various intervals during this period. Figure 54A shows a decalcified cell before resuspension in the growth medium, with no coccoliths being evident on the cell surface. Figure 54B shows a cell after two hours in the recalcification (growth) medium. Approximately nine coccoliths are present on the cell surface at this time (four of which can be observed in this optical section - arrows) and they tend to be clustered at the anterior end of the cell near the region of the flagellar and haptonemal insertion. Figures 54C and 54D illustrate later stages of recalcification (three and four hours, respectively) in which the coccoliths appear spread out in a rather random fashion over the cell surface.

Effect of Light (Dark) and Heterotrophic Nutrition on Coccolithogenesis

Figure 55 illustrates the effect of light vs dark incubation on the kinetics of coccolith formation. The rate of coccolith formation in the standard growth medium by cells incubated in the light averaged ten coccoliths/cell/hour, whereas those incubated in the dark averaged 3.8 coccoliths/cell/hour (Figure 55A). In a parallel experiment conducted on cells incubated in the growth media minus lactate, but supplemented with $10^{-3}$ M Na$_2$CO$_3$, the corresponding rates of coccolith formation were ten in the light and 3.5 in the dark (Figure 55B). The dark-to-light kinetic ratio was therefore 0.38 in the normal growth medium and 0.35 in the lactate deficient growth medium. Counts indicate that cell division did not take place during the course of this experiment.
Effect of Ca$^{++}$ Concentration on Growth and Coccolithogenesis

Figure 56 describes the effect of Ca$^{++}$ concentration in the growth medium on cell division. The cells used in this experiment were decalcified prior to inoculation into the test medium. While the data is inadequate to completely characterize the growth curve at the various concentrations, it does, however, indicate certain features. At concentrations of $10^{-2}$ to $10^{-5}$ M, cell populations increase with increasing Ca$^{++}$ concentration. This increase is not first order with respect to Ca$^{++}$ concentration; after three days incubation for instance, a ten-fold increase in Ca$^{++}$ results in only a 50 to 60% increase in cell population. The growth pattern in which $10^{-6}$ M Ca$^{++}$ was added is essentially indistinguishable from the medium in which no Ca$^{++}$ was added.

The Ca$^{++}$ concentration has a definite effect on the kinetics of coccolith formation between $10^{-2}$ M and $10^{-4}$ M. As illustrated in Figure 57, coccolith formation appeared to approximate first order kinetics with respect to Ca$^{++}$ between these concentrations. The slopes of the kinetic curves were 11.6 coccoliths/cell/hour at $10^{-2}$ M and 1.1 coccoliths/cell/hour at $10^{-3}$ M Ca$^{++}$ between the first and fifth hours, and 0.12 coccoliths/cell/hour at $10^{-4}$ M Ca$^{++}$ between the second and fifth hours. When $10^{-5}$ M or less Ca$^{++}$ was added to the medium, external coccoliths were only rarely observed on the cell surface, and those that were observed often appeared indistinct.

Ultrastructural observations on cells even at the lowest concentration of Ca$^{++}$, "0" M (i.e. no added Ca$^{++}$, so that the only Ca$^{++}$ present was derived from contamination of glassware or reagents), showed internal calcified structures (e.g. Figures 58 through 60). However, as compared to
cells grown under normal conditions i.e., $10^{-2}$ M Ca$^{++}$ (e.g. Figure 22), few cells grown at "0" or $10^{-6}$ M Ca$^{++}$ contained calcified structures and rarely was more than one calcified coccolith observed per cell. No well formed uncalcified matrices were observed, and completely calcified rim elements were essentially absent (Figures 58 through 60, 61). The Golgi apparati did not appear reduced (e.g. Figure 61), and several incompletely formed coccoliths were frequently observed within them (e.g. Figure 58).

In cells grown at $10^{-5}$ M Ca$^{++}$, well-calcified internal coccoliths appeared commonly, and multiple intermediate stages of calcification were often observed (e.g. Figure 62). In general, the ultrastructural aspects of the coccolithogenic system appeared relatively normal in cells grown in Ca$^{++}$ concentrations between $10^{-2}$ and $10^{-5}$ M.

The Effect of Sr$^{++}$ on Growth and Coccolithogenesis and Comparison with High Molarity (0.1 M) Ca$^{++}$ and Mg$^{++}$

The effect on the kinetics of coccolith formation of the addition of Sr$^{++}$ to the normal growth medium was examined. Figure 63 illustrates representative concentration curves taken at two and seven hours of incubation in Sr$^{++}$ containing medium. A reduced rate of coccolith formation is observed at Sr$^{++}$ concentrations of greater than $10^{-5}$ M, with complete inhibition evident at $10^{-1}$ M Sr$^{++}$. As illustrated particularly well in the seven hour curve, the inhibition of coccolithogenesis by Sr$^{++}$ cannot be expressed as a linear process, but rather represents a more complex relationship.

The comparative effect of high molarity (i.e. $10^{-1}$ M) concentrations of Sr$^{++}$, Mg$^{++}$, and Ca$^{++}$ on cell division (Figure 64) and the kinetics of coc-
colith formation (Figure 65) were determined. The growth curves for Sr$^{++}$ and Mg$^{++}$ are very similar to that for the normal growth media. The cells in $10^{-1} \text{M Ca}^{++}$ on the other hand showed a marked reduction in growth, reaching a final population after four days of only about half of that for the control.

The effect of divalent cations on the rate of coccolith formation is illustrated in Figure 65. As compared to the control medium, the rate of coccolith formation in the medium containing $10^{-1} \text{M Mg}^{++}$ was reduced by about 14%, while the comparable reduction for $10^{-1} \text{M Ca}^{++}$ was 47%. Coccolith formation appeared completely inhibited by $10^{-1} \text{M Sr}^{++}$.

Ultrastructural examination of $10^{-1} \text{M Sr}^{++}$ inhibited cells after 12-hour incubation revealed the presence of calcified intracellular coccoliths in only two of the several hundred organisms observed in section. Cells examined at 24 hours and 31 days after exposure contained no intracellular calcified coccoliths. In most cells incubated for 12 hours (Figure 66), and in no cell incubated for longer periods of time (Figures 67 through 69) were any stages of coccolithogenesis more advanced than the coccolithosome-base association (A-1, Figure 26) observed. Matrices were not formed and CaCO$_3$ crystallization was not evident. In addition extracellular remnants of decalcified coccoliths or scales (see Figure 49), or partially formed coccoliths (i.e. bases of inhibited coccoliths) were not evident on the surface of cells subcultured in $10^{-1} \text{M Sr}^{++}$ media for the 31-day inhibition period.

Cells grown in medium containing $10^{-2} \text{M Sr}^{++}$, but no Ca$^{++}$, other than that contaminating glassware and reagents fail to form extracellular coccoliths, though they will revert to coccolithogenic forms upon transfer to
the standard growth conditions. Ultrastructural observations of this material have been somewhat hampered by the relatively poor preservation by either the standard procedures (Appendix B) or by the substitution of equimolar SrCl₂·6H₂O for CaCl₂ into the standard fixation solutions, reflecting perhaps the physiological condition of the organisms. It does, however, suggest that the cells are generally unable to form coccolith components (Figures 70 through 72). Neither body scales nor coccolith bases are observed, either within the Golgi complex, or on the cell surface (Figures 70 through 72), and neither matrix formation or the presence of crystalized CaCO₃ was evident. Coccolithosomes, however, are encountered in Golgi cisternae in a small percentage (less than 10%) of cells (Figure 72).

Effect of Acetazolamide on Growth and Coccolithogenesis

The effect of acetazolamide, an inhibitor of the enzyme carbonic anhydrase, was investigated with regard to cell growth and coccolith formation, both in the standard growth medium and also in a growth medium in which lactate was omitted and 10⁻³ M Na₂CO₃ added. Figure 73 represents the effect of acetazolamide concentration on net cell growth after a period of four days incubation in the test medium. Acetazolamide concentrations of less than 10⁻⁶ M seemed to have little effect on growth. Cultures grown in 10⁻³ M acetazolamide were moderately affected, and 10⁻² M acetazolamide seemed to completely inhibit all growth. The effects of acetazolamide on cell division did not seem to be greatly influenced by the presence or absence of lactate in the test medium.

Figure 74 illustrates the effect of acetazolamide concentration on the rate of coccolith formation in test medium at representative intervals of
two and four hours. The curves tend to mimic the growth curves, except that complete inhibition of coccolith formation at $10^{-2}$M acetazolamide was not obtained. Figure 75, however, illustrates an experiment in which cells incubated in $10^{-2}$ and $10^{-3}$M acetazolamide for a period of 28 hours in both normal and lactate deficient media were redecalcified and then resuspended in new acetazolamide-containing media of the original concentration. Cells suspended in acetazolamide at $10^{-3}$M resumed making coccoliths at about the same rate as previously, while coccolith formation in cells incubated in $10^{-2}$M acetazolamide was almost completely inhibited. Graph points reflect average values, though population divergence with respect to coccolith formation was quite evident. Approximately 85% of the redecalcified cells incubated in $10^{-2}$M acetazolamide failed to form any coccoliths after 17 hours of incubation in either normal or lactate deficient medium as compared to a value of less than 5% under normal (i.e. control) circumstances. Of the cells forming coccoliths in $10^{-2}$M acetazolamide, the number of coccoliths per cell ranged from one to greater than 40. When cells inhibited by acetazolamide for ten days were transferred to media (with or without lactate as appropriate) not containing acetazolamide (approximately 1:100 dilution) both growth and coccolithogenic abilities were restored.

Ultrastructural examination of $10^{-2}$M acetazolamide inhibited cells after 24 hours (e.g. Figure 78) or 48 hours (Figures 76, 77, 79 through 81) either in the presence (Figures 76, 78, 79, 81) or absence (Figures 77, 80) of a heterotrophic nutritional source (i.e. lactate) indicated that all intermediate stages of coccolith formation, including fully calcified coccoliths, were present within the cells. With respect to the coccolithogenic system, no ultrastructural differences were observed between control
and inhibited cells, except in the case of "redecalcified" cells where external coccoliths were not observed.

Effect of Colchicine on Growth and Coccolithogenesis

Figure 82 illustrates the effect of colchicine, an inhibitor of microtubule activity, on coccolithogenesis. Decalcified cells were suspended in growth medium containing $10^{-3}$ M (0.04%) or $10^{-2}$ M (0.4%) colchicine. Figure 82 illustrates the recalcification of decalcified cells at a representative time of 7 hours. The control value was approximately 70 coccoliths/cell. Coccolith formation was partially inhibited at a colchicine level of $10^{-3}$ M, and completely inhibited at $10^{-2}$ M. A similar dose response was evident for both growth and motility. Cells became non-motile within 15 minutes after inoculation into the $10^{-2}$ M colchicine-containing media, and failed to grow or form coccoliths at incubation times of up to 30 days. Cells in $10^{-3}$ M colchicine on the other hand were normally motile, formed coccoliths, and grew, though rates of growth and coccolithogenesis were somewhat reduced as compared to cells incubated in the absence of colchicine.

The results of these colchicine inhibition experiments must be interpreted cautiously, since adequate physiological data indicating the ability of cells to perform functions not directly related to coccolithogenesis (e.g. respiration, photosynthesis) are lacking. However, the effect of colchicine on cell function is generally attributed to its effect on microtubules (12, 148), and cell functions not associated with these structures are probably unaffected by short-term colchicine exposure (12).

The microtubule system of hymenomonads has been described previously by Manton and Peterfi (90), Leadbeater (62, 63), and Brown and Franke (16).
Microtubules can be conveniently divided into three closely associated groups - the flagellar, the haptonemal, and the cytoplasmic microtubular complexes. The flagellar system (Figures 83 through 86, 89) consisting of a typical doublet-9 + 2 pattern in the flagella and a triplet-9 + 0 in the basal body is common to most eukaryotic organisms. The haptonemal system, on the other hand, is unique to the Haptophycean algae. In Hymenomonas, the haptonema appears to be of a somewhat degenerate form (90) which can be observed exteriorly as a short bulbous appendage located between the two flagella (h, Figures 3 and 54C). The intracellular microtubular portions of the haptonema can be seen in transverse section in Figure 85 and longitudinal section in Figure 84. The cytoplasmic microtubular system, described by Manton and Peterfi (90) as a flagellar "root" system, consists of a pair of sheets formed by a single row of closely aligned microtubules (ms. Figures 83 through 87, 89) which are closely associated with the basal bodies and close-packed bundles of microtubules which arise at approximately right angles to the microtubular sheets (mb, Figures 83 through 87, 89).

While the precise three-dimensional relationship between the microtubular systems and other cell components is not known, Figures 83 through 98 show some features which have been observed, and Figure 93 shows a hypothetical diagram of these microtubular systems. The haptonema and flagella arise at the anterior end of the cell in a region between the tips of the two plastids. The haptonema lies in a plane between the two flagella, though displaced somewhat laterally from a line drawn between the axes of the two flagella (see cross-section in Figure 85). The bulbous extension of the haptonema contains elements of the smooth endoplasmic reticulum (87) and five to six centrally placed microtubules. In the haptonemal base the
number of microtubules increases to seven or eight. The bases of the flagella and haptonema appear to lie in close association, though anterior to the immature cisternae, and lateral to the mature, coccolith-containing vesicles (Figure 83).

The paired microtubule sheets are closely associated with the basal bodies where the plane of one sheet appears to pass into the region between the two basal bodies, and the plane of the other to one side of them (Figures 85 and 93). The sheets, normally consisting of 30 to 40 tubules pass from a position somewhat posterior to the basal bodies through the basal body region and then curve along the lower surface of the smooth endoplasmic reticulum associated with the haptonema (see Manton and Peterfi - 90; Figures 86 and 93). The microtubule bundles, containing from 20 to several hundred microtubules, arise at about right angles to the microtubular sheets, pass laterally beneath the plasmalemma, then curve posteriorly along the inner surfaces of the plastids (Figure 93). Microtubular sheets and bundles are commonly observed in sections passing through the anterior end of the organism (e.g. Figures 17, 83 through 92), and, it is possible to trace the microtubular bundle through relatively great distances within the cell (e.g. Figure 83). They are virtually constant components of sections passing through the two basal bodies (Figures 83 through 87, 89). While their position in the anterior end of the cell places all of the microtubular components near Golgi cisternae, the microtubular bundles and sheets in particular are seen in close contact with vesicular components of the cell (arrows, Figures 83 through 87, 89), and sections through bundles of microtubules at the periphery of the Golgi are observed quite commonly (Figures 17, 87 through 92). The microtubule bundles associated with the
Golgi complex often contain fewer microtubules than those observed at the junction of the bundles and sheets, and it is possible that branching of this system occurs (Figure 93).

As illustrated in Figures 94 through 98, all stages in coccolithogenesis can be observed in $10^{-2}$ M colchicine inhibited material, even after as long as a 24-hour incubation period in the inhibitor. The number of cells exhibiting intracellular coccoliths however appears to be somewhat reduced in comparison with the non-colchicine control material. There is no evidence of an abnormal accumulation of intracellular coccoliths in the cell, and the stacked configuration of Golgi cisternae seems to be maintained (e.g. Figures 94 and 95).

Figures 99 through 106 illustrate various aspects of the intracellular microtubular systems in $10^{-2}$ M colchicine inhibited cells. The organization of the basal body can best be seen in Figure 99 where the view approaches a cross-section through one of these organelles. In this section, as well as other similar cross-sectional views that have been observed, the basal body organizational pattern appears normal. Details of the haptonemal base have not been adequately demonstrated in colchicine inhibited material, though tangential sections through this organelle can be seen in Figures 99 and 104.

Microtubular sheets in colchicine inhibited material, as in controls, are invariably associated with the basal bodies (ms, Figures 99 through 104). While an accurate cross-section of the sheet has been approximated only in Figure 102, the organizational pattern of these sheets in the inhibited material does not appear to differ from that observed in control material.
The occurrence of microtubule bundles in inhibited material was strikingly reduced as compared to control material. Of the several hundred cells examined in section, less than a dozen contained microtubule bundles. Figures 99 through 101 are sections through basal bodies and microtubular sheets, and, as is typical of inhibited material, no bundle microtubules are evident. In those cases where bundle microtubules were observed in association with basal bodies, they appeared to be reduced in number in comparison with control material (compare Figures 102 through 104 with Figures 83 through 86). While extensive microtubular arrays were observed commonly in control material, in only two instances (Figures 105 and 106) were similar arrays observed in colchicine inhibited cells.
Figure 1. Growth curve for *H. carterae* illustrating division periodicity when grown under standard conditions. Shaded area indicates period of dark growth. Arrow indicates time when large number of small swarmer cells (meiospores?) were observed.
Figures 2-4. Nomarski differential interference contrast light micrographs of living *H. carterae*. X 1250

2. Surface view of cell in which coccoliths appear as elliptical rings with major and minor diameters of about 2 \( \mu \) and 1.5 \( \mu \) respectively

3. View of cell showing the haptonema (h) and two flagella (f)

4. Optical section through the same cell as illustrated in figure 2. The lateral plastids and posterior vacuolar region are quite prominent. In the anterior region near the flagellar insertion, two intracellular coccoliths can be seen (arrows)
Figures 5-16. Electron micrographs of coccoliths and scales

5. Unshadowed whole-mount of coccolith showing the two types of calcified elements (A and B). X 20,000

6. Pt-shadowed whole-mount of coccolith illustrating the upper (distal) surface of base (b). X 20,000

7. Pt-shadowed whole-mount of coccolith illustrating the lower (proximal) surface of the base (b). X 20,000. Arrow indicates area of metal "shadow"

8. Pt-shadowed whole-mounts of the two types of unmineralized scales. X 20,000

9. Section through coccolith on surface of cell showing base (b), A and B elements, hook region of A element (h), and the electron-opaque appearance of the calcified (A and B) elements

10. Section through coccolith decalcified with 0.02 M uranyl acetate in ethanol (Table 1-10) showing base (b), base rims (r); upper (uA), lower (lA), and hook regions of A element, and inner (iB) and outer (oB) regions of B element. Stained with uranyl acetate and lead citrate. X 37,000

11 and 12. Pt-shadowed carbon replicas of whole-mount coccoliths illustrating the upper (distal) surface of base, inner (iB) and outer (oB) regions of B elements, and upper (uA) region of A elements including the "hook" structure (h). X 20,000

13 and 14. Pt-shadowed carbon replicas of whole-mount coccoliths illustrating the lower (proximal) surface of the base, upper (uA) and lower (lA) regions of the A elements. X 20,000

15. Section through intracellular, calcified coccolith showing granular matrix (arrows). Stained with uranyl acetate and lead citrate. X 70,000

16. Section through extracellular coccoliths decalcified with 0.02 M uranyl acetate (Table 1-10) showing granular matrix. Stained with uranyl acetate and lead citrate. X 70,000
Figures 17-21. Electron micrographs of sectioned material showing several aspects of sequential coccolith morphogenesis. Cells decalcified in 0.02 M uranyl acetate (table I-10) and stained with uranyl acetate and lead citrate.

17 and 18. Views of the polarized Golgi apparatus illustrating cisternal dilation (cd), coccolithosomes (c), residual coccolithosomes (rc), bases or scales (b), and an early stage in scale-base formation (eb), as well as several other stages in coccolith formation (w-z). Also evident are a microtubular bundle (mb), vacuolar region (v), and nucleus (N) intracellularly, and coccoliths (C), scales (S), and columnar material (cl) extracellularly. X 25,000

19-21. Micrographs showing coccolithosomes (c) present in same Golgi cisternae as early stages in base formation (eb)

19. X 35,000

20 and 21. X 60,000
Figures 22-24. Sections through forming coccoliths illustrating several stages, including calcification. Large arrows indicate areas of membrane-coccolith interaction. Stained with uranyl acetate and lead citrate. X 60,000

22. Three sequential stages of coccolithogenesis (w, x, and y) as well as part of a fourth (z) are shown; w is the earliest stage and z is the most mature. Small arrows in the stage labeled w indicate areas where the matrix has outlined the shape of the rim element, but calcification has not yet taken place.

23 and 24. Two serial sections through a forming coccolith showing stages ranging from incomplete matrix formation to partial calcification.

Figure 25. Section through uranyl acetate decalcified material (table 1-10) showing the simultaneous presence of a coccolith (C) and scale (S) within cell.
Figure 26. Interpretive drawing of coccolith morphogenesis based on ultrastructurally demonstrable steps. See text for details.
Figures 27-34. Comparison of processing procedures for ultrastructural observation of forming coccoliths

27. Unstained section floated on distilled water. Electron lucent areas of calcite dissolution indicated by d. X 35,000

27A. Enlarged view of area outlined in figure 27. Granularity of coccolithosomes evident (arrows). X 100,000

28. Unstained section floated on cacodylate-CaCl₂ solution. Electron dense calcite (Ca) and areas of dissolution (d), along with base (b), coccolithosomes (arrows), and matrix (m) evident. Areas of granularity in coccolithosomes and matrix present (arrows and double-headed arrows). X 100,000

29 and 30. Uranyl acetate stained sections floated on cacodylate-CaCl₂ solution. Calcite (Ca), base (b), coccolithosomes (arrows), and matrix (m) evident. Areas of granularity in coccolithosomes and matrix present (arrows and double-headed arrows). X 100,000

31. Lead stained section floated on cacodylate-CaCl₂ solution illustrating enhanced granularity of coccolithosomes (arrows) and matrix (m). X 100,000

32. Section floated on distilled water then stained with uranyl acetate and lead citrate, illustrating the matrix (m) which remains after calcite has been removed. X 35,000

33. Section through cell fixed only in glutaraldehyde solution showing base (b), matrix (m), and coccolithosome (arrows) structure like that of cells fixed by standard procedures. Floated on cacodylate-CaCl₂ solution. Lead stained. X 60,000

34. Section through cell fixed only in OsO₄ solution showing base (b), matrix (m), and coccolithosome (arrows) structure like that of cells fixed by standard procedures. Floated on cacodylate-CaCl₂ solution. Lead stained. X 60,000
Figures 40-47. Effects of decalcification procedures on the components of the coccolithogenic system. Cells decalcified following sequential fixation in glutaraldehyde and osmium solutions. Lead stained. X 60,000

40-41. EDTA chelation, aqueous solution, pH 5.0. Table 1-5. Neither matrix or coccolithosomes evident

42. EDTA chelation, ethanolic solution, pH 5.0. Table 1-6. Degraded matrix at dm

43. EDTA chelation, aqueous solution, pH 7.4. Matrix (m) and coccolithosomes (arrows) present. Table 1-7

44. Low pH and Ca^{++} deficiency, aqueous solution. Table 1-8. Matrix and coccolithosomes present

45-46. Low pH and Ca^{++} deficiency, ethanolic solution. Table 1-9. Matrix and coccolithosomes present

47. Uranyl acetate, ethanolic solution, pH 4.5. Table 1-10. Matrix and coccolithosomes present
Figures 48-53. Effects of decalcification procedures on the components of the coccolithogenic system. X 25,000

48. Control material exposed to 0.1 M cacodylate, pH 7.4 after standard fixation. Table 1-11. Calcified coccoliths (C), scales (S), and columnar material evident on cell exterior. Stained with uranyl acetate and lead citrate.

49. Cell decalcified "in vivo." Calcite absent and matrix degraded (dm) on external coccoliths. Internal components of coccolithogenic system unaffected and electron opaque calcite evident in rim elements of internal coccolith (C). Lead stained.

50. Uranyl acetate decalcified material. Table 1-10. Calcite removed leaving matrix in form of rim elements. Orientation of coccoliths, scales, and columnar material on cell surface normal. Stained with uranyl acetate and lead citrate.

51. Low pH and Ca^{++} deficiency, ethanolic solution, after standard fixation. Matrix of extracellular coccoliths degraded (dm) and amount of columnar material (cl) reduced. Table 1-9. Lead stained.

52. Control material showing effects of Ca^{++} deficiency in solutions. Coccoliths and scales not present on cell surface, and only remnants of columnar material (cl) remain. Table 1-3. Lead stained.

53. EDTA chelation, ethanolic solution, pH 5.0, after standard fixation. Coccoliths and scales not present on cell surface, and only remnants of columnar material remain. Table 1-6. Lead stained.
Figure 54. Kinetics of coccolith formation. Plot illustrates the rate of recalcification of \textit{in vivo} decalcified cells under standard conditions of growth. Rate is about ten cocoolith/cell/hour.

A-D. Nomarski differential interference contrast light micrographs of living cells at 0 hour (A), 2 hours (B), 3 hours (C), and 4 hours (D) after suspension of \textit{in vivo} decalcified cells in growth media. Arrows (B) indicate extracellular coccoliths on anterior surface of cell. The bulbous haptonema (h) can be seen between the flagella in C. X 1,350
Figure 55. Effect of light (dark) and heterotrophic nutrition on the rate of coccolith formation

A. Comparison of light (●) and dark (★) rates in normal growth medium. Dark to light kinetic ratio is about 0.38

B. Comparison of light (●) and dark (★) rates in lactate-deficient, $10^{-3}$ M NaCO$_3$ supplemented growth medium. Dark to light kinetic ratio is about 0.35
Figure 56. Effect of Ca$^{++}$ concentration on growth. Comparative growth curves over a seven-day period in growth media to which Ca$^{++}$ was added at concentrations of $10^{-2}$ M ($\bullet$), $10^{-3}$ M ($\times$), $10^{-4}$ M ($\bullet$), $10^{-5}$ M ($\square$), $10^{-6}$ M ($\ast$) and "0" M ($\ast$). Population density varies with Ca$^{++}$ concentration between $10^{-2}$ and $10^{-6}$ M, but is not directly proportional to it. Essentially no difference was noted between $10^{-6}$ M Ca$^{++}$ and "0" M Ca$^{++}$ samples.
Figure 57. Effect of Ca^{++} concentration on the rate of coccolith formation. Comparative kinetic curves for $10^{-2}$ M (A), $10^{-3}$ M (B), and $10^{-4}$ M (C) Ca^{++}. Following an initial lag phase, the rate of coccolith formation appears to be directly proportional to the concentration of Ca^{++} in the medium through at least the first five hours of recalcification. The decline in this rate between the fifth and twelfth hours may reflect a decline in Ca^{++} concentration in the medium as it is used to form coccolith calcite.
Figures 58-60. Effect of Ca\(^{++}\) concentration on coccolithogenesis. Lead stained

58-60. Sections through \textit{in vivo} decalcified cells after 48 hours in growth medium without added CaCl\(_2\)

58. Three stages in coccolithogenesis (x, y, and z) illustrating reduced calcification (Ca) even in the most mature stage (z). X 50,000

59. Coccolith in "mature" vesicle. Note reduced areas of calcite deposition (Ca). X 25,000

60. Section through intracellular coccolith illustrating the most extensive degree of calcite deposition noted in "0" M material. X 25,000

61. Section through \textit{in vivo} decalcified cell after 48 hours in 10\(^{-6}\) M Ca\(^{++}\)-containing growth medium. A morphologically normal Golgi complex with one incompletely calcified internal coccolith is present. X 25,000

62. Section through \textit{in vivo} decalcified cell after 48 hours in 10\(^{-5}\) M Ca\(^{++}\)-containing growth medium. Several stages in coccolith formation are evident (w, x, y, and z), at least two of which are partially calcified (y and z). X 25,000
Figure 63. The effect of Sr$^{++}$ on the rate of coccolith formation.

A. Plot relating number of coccoliths/cell to Sr$^{++}$ concentration after incubating in vivo decalcified cells for two hours in recalcification medium.

B. Plot relating number of coccoliths/cell to Sr$^{++}$ concentration after incubating in vivo decalcified cells for seven hours in recalcification medium.

Inhibition of coccolith formation was apparent at Sr$^{++}$ concentrations of greater than $10^{-5}$ M, with complete inhibition at $10^{-1}$ M.
NO. COCCOLITHS/CELL

A

Sr²⁺ conc. (m)
0
10⁻¹
10⁻²
10⁻³
10⁻⁴
10⁻⁵

B

NO. COCCOLITHS/CELL

Sr²⁺ conc. (m)
0
10⁻¹
10⁻²
10⁻³
10⁻⁴
10⁻⁵

Effect of high molarity (0.1 M) divalent cation concentration on growth. Four-day growth curves.

A. Standard growth medium.

B. Sr$^{++}$ added to a concentration of 0.1 M in the standard growth medium.

C. Mg$^{++}$ added to a concentration of 0.1 M in the standard growth medium.

D. Ca$^{++}$ added to a concentration of 0.1 M in the standard growth medium.

Compared to the standard medium, Mg$^{++}$ and Sr$^{++}$ had little effect on the growth rate. High molarity Ca$^{++}$ on the other hand reduced the growth rate to about one-half of that observed in the standard medium.
Figure 65. Effect of high molarity (0.1 M) divalent cation concentration on the rate of coccolith formation. Sr$^{++}$ (○), Ca$^{++}$ (×), and Mg$^{++}$ (□) added to concentrations of 0.1 M in standard growth medium compared to the rate in standard growth medium alone (●). Compared to the rate observed in standard medium, 0.1 M Mg$^{++}$ reduced the rate by about 14%, 0.1 M Ca$^{++}$ by about 47%, and 0.1 M Sr$^{++}$ completely inhibited coccolith formation.
Figures 66-69. Sections through stages of coccolithogenesis in cells inhibited by 0.1 M Sr$^{++}$. Coccolith formation did not progress beyond the point of association between coccolithosomes and base periphery. Matrix formation did not occur and crystallization of CaCO$_3$ was not evident.

66. Section through in vivo decalcified cell after 12 hours in 0.1 M Sr$^{++}$-containing medium. Lead stained. X 45,000

67-68. Sections through cells after 24 hours in 0.1 M Sr$^{++}$-containing medium. Lead stained. X 60,000

69. Section through cell after 31 days in 0.1 M Sr$^{++}$-containing medium. Lead stained. X 60,000
Figure 73. The effect of acetazolamide on growth. Curves relating net cell growth to acetazolamide concentration in standard (A) and lactate-deficient, $10^{-3}$ M Na$_2$CO$_3$ supplemented (B) medium after four day's growth. Acetazolamide at $10^{-3}$ M partially inhibited growth, while $10^{-2}$ M completely inhibited growth in either medium.
Figure 74. The effect of acetazolamide on the rate of coccolith formation. Plots illustrate the effect of acetazolamide concentration on recalcification of cells both when added to the standard growth medium (A) or to lactate-deficient, 10^{-3} M \text{Na}_2\text{CO}_3 \text{ supplemented medium (B). Counts made at two hours (○) and four hours (●). Partial inhibition of coccolithogenesis is evident both at 10^{-2} and 10^{-3} M acetazolamide in either medium. Complete inhibition was not observed during this initial four-hour period}
Figure 75. The effect of acetazolamide on the rate of coccolith formation. Recalcification of cells in standard growth media (●), standard growth media containing $10^{-3}$ M (★), or $10^{-2}$ M (●) acetazolamide, as well as lactate deficient, $10^{-3}$ M Na$_2$CO$_3$ supplemented growth medium containing $10^{-3}$ M (□) and $10^{-2}$ M (×) acetazolamide is illustrated. After 28 hours, the acetazolamide exposed cells were redecalcified for one hour at low pH (dashed lines) and then re-exposed to the same concentrations of acetazolamide. The kinetics of coccolith formation in redecalcified cells is indicated. Partial inhibition is evident both at $10^{-2}$ M and $10^{-3}$ M acetazolamide concentrations in either medium during the initial 28 hour period. Following redecalcification, cells incubated in $10^{-3}$ M continued to produce coccoliths at about the same rate as before, while coccolith formation in cells incubated in $10^{-2}$ M acetazolamide was almost completely inhibited.
Figures 76-81. The effect of acetazolamide on coccolithogenesis. In vivo decalcified cells recalcified in the presence of $10^{-2}$ M acetazolamide. Lead stained. X 60,000

76. Section through early stage in matrix formation. Cell incubated 48 hours in acetazolamide-containing standard growth medium

77. Early stage in matrix formation. Cell incubated 48 hours in acetazolamide-containing lactate deficient, Na$_2$CO$_3$ supplemented medium

78. Early stage in matrix formation. Cell incubated 24 hours in acetazolamide-containing standard growth medium

79. Calcified internal coccolith. Cell incubated 48 hours in acetazolamide-containing standard growth medium

80. Two stages in coccolith formation. In x, the matrix has not yet formed; in y, calcification has begun. Cell incubated 48 hours in acetazolamide-containing, lactate deficient, Na$_2$CO$_3$ supplemented medium

81. Two stages in coccolith formation. In x, matrix formation has begun, but calcification is not yet evident; in y, calcification appears complete. Cell incubated 48 hours in acetazolamide-containing standard growth medium
Figure 82. Effect of colchicine on coccolithogenesis. Plot relates the number of coccoliths/cell after seven hours recalcification in growth medium containing $10^{-3}$ M or $10^{-2}$ M colchicine. Coccolithogenesis was inhibited partially at $10^{-3}$ M and completely at $10^{-2}$ M colchicine.
Figures 83-86. Sections through cells illustrating various aspects of the microtubular systems. Arrows indicate areas of proximity between microtubule and vesicular structures.

83. Cross section through basal plate region of flagella showing an extensive array of bundle microtubules. Uranyl acetate decalcified cell. Stained with uranyl acetate and lead citrate. X 16,500

84. Section through flagellar and haptonemal bases. The haptonema (h) and one basal body (f, left) are sectioned longitudinally, while the other basal body is seen in tangential section (f, right). The microtubular sheet (ms) and one of the bundles (mb, lower) are seen in cross section, while the other microtubular bundle is seen in longitudinal section. Lead stained. X 40,000

85. Cross section through basal bodies (f) and haptonemal base (h), with both microtubular sheets (ms) and bundles (mb) approaching a longitudinal view. Lead stained. X 40,000

86. Section showing basal bodies (f), microtubular sheets (ms), and bundles (mb) in relation to the cell surface. The section includes a portion of the haptonemal appendage (h) and the smooth endoplasmic reticulum (ser) associated with it. Lead stained. X 40,000
Figures 87-92. Sections illustrating the relationship between the Golgi apparatus and various microtubular complexes - basal bodies (f), microtubular sheets (ms), and bundles (mb). Uranyl acetate decalcified. Stained with uranyl acetate and lead citrate. X 25,000
Figure 93. Interpretive drawing of the microtubular system based on ultrastructural observations. See text for details

f = flagella
h = haptonema
ms = microtubular sheet
mb = microtubular bundle
ser = smooth endoplasmic reticulum
Figures 94-98. Section through cells illustrating the presence of various stages of coccolithogenesis in cells inhibited by $10^{-2}$ M colchicine. Lead stained. X 25,000

94. Section passes through Golgi region and a mature intracellular coccolith. Incubated in colchicine-containing medium for 24 hours

95. Section through parts of Golgi complex and one partially calcified coccolith. Incubated in colchicine-containing medium 12 hours

96. Two stages in coccolith formation, one early in matrix formation (x) and the other (y) already calcified. Incubated in colchicine-containing medium 24 hours

97. Two intermediate stages in coccolith formation (x and y). Incubated in colchicine-containing medium 12 hours

98. Three stages in coccolith formation, one very early (w), and two (x, y) already well calcified. Incubated in colchicine-containing medium 24 hours

Figures 99 and 100. Sections through microtubular systems of cells inhibited by colchicine basal bodies (f), haptonemal base (h), and microtubular sheets (ms) present. Bundle microtubules not evident. Lead stained. X 40,000

99. Incubated in colchicine-containing medium 12 hours

100. Incubated in colchicine-containing medium one hour
Figures 101-104. Sections through microtubular systems of cells inhibited by colchicine. Basal bodies, haptonema (h), and microtubular sheets (ms) present. Microtubular bundles, if present, appear reduced. X 40,000

101. Incubated in colchicine-containing medium 24 hours. Microtubular bundles not evident

102. Incubated in colchicine-containing medium 12 hours. Bundle microtubules (mb) present, but not oriented perpendicular to microtubular sheet (ms)

103-104. Incubated in colchicine-containing medium 12 hours. Bundle microtubules (mb) present, but reduced in number as compared to control material

Figures 105-106. Micrographs show the only two examples of extensive arrays of microtubular bundles (mb) observed. Microtubular sheet evident in figure 106. In addition, sections pass through several stages in coccolith formation. Incubated in colchicine-containing medium 12 hours. Lead stained. X 16,500
DISCUSSION

Structural Aspects of Coccolith Formation

The results of ultrastuctural investigations, reported here and in several previous publications (106, 107, 163, 164), show that coccolith assembly is a well ordered intracellular process which occurs in a complex, though defineable sequence within the Golgi apparatus. It seems to proceed in a manner analogous to that shown for other secretory processes (5, 32), with appropriate modifications being made so that the cell can exert the necessary control over the architecture of the coccolith.

Coccolithogenesis as a model for intercisternal differentiation

The concepts of Golgi polarity and membrane flow are strongly supported by morphological evidence. It is often clear for instance that one face of the Golgi apparatus is differentiable from the other by the presence of cisternal distensions, dense staining intracisternal materials, and association with "secretory granules" (5, 32). At least in some cases (e.g. 127, 158, 166) this polarity has been observed to be accompanied by staining gradients of intracisternal material across the Golgi stack. In addition, appropriate use of a permangenate staining reaction has revealed a membrane structural gradient within the Golgi field (39). The Golgi polarity and cisternal maturation evident during coccolith formation are particularly convincing however, since their observation is not dependent upon an indirect quantification of staining reactions, but rather upon an obvious and distinct maturation sequence of the coccolith structure. In that it is difficult to imagine the complex coccolith structures passing from one cisterna to another, it seems clear that sequential coccolith formation
must be accompanied by cisternal maturation and flow from the proximal to distal Golgi pole and then to the plasma membrane.

The role of intracisternal differentiation in coccolith formation

In addition to intercisternal differentiation, intracisternal differentiation is also quite evident, and its involvement both in the development of coccoliths and other complex algae secretory products has been suggested by several workers (17, 82, 107). Manton has pointed out that the Golgi cisternae are dorsoventrally (proximodistally) differentiated (78-80). Moreover, the structural bases for this dorsoventrality reside either in the membrane or the contents of the lumen, since displacement from the Golgi stack does not alter the functional polarization of the cisterna. Brown et al. (17) have suggested that the initial site of cellulose polymerization during scale (base) formation may occur along the thickened inner surface of cisternal membranes in the region of the central dilations, thus hypothesizing functional as well as structural centrapeipheral cisternal differentiation. The continued involvement of cisternal membranes in scale formation is particularly well illustrated in the study of Manton and Ettle on Mesostigma (82), where the bounding membranes clearly play an "active role" in laying down the various parts of the complex basket-shaped scale.

Coccolithogenesis involves added structural complexity, and this study has revealed an even greater capacity for intracisternal differentiation within the Golgi system. As illustrated in Figures 19 through 21 not only are the proximodistal and centroperipheral cisternal differentiations needed for base formation evident, but an additional centroperipheral dif-
differentiation which allows both base and coccolithosomes to be formed within the same cisterna is observed. Even greater differentiation must be present during matrix formation, where the cisternal membranes appear to be involved in the laying down of matrix material into the contoured form of the calcified elements. Calcification itself involves a differentiation of the intracisternal space, since only the areas delimited by matrices calcify.

**Base formation** The formation of coccolith bases can be envisioned to occur in a manner similar to that hypothesized by Brown *et al.* (17) for *Pleurochrysis* scales. Though it is not possible in our study to consistently differentiate between early bases and scales because of their similar appearance in section, the presence of coccolithosomes in peripheral pockets of cisternae in several instances (e.g. Figures 19 through 21) presumably denotes base formation, and, while we cannot confirm any of the speculation of Brown and his co-workers, we can confirm the observations which led to these speculations. Thus, the similarity between our observations on "base" formation and those of Brown *et al.* on "scale" formation supports suggestions of homology between haptophycean scales and the coccolith base.

The proposals of Brown and his co-workers regarding scale morphogenesis provide few clues as to how the cell might control scale morphology. They suggest that the cellulosic fibrils are formed in two layers and converted to one layer, at which time scale morphology becomes evident. During the conversion of the double layer to a single layer, the cell must presumably orient the fibrils into the whorled (concentric) or radiating morphology. Their proposal also fails to account for the ultimate presence
of not one but two layers of fibrils. As an alternative hypothesis, it would seem that the cell might synthesize the two fibril layers separately, one at the upper (distal) and one at the lower (proximal) cisternal membrane surfaces, with the subsequent single-layered appearance resulting from a close appression of the two. Thus, the distinct morphologies of the two layers might evolve at their respective sites of synthesis rather than at a later time. This scheme would also account for the very early establishment of intracisternal polarity suggested by Manton (80).

**Coccolithosome formation**  
Centroperipheral differentiation, which results in the localization of coccolithosomes in pockets at the cisternal perimeter, is uniquely evident during cricolithogenesis. Though it is probable that at least some of the material which contributes to the formation of coccolithosomes is derived from the endoplasmic reticulum, the assembly, or polymerization of this material into distinct morphological units is limited to the cisternal pockets. The continued localization of coccolithosomes at the cisternal periphery appears to be accomplished by a constriction of the cisterna between the pockets of coccolithosomes and the central base-containing region (Figures 17 through 24).

While morphological observations clearly indicate the site of coccolithosome formation, they fail to suggest the roles that might be played by cisternal constituents in this process. It is not clear for instance whether assembly might occur spontaneously as a result of addition of material into the cisternal milieu, perhaps as a result of fusion with endoplasmic reticulum derived vesicles, or whether the process might involve some form of specific enzyme localization within the intracisternal space. If the former case is true, then the centroperipheral differentiation might
result primarily through localization of the assembly products at their sites of formation (i.e. the Golgi periphery) via cisternal constriction. If the latter case is true however, an additional form of intracisternal differentiation would need to be present, localizing the enzymatic components to a particular Golgi region, with a region of cisternal constriction serving as a secondary form of intracisternal differentiation. In either case, however, some additional form of control must be present to release coccolithosomes into the central cisternal region at the appropriate time for matrix formation.

Matrix formation It seems clear, on morphological grounds, that coccolithosomes are involved in matrix formation (106, 107, 163, 164). They appear to exist as a morphologically heterogeneous population, with particle diameters ranging from 15-35 nm in calcified material (107). There is no evidence as to whether the coccolithosomes are or are not in some way differentiated to form A vs. B elements, or, curved vs. planar surfaces, though it seems unlikely that they contain sufficient information to form the matrix without some outside influence. It does seem likely however that they do contain assembly potential, such that when placed into the appropriate environment they will polymerize in such a manner as to form sheets of matrix material, with the architectural control of matrix form being carried out through some type of intracisternal differentiation.

The base of the coccolith undoubtedly plays an important role in matrix formation, and the accumulation of coccolithosomes at the rim of the base is the first recognizable stage of matrix formation (stage A-1, Figure 26). Even at this time however, a role for cisternal membranes in matrix orientation is suggested by the association of the membrane with the
periphery of the base (Figures 22, 26). The role of membranes is additionally supported by the continued association of the cisternal membrane with specific sites on the developing matrix. While the base, perhaps in conjunction with the membrane, would seem to be most intimately associated with initiation of matrix formation and perhaps nucleation of A vs. B elements, the observation of contact points between membranes and the more distal portions of the developing matrix would suggest that they might be more closely involved in controlling matrix contour. Our micrographs suggest that this control does not take place by simply laying the matrix down along a precontoured membrane mold, but rather that membrane-matrix contact is discontinuous, occurring only at specific sites at any given moment.

**Calcification**

Calcification, the final stage of cricolith morphogenesis, appears to be under no less stringent control than the previous phases of development. Calcite crystallization occurs only in those areas predelineated by matrix material. Thus, the shape and position of the matrix predetermines the shape and position of calcite deposition. While the matrix clearly controls mineral form, its function relative to the actual crystallization is not completely understood. Presumably it provides an appropriate microenvironment at its inner surface, perhaps in the form of nucleation sites, so that crystallization is initiated, and then it maintains some form of intracisternal differentiation so that crystal growth is limited to the area within its boundaries.

**The role of cisternal membranes in coccolith formation**

Two factors suggest that the cisternal membranes play a primary role in the translation of genetic information into biological form during coc-
colith formation. The membranes are consistently involved with the various formative stages e.g. fibril formation, cisternal constriction, and matrix formation, and within the limitations of the electron microscopic preparative techniques and resolution, they are the only cisternal component that possesses sufficient architectural integrity to contain conformational information. In that the ultimate source of information for colith formation is presumably nuclear DNA, it is interesting to consider that the argument for a role for membranes as carriers of genetic information is strengthened by the concept that membrane flow occurs in a nuclear (proximal) to plasmalemma (distal) direction, with membrane continuity between various organelles being evident (31, 32, 33, 34, 39, 57, 92, 134, 135, 159). Kessel (57) has pointed out, for instance, that in many cells blebbing of the nuclear envelope is clearly involved in the morphogenesis of specific membraneous organelles and that inherent in such activity is the notion that the membranes contain information to control their activity or fate. The involvement of cisternal membranes in colith formation would certainly seem to support this concept. While any suggestion as to the chemical or physical nature of the information or the mode of "implantation" into the membrane requires the apposition of speculation upon speculation and has therefore been avoided, it is clear that if membranes are carriers of information, then during colith formation this information must not only be able to code for several different activities but also to localize these activities to particular regions of the cisterna.
Comparison of Cricolithogenesis with Other Coccolithogenic Systems

Three coccolithogenic systems have been studied in some degree of detail at the ultrastructural level - cricolith formation, placolith formation, and crystallolith formation. It is evident from this study, as well as the previous reports of Outka and Williams (106, 107, 163, 164) and Manton and Leedale (87) that cricolith formation is a Golgi associate secretory process, and the postulated involvement of intricate specialized organelles is probably erroneous. The suggested reassignment of the intracellular coccolith precursor body of Isenberg et al. (45, 46) and Pienaar (123) to a degenerative or autophagic stage has already been dealt with in the Literature Review.

The work of Manton and Leedale (87) on placolith formation in *C. pelagicus* is sufficiently similar to our study to suggest that placolithogenesis too involves only slight modification of a "typical" secretory pattern, with the Golgi serving as a site for packaging and assembly of the secretory product. The earlier proposals of Wilbur and Watabe (161) for placolith formation in *C. huxleyi* differed in that they suggested the involvement of a specialized matrix region and reticular body in coccolithogenesis, however, their published micrograph, along with the more recent pictures of Klaveness and Paasche (58) should probably be reinterpreted in light of the more extensive information now available from studies of *H. carterae* and *C. pelagicus*. The matrix region- reticular body complex, for example is probably equivalent to a single Golgi cisterna and its associated anastomosing network (compare Figures 12 and 13 reference 63 with Figure 18 in reference 5), and the structure connecting the centers of cal-
cification, termed a "membrane" by Wilber and Watabe, may be a composite formed by the base and sheath-like matrix. Thus, the three heterococcolithophorid species studied at the ultrastructural level, *H. carterae, C. pelagicus, C. huxleyi* probably all follow a similar pattern of coccolith formation and secretion.

Pertinent studies of holococcolithogenesis have been limited to one cell type, the motile "crystallolithus" phase of *C. pelagicus*. The early work of Parke and Adams (119) indicated that holococcoliths are formed by the pattern specific deposition of calcite microcrystals on an organic scale. The system was subsequently examined at the electron microscopic level by Manton and Leedale (86). Though interpretation of this work is complicated by the lack of adequate culture conditions and limited techniques then available for preparing cells for fine structural examination, several interesting and perhaps important observations were recorded.

In particular, calcification was not observed intracellularly, but between the plasmalemma and an external "skin," and a three-dimensional matrix was not observed about the calcite elements. If these observations do reflect the actual biological situation, then it is worthwhile to speculate about how holococcolithogenesis differs from heterococcolithogenesis. As pointed out by Black (9) the calcite rhombs forming the holococcolith may differ little from crystals formed in a test tube, thus the cell need not control crystal morphology as it does in heterococcolith development but only crystal distribution and size. Crystal distribution on the coccolith might be accomplished by a two-dimensional "matrix" rather than the more typical three-dimensional matrix, with the coccolith base, or a closely apposed surface layer, serving in this capacity. Control over
crystal size might also be accomplished by way of a surface matrix, perhaps in conjunction with the external "skin." Holococcolithogenesis probably represents a simpler system than heterococcolithogenesis, with "matrices" formed in the Golgi, secreted, and then calcified, with the cell exerting only minimal control over crystal morphology.

Comparison of Coccolithogenesis with Metazoan Calcification Systems

Coccolithogenic calcification holds an unique position among all forms of biological mineralization. In no other mineralizing system is crystal morphology so subjugated to cellular control as it is among the more highly evolved heterococcolithophorids, such as Hymenomonas, and yet, within this same class of organisms exist cells which exert but minimal control of mineralization such as the motile phase of C. pelagicus. Thus, within a limited taxonomic range, biological calcification can be studied over a broad spectrum of complexity.

It is evident from developmental data that crystallolithogenesis is functionally closer to metazoan calcification than either placolithogenesis or cricolithogenesis. Like most metazoan systems studied, the crystallolith matrix is apparently formed intracellularly and then secreted outside the plasmalemma where it is subsequently calcified. With respect to metazoan systems, cellular control of crystallolith mineralization appears to hold an intermediate position, being somewhat more advanced than calcification of crustacean exoskeleton where the matrix exerts little control over crystal size or orientation, but certainly less advanced than other systems cited by Travis (149) which strictly control crystal size and orientation and act as well to modify crystal form. The major developmental difference
between holococcolithogenesis and heterococcolithogenesis, and likewise heterococcolithogenesis and metazoan calcification, is that crystal form is much more highly controlled in heterococcolithogenesis, and calcification occurs intracellularly. That there is a causal relationship between these two seems quite probable. It seems likely that in order for the cell to exert sufficient control over crystal morphology to form the contoured surfaces of the heterococcolith, it must regulate the crystallization environment to a degree not possible in the extracellular milieu. That the cell exerts this control via the organic matrix, and thus clearly indicates the potential of matrix regulation in other mineralization systems, is evident in this study.

Coccolith Evolution

While a detailed discussion of coccolith evolution is beyond the scope of this work, certain features of the coccolith morphogenic sequence have important evolutionary implications. The use of the paleontological record in attempts to provide a direct indication of coccolith evolution is severely limited by the inability of the microcrystalline elements to withstand geological stress over long periods of time (10). Thus, while the paleontological history of coccoliths extends as far back as the Mesozoic era, even in the earliest recognizable deposits, very complex, highly evolved forms are found. In addition, the organic portions of the coccolith, even in relatively recent deposits, are not evident. Comparative study of coccolith structure and morphogenesis on the other hand can be used to provide a significant body of information from which we can infer certain things about their evolution.
Recent investigations (17, 87, 107, 118, 119, 122) have suggested a close relationship between the coccolith base and unmineralized scales found on the surface of non-coccolithophorid members of the Haptophyceae (64, 78-85, 88, 89, 91). In that it is apparent from morphogenic evidence that the base plays an important role in the positioning of the calcified elements, it seems likely that scales must have preceded the coccolith in evolutionary lineage. It is similarly tempting to suggest that heterococcoliths were derived from holococcolith-like structures through the evolution of a three-dimensional matrix to control crystal morphology. On developmental grounds this would seem to be a valid supposition, with a more complex developmental sequence evolving from a simpler one. In addition, Black (9) has pointed out that a spectrum of intermediate stages of crystal forms exists between that of the simple holococcolith, such as the crystallolith, and that of the more complex forms, such as the cricolith. In the three systems whose morphogenesis has been studied, the monomorphic placolith can easily be imagined to have evolved from the crystallolith, and the dimorphic cricolith to have evolved from the placolith.

Black (9) cautions against the unqualified acceptance of hypothetical evolutionary schemes based primarily on structural comparisons by noting that the simplest of all known coccoliths are borne by plants living today, whereas many of the most elaborate are found only in the fossil record. It is additionally true that all of the developmental data currently available has been obtained from a very restricted number of closely related organisms. Thus, for instance, in the first place, holococcoliths may be a product of regressive rather than progressive evolution, and secondly, the
development of cricoliths, placoliths, and crystalloliths may not necessarily be representative of all coccoliths.

However, within these restrictions the following considerations would seem relevant. Coccolith formation must initially have involved the ability of cells to form calcite crystals, and their capacity to provide an appropriate substrate to retain them at the cell surface. Thus one might speculate that the evolution of coccoliths involved the sequential ability of ancestral cells to (1) form base plates (the "scale" stage), and perhaps independently to precipitate simple calcite crystals, (2) "mount" the simple crystals upon the base plates, (3) organize the crystals on the surface of the base plate (the holococcolith stage?), and (4) control crystal morphology via a three-dimensional matrix (the heterococcolith stage).

This system is based upon the initial presence of a scale (base), with coccolith evolution dependent upon the subsequent modification of that scale. The exact role of the calcification matrix in the early stages of this evolutionary scheme is purposely left open. Whether the initial crystallization of calcite could occur in a "test tube" environment between a skin and plasmalemma as suggested by Manton and Leedale (86) or whether a preformed protein-polysaccharide matrix would need to be present as is seen in metazoan systems is unclear. Alternatively, would it not be possible, for instance, that the initial nucleation sites developed as a result of some modification of the scale pectin-like layers, with this material then serving as a primitive matrix? It is clear that one approach to answering these questions about coccolith evolution is to re-examine the crystallo-lithogenic system, as well as other of the less complex coccolithophorids,
and more carefully evaluate the role of a matrix in these seemingly primitive systems.

Experimental Modification of the Coccolithogenic System

Evaluation of the experimental system

Growth conditions and analytical system One of the primary difficulties involved in evaluating research reports on coccolithogenesis has been the inadequacy of information concerning the growth condition and physiological state of the organisms at the time of experimentation. This is particularly true of structural studies where growth data has often been limited to a minimal characterization of the physical and chemical environment (86, 87, 119, 123, 161). The importance of controlled cultural conditions can be clearly seen in Figure 1, where division and non-division phases are separable through control of the diurnal-nocturnal cycle, and a uniform cell-type can be obtained by maintenance of the chemical environment via a 48 hour transfer schedule. The inadequacy of culture controls in previous reports has not gone entirely unnoticed. Manton and Leedale (87) for instance site suboptimal growth conditions as a possible source for failure of their organisms to form completed coccoliths, and it follows, for their inability to describe the sequence of coccolithogenesis. Isenberg and his co-workers, on the other hand, while exploring the effect of the chemical environment on coccolithogenesis in relatively great depth, have failed to correctly correlate growth and culture "age" with coccolith formation. Whether this is due to their handling of the organisms, or to some peculiarity of the analytical system is not evident in their publications (49, 52).
As compared to the relatively indirect analytical techniques employed by Isenberg and his co-workers (49) and the C-14 or Ca-45 radiotracer techniques employed by Paasche (110) and Crenshaw (21) to attempt to quantitate coccolithogenesis, the optical techniques employed in this study provide a precise and reproducible measure of coccolith formation with a minimum of procedural manipulation. A particular advantage of this methodology is that it quantitates coccolithogenesis by a direct measure of coccolith formation, i.e., by the appearance of the coccoliths themselves at the cell surface, rather than indirectly through measures of Ca\(^{++}\) or CO\(_3^-\) metabolism.

**Specimen preparation** Under appropriate conditions, limited modification of normal specimen preparation procedures for ultrastructural examination has little effect on the observed sequence of coccolithogenesis except in regards to decalcification of specimens and enhancement of matrix and coccolithosome granularity. These two factors are important. The characteristic granularity observed specifically in both the coccolithosome and matrix upon lead staining is a major factor in implicating the coccolithosome as the primary source of matrix material. The ability to maintain calcite *in situ* has allowed us to determine when calcification occurs in the coccolithogenic sequence and also to document the role that the matrix plays in the mineralization process. On the other hand, the use of appropriate decalcification procedures has helped immeasurably in the dissection of the earlier phases of coccolith formation. For technical reasons, primarily associated with the great care required in maintaining calcite in sections and the resulting damage caused by its loss, productivity is greatly enhanced by decalcification prior to embedding. As indicated by Figures 35 through 53 however, the methods used in decalcification are
extremely important. In general, decalcification following postfixation is superior to that during glutaraldehyde fixation. Of the several procedures tried, 0.02 M uranyl acetate in 30% ethanol, pH 4.5 appeared to cause the least amount of structural modification, while doing a sufficiently adequate job of decalcification. It is however additionally interesting to note that while the base structure was unaffected by any of the decalcification procedures, both coccolithosome and matrix were often affected similarly, though coccolithosome structure appeared to be somewhat less sensitive to decalcification than matrix structure.

Effect of light

Our results concerning the effect of light, or more appropriately the absence of light, on coccolithogenesis are in disagreement with those of Crenshaw (21) who found complete inhibition of coccolith formation in the absence of light. Our data indicate that, while the rate of coccolith formation is significantly inhibited by the absence of light in either lactate or carbonate supplemented medium (rate reduced to about one-third in the dark), complete inhibition was not obtained. These results are thus in somewhat closer agreement with those of Paasche (110) who found a reduction, but not complete inhibition of coccolith formation in C. huxleyi. In any case however there can be little doubt that coccolith formation is affected significantly by the presence of light.

Effect of divalent cations

Calcium concentration has a profound effect on both coccolithogenesis and growth. As illustrated in Figures 56 and 57, reducing the $Ca^{++}$ concentration to levels below $10^{-2}$ M (the approximate level of sea water) resulted
in a reduction of both growth and coccolithogenesis, with the rate reduction of coccolith formation being directly proportional to that of calcium concentration between $10^{-2}$ M and $10^{-4}$ M. While the precise level of Ca$^{++}$ in solution was not determined (data are in terms of added Ca$^{++}$), kinetics data (Figure 57) suggest that in concentrations of $10^{-2}$-$10^{-4}$ M, Ca$^{++}$ depletion during the first 5 hours was not sufficient to affect the linearity of the curves, however, deviation from the kinetic pattern over an extended period (12 hour count) may reflect an experimentally significant reduction in available Ca$^{++}$ due to precipitation in the form of coccolith calcite. In addition, growth data (Figure 56) indicate that the "0" M level of Ca$^{++}$ in the medium was less than $10^{-5}$ M, with growth response being about the same as at $10^{-6}$ M. At the ultrastructural level, Ca$^{++}$ depletion seems to affect both crystallization and matrix formation. Increase in Ca$^{++}$ concentration to $10^{-1}$ M resulted in a decrease in both growth and coccolithogenesis of about 50%, suggesting an effect on some general system rather than one specifically associated with coccolithogenesis.

Our results with reference to the effect of divalent cations other than Ca$^{++}$ are not in complete agreement with the conclusions of Isenberg et al. (49). Our data are in agreement with their contention that Sr$^{++}$ can substitute, at least in part, for Ca$^{++}$ as a growth requirement, and that under these conditions coccoliths are not formed. However in contrast to their work, we found that the Sr$^{++}$ had a significant inhibitory effect on coccolithogenesis in the presence of normal calcium concentrations (Figures 63 and 65). Only minimal inhibition of coccolithogenesis was observed in the presence of increased Mg$^{++}$ concentrations, and neither Sr$^{++}$ or Mg$^{++}$ in high concentrations had a significant effect on growth. Interestingly
enough, ultrastructural observations of Sr$^{++}$ inhibited cells mimicked the observations of cells grown in Ca$^{++}$ depleted medium, though the effect was more pronounced. In Ca$^{++}$ depleted cells, matrix formation and crystallization were greatly reduced, whereas in Sr$^{++}$ inhibited cells neither formed matrices or calcite deposits were observed at all. Since it seems reasonable to assume that Sr$^{++}$ is competing with Ca$^{++}$, the results suggest that Ca$^{++}$ is necessary not only for crystallization, but also for matrix formation and probably for adhesion of coccoliths to the cell surface as well.

**Effect of acetazolamide**

The carbonic anhydrase inhibitor, acetazolamide, has been used to inhibit calcification in several invertebrate systems (e.g. 20, 37, 41, 60) as well as two coccolithophorid systems (51, 110). In the case of *C. huxleyi* (110), Paasche demonstrated an almost equal effect on both photosynthesis and coccolith formation, and was therefore unable to determine if any specific effect on coccolithogenesis, as opposed to a general effect on photosynthetic metabolism, was taking place. Isenberg et al. (51) on the other hand suggested that in *H. carterae* (Plymouth Culture Collection #156) acetazolamide had the specific effect of completely inhibiting coccolithogenesis while reducing growth by only one third. Our results were essentially similar to those of Paasche, though relating coccolith formation to growth rather than photosynthesis, and, like Paasche, we are unable to link a specific carbonic anhydrase to coccolithogenesis, as opposed to a more general metabolic pathway, nor were we able to demonstrate any specific inhibition of coccolithogenesis at the ultrastructural level as has also been suggested by Isenberg and his co-workers (46).
Effect of colchicine

The colchicine experiments were prompted by the recent work of Brown and Franke (16) suggesting that the complex microtubular array of Pleurochrysis may be involved in scale secretion, along with suggestions of similar microtubule involvement in other secretory processes (61, 100, 126, 136, 165). The results of these experiments indicate the 10^-2 M colchicine inhibits both cell division (mitotic activity) and motility (flagellar activity) as well as coccolithogenesis, and that these activities can be restored by subsequent dilution of the inhibitor. Thus it appears that microtubular activities may be involved in coccolithogenesis. In the absence of more detailed physiological information about coccolithogenesis however, the question arises whether the effect of colchicine on coccolithogenesis is related directly to formation or transportation of coccolith materials, or indirectly related through an effect on other microtubular functions.

It is highly unlikely that inhibition of coccolithogenesis is linked to interference with either mitosis or cell motility since coccolith formation is observed under normal circumstances both in non-dividing and non-motile cells. An alternative site of colchicine activity with regards to inhibition of coccolithogenesis may be the cytoplasmic microtubules described by Manton and Peterfi (90) as a cytoskeletal system. This suggestion is partially supported by the structural observations which indicate that inhibition results in a loss of bundle microtubules. As has been suggested for other secretory systems, microtubules are probably associated with coccolithogenesis in some direct manner and the cytoplasmic microtubules, particularly those forming the bundle pattern, are a likely candi-
date for this association. It is not clear whether these microtubules might be functioning in an active role, as has been suggested in axoplasmic transport (136) or a more passive role such as that hypothesized for plant wall thickening (76). The latter role would seem more likely however since an intimate vesicle to tubule relationship such as that seen in axoplasmic transport is not observed, but rather the association seems to be between bundles of tubules and the Golgi region in general. Thus the concept of a cytoskeletal guidance system might be more appropriate.

Conclusion

The experimental manipulation of the coccolithogenic system has provided us not only with pertinent information about the system itself, but perhaps more importantly, the manipulative potential may allow us to further elucidate more general aspects of calcification, secretion, and cellular control. While several studies have indicated a relationship between Golgi function and biological mineralization, probably none is as distinct as the association that is evident during coccolith formation. Coccolithogenesis is a system in which an extremely complex structure is formed within Golgi cisternae by a very precise and orderly assembly of component parts. It illustrates the translation of genetic information, probably by way of cisternal membranes and the organic matrix, which results in the directed crystallization of inorganic material to form highly contoured structures. The secretory sequence appears to be specifically affected both by divalent cations and by the microtubule inhibitor colchicine. In addition, the appropriate use of light (21, 107, 110, 112, 113, 114, 116), temperature (157), organic nutrients (52) and specific metabolic inhibitors
(21, 46, 47, 51, 110, 111) also is a potential means of manipulating this system. Because of the distinct morphology of the forming coccolith elements during their sequential development within the polarized Golgi apparatus, they provide a morphological assay for evaluating the action of various effectors on calcification, secretion, and the cellular control system.


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APPENDIX A

Culture Media and Antibiotic Mixture

### Standard Growth Medium

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<thead>
<tr>
<th>Component</th>
<th>Amount</th>
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<tr>
<td>NaCl</td>
<td>20.0 g</td>
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<tr>
<td>KCl</td>
<td>0.6 g</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>2.465 g</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>1.11 g</td>
</tr>
<tr>
<td>Na·glycerophosphate</td>
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</tr>
<tr>
<td>NaNO₃</td>
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</tr>
<tr>
<td>Tris (2-amino-2-(hydroxy-methyl)-1,3-propanediol)</td>
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</tr>
<tr>
<td>Vitamin B₁₂</td>
<td>3 x 10⁻⁶ g</td>
</tr>
<tr>
<td>Thiamine HCl</td>
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</tr>
<tr>
<td>Na·lactate</td>
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</tr>
<tr>
<td>Na₂CO₃</td>
<td>1 x 10⁻⁴ g</td>
</tr>
<tr>
<td>Na₄·EDTA</td>
<td>0.1 g</td>
</tr>
<tr>
<td>Trace Metals*</td>
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</tr>
<tr>
<td>Distilled H₂O</td>
<td>q.s. to 100 ml</td>
</tr>
</tbody>
</table>

pH 7.7-7.9

autoclave at 15 lbs of pressure for 15 minutes

*Trace Metals

<table>
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</tr>
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<tr>
<td>Versen-01(Na₃·N-hydroxyethylethylene diamine Triacetate)</td>
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<tr>
<td>FeSO₄·7H₂O</td>
<td>0.44 g</td>
</tr>
<tr>
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<tr>
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<tr>
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<td>CuCl₂</td>
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<td>Na₂MoO₄</td>
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<tr>
<td>H₃BO₃</td>
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<td>Distilled H₂O</td>
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### Antibiotic Mixture PNFB

<table>
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<tr>
<th>Component</th>
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<tbody>
<tr>
<td>Penicillin G, Bufford Potassium (Lilly)</td>
<td>2000 units/ml</td>
</tr>
<tr>
<td>NegGram (Winthrop Laboratories)</td>
<td>2 mg/ml</td>
</tr>
<tr>
<td>Polymyxin B sulfate (CALBIOCHEM)</td>
<td>100 units/ml</td>
</tr>
<tr>
<td>Bacitracin (CALBIOCHEM)</td>
<td>20 units/ml</td>
</tr>
</tbody>
</table>
APPENDIX B

Procedures for the Preparation of Sectioned Material for Ultrastructural Observation

Fixation and Rinse Procedures

Glutaraldehyde solution two changes 2-4 hours
initiated at room temperature
then placed at 4 °C

In experiments where sampling occurred over extended periods, material was stored in the glutaraldehyde solution for up to 48 hours

Rinse solution three rinses 10 minutes each
4 °C

Osmium solution two changes 2-19 hours
4 °C

Fixation and Rinse Solutions

Glutaraldehyde solution
0.1 M sodium cacodylate
0.01 M CaCl₂
0.4 M glutaraldehyde
0.17 M sucrose
pH 7.4-7.8

Rinse solution
0.1 M sodium cacodylate
0.01 M CaCl₂
0.59 M sucrose
pH 7.4-7.8

Osmium solution
0.1 M sodium cacodylate
0.01 M CaCl₂
0.06 M OsO₄
0.53 M sucrose
pH 7.4-7.8
Dehydration

Graded series (30%, 50%, 75%, 85%, 95%, 100%; % V/v) ethanol or acetone
all except last step at 4 C
last step at room temperature

Followed by two rinses of propylene oxide when ethanol was used for dehydration

Embedding

Embed in maraglas (139), or
ERL (140) resin mixtures
1 pt solvent (acetone or propylene oxide) to 1 pt resin room temperature 1 hour
1 pt solvent to 3 pt resin room temperature 3 hours
pure resin 4 C 12-18 hours
Sample placed in capsules and polymerized at 60C (Maraglas) or 70C (ERL)

Sectioning

Sections were cut with a diamond knife on either an LKB Ultratome I or a Reichart Om-U2 microtome and floated on distilled water or a 0.1 M cacodylate 0.01 M CaCl\textsubscript{2} solution (pH 7.8-8.0). The sections were picked up on parlodion-carbon coated or uncoated grids.

Staining

Sections were stained with 1% (V/v) uranyl acetate (UA) in 50% (V/v) ethanol or 5% UA in absolute methanol, in 0.2-0.3% (V/v) lead citrate (151), or sequentially in UA then lead citrate.