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Mechanism of Calcification in the Marine Alga Emiliania huxleyi [and Discussion]

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Mechanism of calcification in the marine alga *Emiliania huxleyi*

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[Plate 1]

Coccoliths are delicate calcified structures produced by marine unicellular algae. In the species *Emiliania huxleyi* the calcium carbonate (mostly calcite) is closely associated with a complex, acidic polysaccharide which binds calcium ions specifically, interferes with the *in vitro* crystallization of calcium carbonate, and appears to be bound to a positively charged protein before the crystallization process is finished. Ultra-high resolution electron microscopy of the coccoliths reveals that the crystallographic structure differs in different parts of the constituent calcite elements. The synthesis of the coccoliths takes place intracellularly, and when this process is ended the coccoliths are extruded and incorporated into the so-called coccosphere surrounding the cell. Transmission electron microscope studies reveal the localization of polysaccharides in the calcifying organelle by means of cytochemical staining technique. The results are combined in a putative scheme describing coccolithogenesis.

Introduction

Emiliania huxleyi (Lohmann) Hay and Mohler is a unicellular marine protoctist that belongs to the phylum Haptophyta (see Margulis & Schwartz 1982). The cell is covered with elaborate calcified structures called coccoliths ($CaCO_3$) (figure 1). This is by far the most common coccolithophorid species in the world ocean and therefore an important constituent of the phytoplankton. It can be considered as the most productive lime-secreting species on Earth. The coccoliths are synthesized inside a specialized vacuolar complex (c.v.–r.b. complex), which appears to be derived from the Golgi apparatus (figure 2). A 'coccolith vesicle' (c.v.) is closely apposed to the nucleus and surrounds the growing coccolith. Continuous with the c.v. is a system of anastomosing tubes forming the 'reticular body' (r.b.). When the calcification process is completed the coccolith is extruded and incorporated into the 'coccosphere', i.e. the cover of coccoliths surrounding the cell.

Calcification in *E. huxleyi* has been studied from various points of view. The kinetics and light-dependence of coccolith biosynthesis have been studied by Wilbur & Watabe (1963), Crenshaw (1964), Paasche (for example, 1964, 1965, 1966, 1967), Steeman Nielsen (1966), De Jong (1975, 1976, 1979), Blankley (1971) and Sikes (1980, 1982). A number of biochemical studies on a polysaccharide that is closely associated with the calcium carbonate have been reported (Westbroek *et al.* (1973); De Jong (1975); De Jong *et al.* (1976, 1979); Borman *et al.*

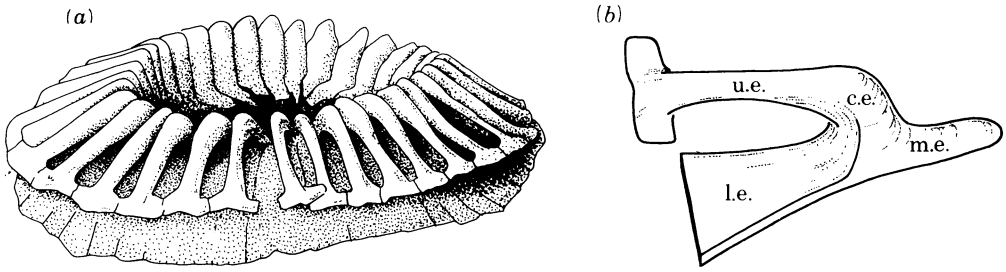

FIGURE 1. (*a*) A coccolith of *Emiliania huxleyi*. The cell is surrounded by about 15 coccoliths. A coccolith is considered to consist of a radial array of segments of calcium carbonate (mostly calcite). (*b*) A segment of a coccolith of *E. huxleyi*. The following elements are distinguished: l.e., flattened radially oriented lower element; u.e., hammer shaped upper element; c.e., central element; m.e., medially directed element.

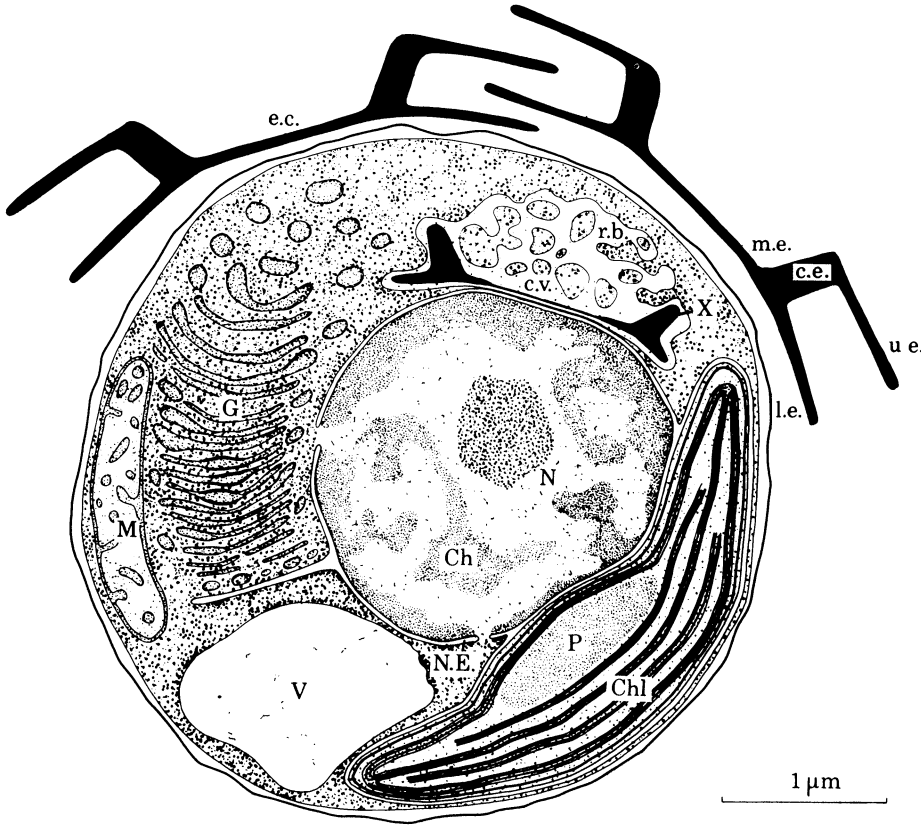

FIGURE 2. Idealized section through the cell of *Emiliania huxleyi*. Ch., chromatin; c.v., coccolith vacuole; e.c., extracellular coccolith with l.e., lower element; u.e., upper element; c.e., central element and m.e., medially directed element; G, Golgi apparatus; M, mitochondrion; N, nucleus; N.E., nuclear envelope; P, plastid; V, vacuole; X, coccolith *in statu nascendi*. Reproduced from Van der Wal *et al.* (1983) by courtesy of Springer-Verlag.

(1982); Fichtinger (1980) and Fichtinger *et al.* (1981)). The fine structure of the coccoliths was studied by Watabe (1967) and recently by Parker *et al.* (1983), and electron microscope studies on the cell morphology in relation to coccolith synthesis were done by Crenshaw (1964), Wilbur & Watabe (1963), Klaveness (1972, 1976), Van der Wal *et al.* (1983) and Van Emburg *et al.* (1983).

This paper summarizes a selection of the available data. Most of the information has been

taken from the literature, and some original data is presented. An attempt is made to formulate a preliminary working hypothesis on the mechanism of coccolith biosynthesis.

Composition of the coccoliths

Coccoliths consist of $CaCO_3$ (mostly calcite) and organic material. When isolated coccoliths are dissolved in 10% (by mass) EDTA (pH = 8) or dilute HCl, an acid polysaccharide is solubilized. The following data suggest that an intimate association exists between the polysaccharide and the crystalline phase. The polysaccharide cannot be extracted from the $CaCO_3$ with dilute NaOH, but, when isolated, it is dissolved in alkaline aqueous media (De Jong 1975). It would follow that the mineral prohibits its dissolution.

To some extent, the polysaccharide is also protected from bacterial as well as physicochemical degradation. Subfossil coccoliths (1000 a B.P.) still contain macromolecular polysaccharide material resembling the recent coccolith polysaccharide in various respects (De Jong 1975). However, the latter can be completely degraded by oxidizing agents when associated with coccoliths. The question whether the polysaccharide is localized on the surface or inside the calcium carbonate crystals is presently under investigation by S. B. Parker and colleagues.

Structure of the coccolith polysaccharide

The primary structure of the coccolith polysaccharide has been partially resolved by Fichtinger-Schepman (1981). The molecule is very complicated; it consists of a mannose backbone with highly branched side chains, which contain at least thirteen different monosaccharides. Some of these monosaccharides are methylated or dimethylated; these are concentrated in the exterior of the side chains. Others contain carboxyl groups or sulphate esters; the carboxyl groups are responsible for the acidic character of the polysaccharide (Borman *et al.* 1982). The molecular mass of the polysaccharide is about 100000 Da, as estimated with light scattering. We are trying to improve on this figure by osmometry and sedimentation analysis.

Ca^{2+} Binding

The binding of Ca^{2+} by the coccolith polysaccharide has been studied by various techniques (De Jong *et al.* 1976). From a $CaCl_2$ solution also containing an excess of Na^+ and Mg^{2+} ions the calcium is preferentially bound. Sr^{2+} is bound to the same extent as Ca^{2+}, whereas La^{3+} is a strong inhibitor of Ca^{2+} binding. The Ca^{2+} is bound primarily by the uronic acids; desulphation of the polysaccharide has no effect on its Ca^{2+} binding capacity (Borman *et al.* 1982).

Effect on $CaCO_3$ precipitation

When solutions of $CaCl_2$ and $NaHCO_3$ are mixed at concentrations exceeding the solubility product of $CaCO_3$, a precipitate is formed that results in a lowering of the pH (figure 3). The rate of decrease in pH is a measure of the rate of $CaCO_3$ precipitation under given conditions. The coccolith polysaccharide is able to delay precipitation when added in very small amounts with respect to the free Ca^{2+} concentration (figure 3). It probably binds to clusters of $CaCO_3$ as well as to growth sites on the crystals, thus preventing crystal growth (Borman *et al.* 1982).

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The uronic acids are primarily responsible for the binding; desulphated polysaccharide inhibits $CaCO_3$ precipitation to the same extent as the native material, whereas chemical conversion of the carboxyl into hydroxyl groups strongly reduces the inhibitory effect (Borman *et al.* 1982).

The native polysaccharide does not inhibit $CaCO_3$ precipitation under all conditions. When ethanol is added to a final concentration of 1% (by volume) to the reaction mixture, no inhibition is observed. Ethanol presumably affects the conformation of the molecules, owing to the presence of the relatively hydrophobic methylated sugars.

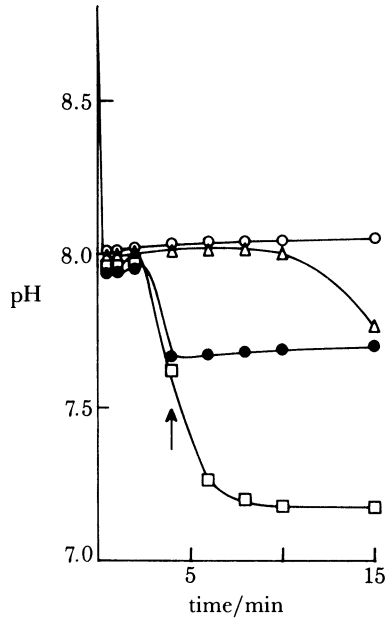

FIGURE 3. Effect of coccolith polysaccharide on $CaCO_3$ precipitation. The rate of $CaCO_3$ precipitation was determined by recording the decrease in pH. Three millilitres of 20 mM $CaCl_2$ (pH approximately 8.7) was added to three millilitres of 20 mM $NaHCO_3$ (pH approximately 8.7) and 0.3 ml of H_2O (□), or 0.3 ml of a solution containing 20 µg (△) or 50 µg (○) of coccolith polysaccharide. In one experiment 50 µg polysaccharide was added 4 min after onset of precipitation (→, ●).

The highly acidic polysaccharide alginic acid (polymannuronic-guluronic acid) inhibits $CaCO_3$ precipitation to about the same extent as the coccolith polysaccharide. However, when alginic acid is coupled to BSA (by carbodiimide) or to sepharose 4B beads (by cyanogen bromide) it has a stimulatory effect on $CaCO_3$ precipitation (not shown). Non-coupled BSA and sepharose beads had no effect on the precipitation. We are now trying to bind the coccolith polysaccharide onto sepharose beads under conditions whereby the functional groups of the former are kept intact as much as possible.

PRECURSORS OF THE COCCOLITH POLYSACCHARIDE

When calcifying cells are labelled with $H(^{14}C)O_3^-$, the label is incorporated in at least two macromolecular fractions, each containing several of the monosaccharides characteristic of the coccolith polysaccharide. When the two fractions are treated with 10% (by volume) TCA at 100 °C and subsequently filtered over Millipore filters part of the radioactivity is retained. When isolated radioactive polysaccharide is treated in the same way, almost no counts are recovered from the filter.

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When cells are dual labelled with [^{3}H]methionine and [^{14}C]galactose for a short period (2 h) both isotopes are incorporated into the two macromolecular fractions. No radioactive coccolith polysaccharide can be detected after 2 h of labelling. After treatment with TCA at 100 °C the [^{3}H]isotope is retained by Millipore filters, whereas the [^{14}C]isotope passes through.

These results indicate that the two macromolecular fractions are polysaccharide precursors associated with a protein moiety. Preliminary results suggest that they either contain a high percentage of basic amino acids or amino sugars, or both. In this respect it is interesting to note that non-calcifying cells (N-cells: see below) excrete a polysaccharide resembling the coccolith polysaccharide in sugar composition. This material probably contains a protein moiety with a basic character (cf. De Jong *et al.* 1979).

Ultrastructure of coccoliths

Watabe (1967) has proposed that the coccoliths consist of a radially arranged array of crystalline segments of $CaCO_3$, each consisting of a flattened radially oriented lower, a hammer shaped upper, a central and a medially directed element (figure 1). Electron diffraction patterns suggested that at least each of these elements, but probably the entire radial segments, behave as single crystals of calcite, with the crystallographic *c*-axis parallel to the morphological elongation.

Both the lower and the upper (hammer shaped) elements have been studied by Parker *et al.* (1983) by means of ultra-high resolution electron microscopy. Their structures were found to differ in that the former consists of a single crystalline sheet of calcite, while the latter is a mosaic of small, microdomain structures of 300–500 Å† diameter with no strong orientation (figure 4 (*a*), (*b*), plate 1).

Cell morphology and polysaccharide localization

Electron micrographs of four successive stages of coccolith biosynthesis are shown in figure 4 (*c*)–(*f*). The preparations of figure 4 (*c*), (*e*) and (*f*) are stained for polysaccharide by the periodic acid-thiocarbohydrazide-silver proteinate technique (Thiéry 1967). In these preparations the $CaCO_3$ has been dissolved. The material shown in Figure 4 (*d*) is obtained by double fixation in glutaraldehyde and OsO_4, and staining with lead; by this procedure the crystals are preserved.

Coccolith synthesis begins with the formation of a small vesicle, similar in position to the c.v., the protococcolith vesicle, against the nuclear surface (Van der Wal *et al.* 1983). Then, a reticular body is added, but as yet no crystalline matter is visible (figure 4 (*c*)). In figure 4 (*d*) incipient coccolith formation is shown; rhombohedral crystallites are formed with an organic 'base plate' subtended between them. The long axis of the rhombohedra is generally found to be perpendicular to the surface of the plate. These incipient crystals are localized at the site where ultimately the central elements will be situated. After their formation they grow out in three directions: radially, to form the lower elements; disto-radially (central and upper, hammer shaped elements); and medially. The organic base plate appears to extend towards the tip of the lower elements (figure 4 (*f*)), although it is not clearly seen at calcified sites. Therefore, it seems to grow radially together with the lower elements.

† $1 Å = 10^{-10}$ m.

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The polysaccharide staining technique led to electron-dense silver deposits at the following four sites in the c.v.–r.b. complex: the outlines of the membranes, fine threads of amorphous material inside the lumen, the base plate and a thin film surrounding the $CaCO_3$ crystals (figure 4 (*a*), (*e*) and (*f*)). The filiform or amorphous material in the lumen is most conspicuous before and at the earlier stages of coccolith formation and extends into the narrow space that always exists between the vesicle walls and the coccolith *in statu nascendi*. It seems to disappear later in the process (Figure 4 (*f*)) and then the staining of the reticular body becomes less pronounced as well.

Degeneration of the c.v.–r.b. complex

When the mineralization process is interrupted, by Ca^{2+}-depletion of the growth medium or prolonged darkness, successive stages in the degeneration of the c.v.–r.b. complex can be visualized by electron microscopy (figure 4 (*h*), (*i*)). First, the reticular body disintegrates, while the coccolith vesicles retains its original form and its position on the cell nucleus. Finally, the coccolith vesicle is detached from the nucleus and disappears. The reticular body appears to be the least stable structure in the c.v.–r.b. complex.

Naked cells

On prolonged cultivation the cells of *E. huxleyi* may lose the capacity to form coccoliths; they become 'naked' (N-cells). It is unknown whether N-cells play a role in the regular cell

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Description of plate 1

Figure 4. (*a*) Ultra-high resolution transmission micrograph of a portion of lower element of a coccolith (cf. figure 1).

(*b*) Ultra-high resolution transmission micrograph of a portion of the upper, hammer shaped element of a coccolith (cf. figure 1), showing its microcrystalline nature.

(*c*), (*d*), (*e*), (*f*) Electron micrographs of sections through the calcifying apparatus (c.v.–r.b. complex) of *Emiliania huxleyi* at four successive stages of coccolith formation. (*c*), (*e*) and (*f*) are obtained after staining for polysaccharide according to Thiéry (1967); $CaCO_3$ is dissolved; (*d*) is stained with lead after fixation in glutaraldehyde and OsO_4; $CaCO_3$ is preserved.

(*c*) Incipient c.v.–r.b. complex with polysaccharide associated with vesicle walls and in lumen. Note close apposition on the nuclear envelope of the coccolith vesicle (c.v.).

(*d*) A base plate (b.p.) is formed with rhombohedral crystallites of calcite along its margin.

(*e*) The crystals grow out radially, disto-radially and in the median direction; a thin film of organic material covers the surface of the crystal at various sites (one such site is marked with an arrow).

(*f*) The coccolith is nearly finished. The thread-like polysaccharide material in the lumen of the reticular body (r.b.) and in the coccolith vesicle (c.v.) is only visible in restricted environments (like that marked by an open arrow). The organic base plate extends to the tips of the lower elements (*a*), (*b*).

(*g*) Coccolith vacuole – reticular body complex of non-calcifying ('naked') cells. Unlike calcifying cells, the coccolith vesicle is not well attached to the nucleus. Its lumen is narrow and the base plate has an uneven appearance. Staining for polysaccharide is according to Thiéry (1967).

(*h*), (*i*) Electron micrographs of cells of *E. huxleyi* after decalcification with CO_2 and prolonged exposure to darkness (16 h), which suggest degeneration of the c.v.–r.b. complex (i.e. the structures that are responsible for coccolith formation). From physiological experiments it is known that coccolith formation is inhibited in the dark. In (*h*) the reticular body (r.b.) is vanished, but the coccolith vesicle (c.v.) is still at its position on the nuclear envelope. In (*i*) the c.v. is detached from the nuclear envelope (N, nucleus; Chl, chloroplast; M, mitochondrion; G, Golgi apparatus). Staining was made with aminotriazole, added to standard OsO_4 fixative. Post-staining was done with lead (see Van Emburg *et al.* 1983).

Figures (*c*), (*e*), (*f*) and (*g*) are reproduced from Van der Wal *et al.* (1983) by permission from Springer-Verlag.

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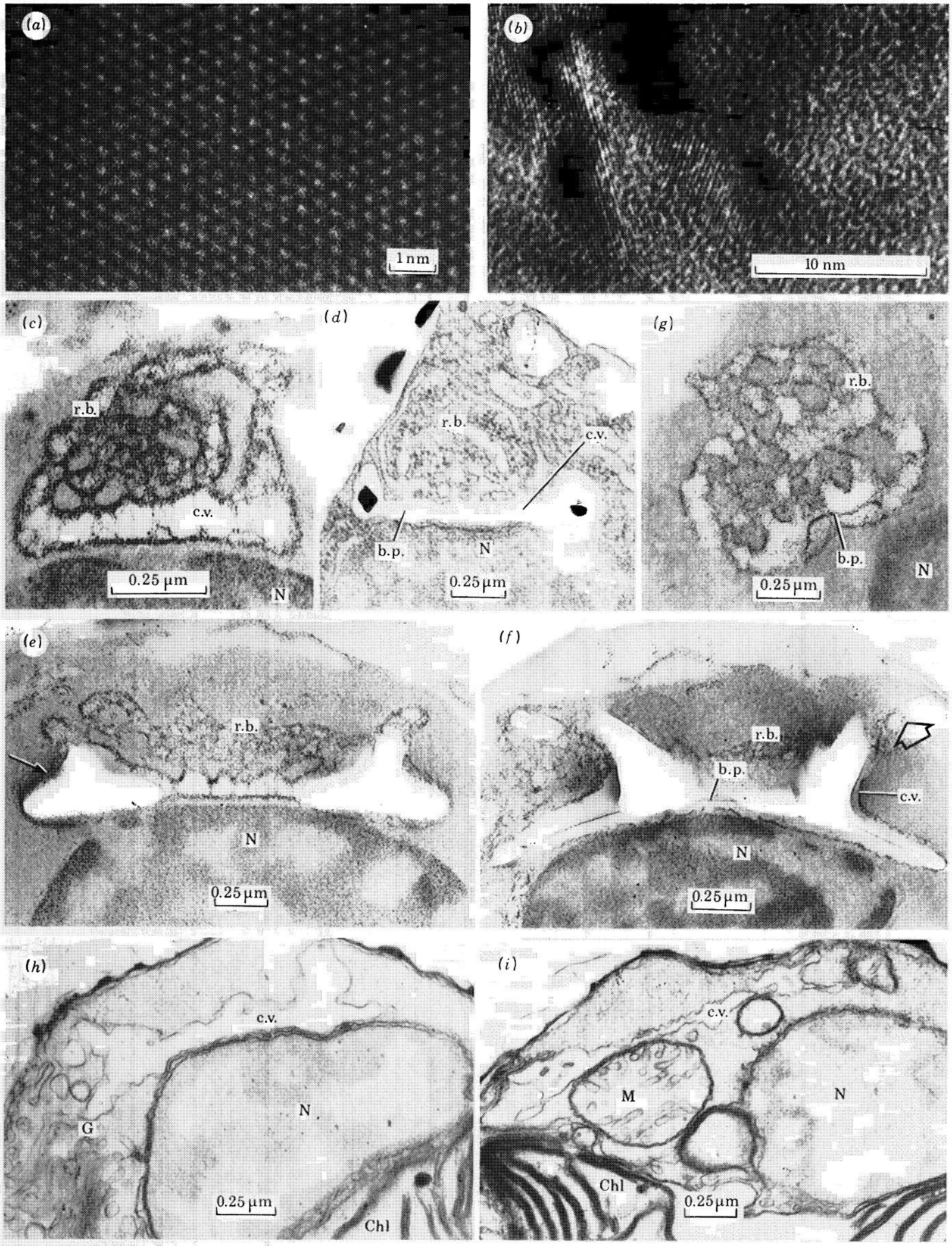

FIGURE 4. For description see opposite.

(*Facing p.* 440)

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cycle of this species, or if they are culture artifacts. The morphologies of naked and coccolith-bearing cells are very similar, but in the former the coccolith vesicle is not well attached to the nucleus. Its lumen remains narrow and the baseplate has a crumbly appearance (figure 4 (*g*)).

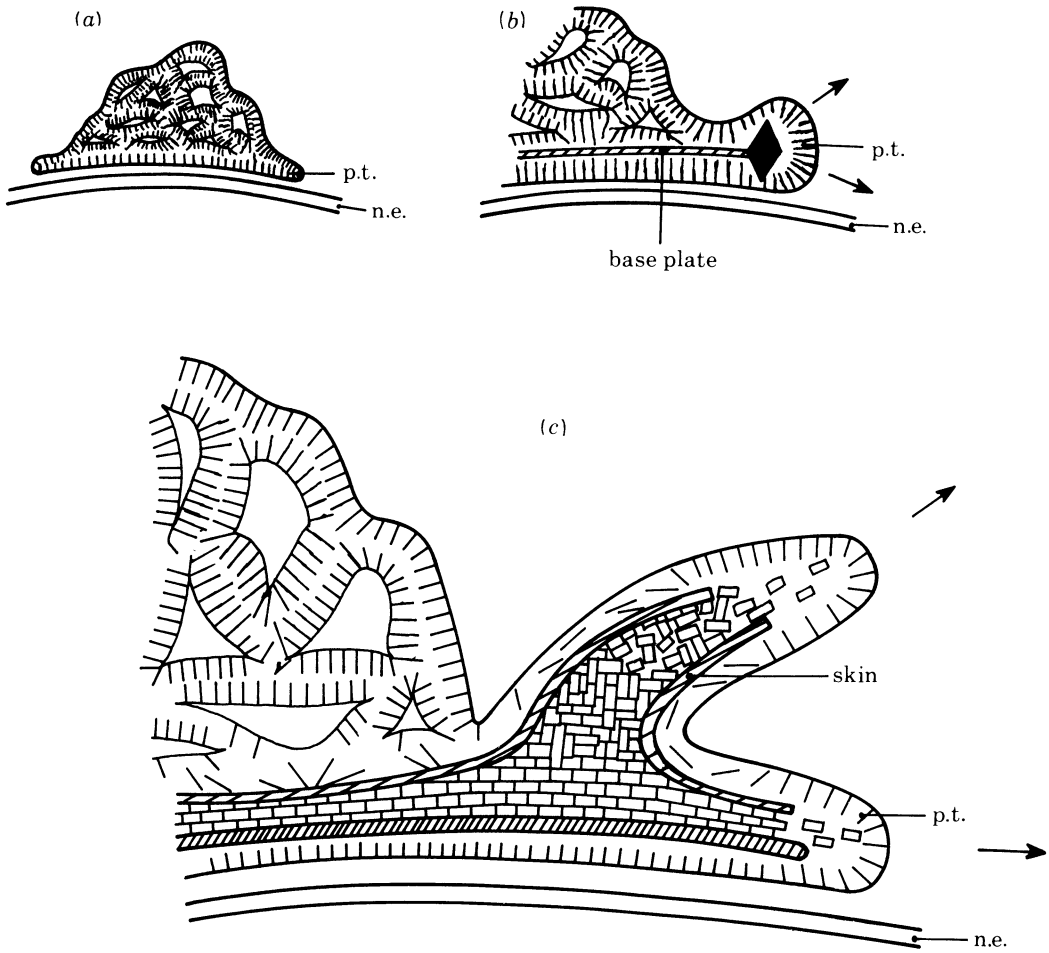

FIGURE 5. Tentative working hypothesis for coccolith biosynthesis. The polysaccharide is supposed to have both an inhibitory and a stimulatory function in crystal nucleation and growth, depending on whether it leaves no space for the development of a nucleus, as in (*a*), or surrounds an 'open' space in a dilated vesicle. Crystal growth is supposed to be terminated when the polysaccharide adheres to the growing crystal surface and is detached from its anchor in the surrounding membrane. The dilation is thought to be caused by pull exerted by the cytoskeleton. For further details see text. Labels: p.t., polysaccharide threads; n.e., nuclear envelope.

WORKING HYPOTHESIS

In an attempt to integrate the data summarized in this paper into a more or less coherent pattern we now propose a working hypothesis for the biosynthesis of coccoliths (figure 5). We start from the assumption that before and at the earlier stages of calcification the polysaccharide molecules are anchored in the membranes of the c.v.–r.b. complex, probably by non-covalent linkage to the positively charged protein, and extend into its lumen. The polysaccharide network is evenly distributed in the lumen of the reticular body and also in that of the c.v. before the

onset of calcification. Even if the c.v.–r.b. complex contains a solution that is supersaturated with respect to $CaCO_3$ no crystallization can take place; the inhibition would result from steric hindrance of the clustering process by the polysaccharide and also from binding of this material to the $CaCO_3$ clusters (figure 5(*a*)).

When alginic acid was coupled to BSA it enhanced the nucleation of calcium carbonate crystals. A similar situation may be created in the coccolith vesicle; when the lumen becomes locally dilated so that an open space is created, crystal formation may be stimulated instead of inhibited by the surrounding anchored polysaccharide (figure 5(*b*)). So, the first crystallites are generated along the rim of the base plate as a result of a dilation of the marginal region of the coccolith vacuole. We suppose that the dilation is caused by pull exerted by the cytoskeleton on the coccolith vacuole. Probably, the orientation as well as the calcite modification of these incipient crystals is determined primarily by a factor associated with the base plate.

Further growth of the coccolith is generated by a coordinated pull of the cytoskeleton on the coccolith vesicle, which results in a dilation of this vacuole in certain well defined directions (figure 5(*c*)). Radial extension leads to coordinated growth of the base plate and the $CaCO_3$. The base plate serves as a substratum for crystal growth and an ordered crystalline layer is formed. Dilation in the disto-radial direction leads to a more disordered crystallization process, because no supporting substratum is available. The polysaccharide then adheres to the surface of the growing crystal, is detached from the membrane and forms a protective cover that inhibits further crystal growth.

At least three different functions are attributed to the polysaccharide in this hypothesis. While it is anchored to the membrane of a narrow vesicle it inhibits crystallization. Where the vesicle becomes dilated it induces crystal formation and growth. And when finally the polysaccharide is detached from the membrane it may cover the crystal surface and inhibit its further growth, in accordance with *in vitro* inhibition studies with free polysaccharide described above. In addition, the polysaccharide may act in inducing crystal agglomeration and in stabilizing the resulting agglomerates, as found in the upper (hammer shaped) elements (figure 4(*b*)) (Bloomen 1983).

The structural integrity of the coccolith vacuole seems to be an essential prerequisite for coccolith formation. The association with the nuclear envelope is particularly important, as evidenced by the aberrant morphology of the c.v. in non-calcifying cells (figure 4(*g*)).

We suggest that during coccolith synthesis, vesicles that are budded off from the trans-side of the Golgi apparatus are added to the reticular body. In its turn this structure donates membranous material, associated polysaccharide and probably Ca^{2+}-ions to the growing coccolith vesicle.

We intend to test the validity of this hypothesis in a series of biochemical and electron microscope experiments, aimed at an inventarization, characterization and localization of the various macromolecular materials involved in coccolith synthesis, with special emphasis on the polysaccharide already under investigation. With immuno-cytochemical techniques, we hope to obtain electron microscope images of the cytoskeleton associated with the c.v.–r.b. complex, thereby revealing its possible role in the calcification process.

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Discussion

S. B. Parker (*Inorganic Chemistry Laboratory, Oxford, U.K.*). The proposed model for calcification does not take into account the presence of polysaccharide within the upper elements. This is important because the high resolution electron micrographs indicate points of nucleation in upper elements (see figure 4 (*b*)), which are not found in the basal plate.

E. W. de Jong. It is true that the model is simplified. Dr Parker is right that polysaccharide may be present within the upper elements, not only because nucleation occurs at several points there, but also because the polysaccharide is expected to stabilize crystal aggregates in the upper elements.

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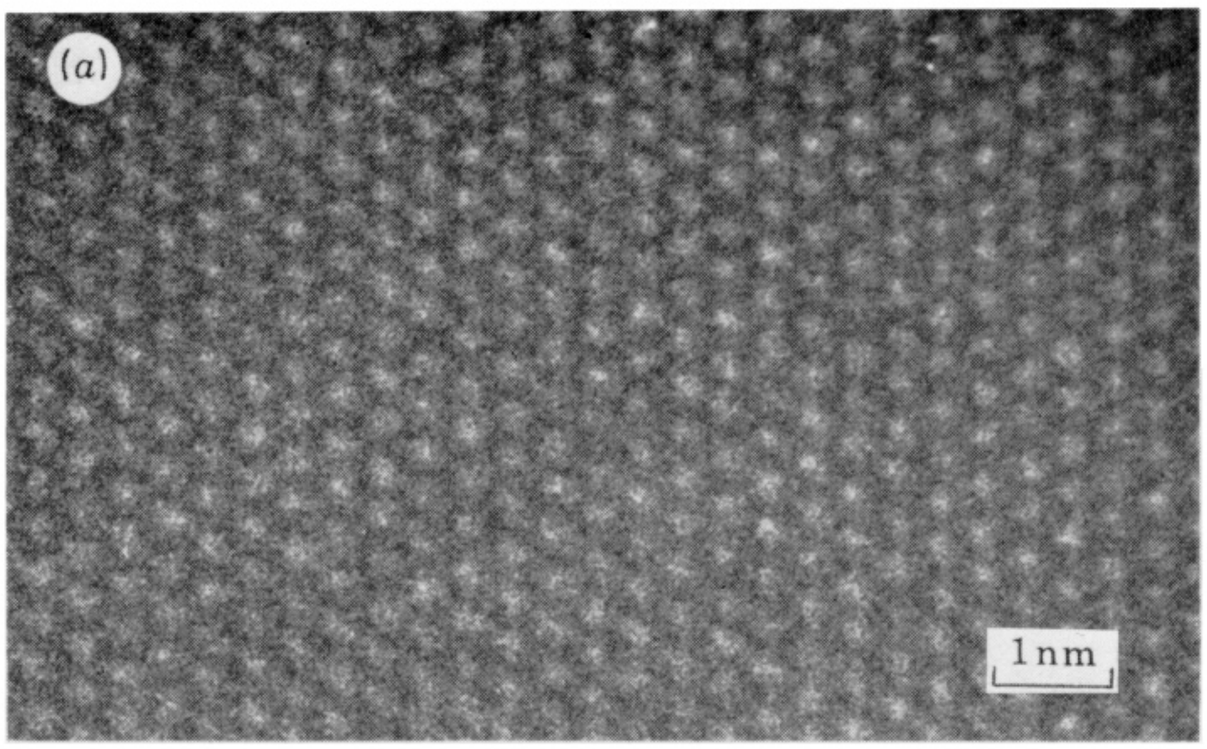

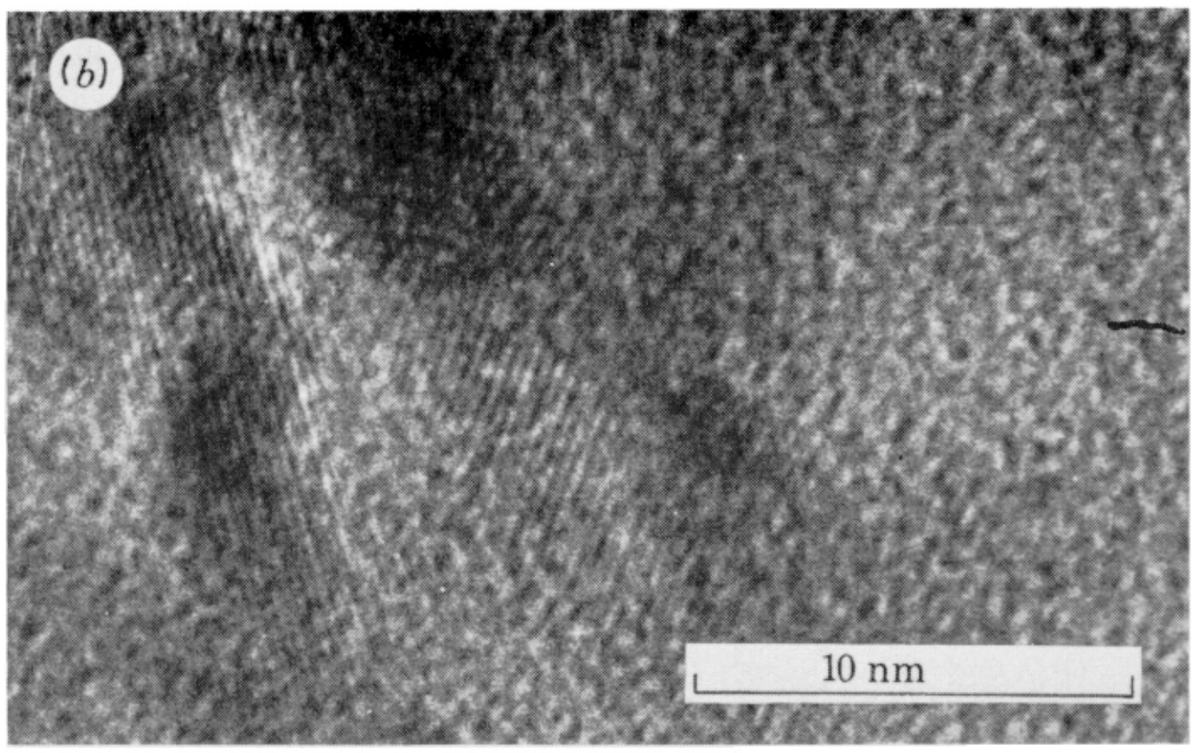

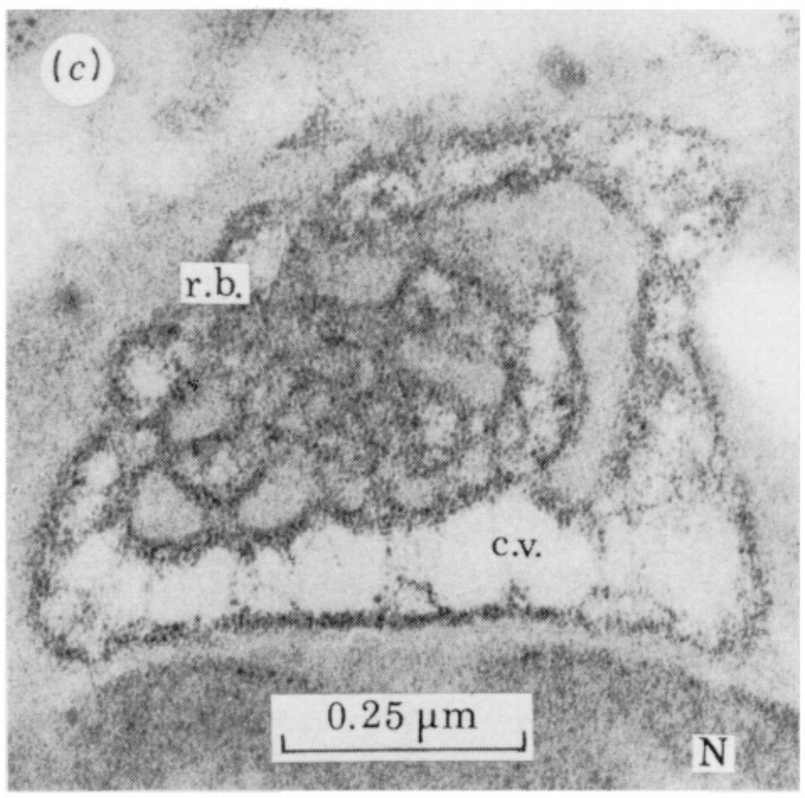

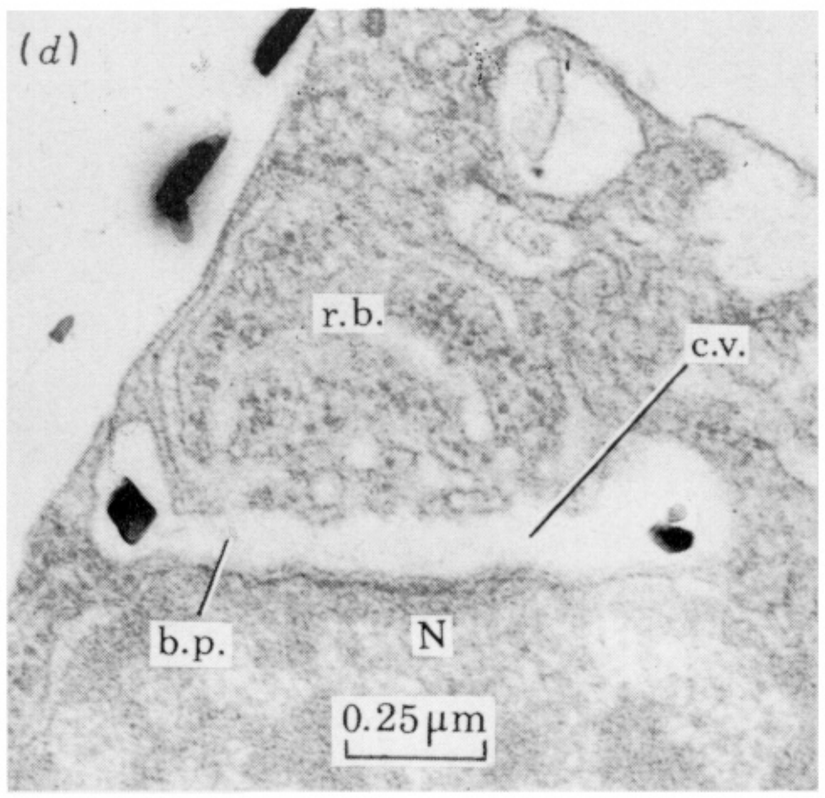

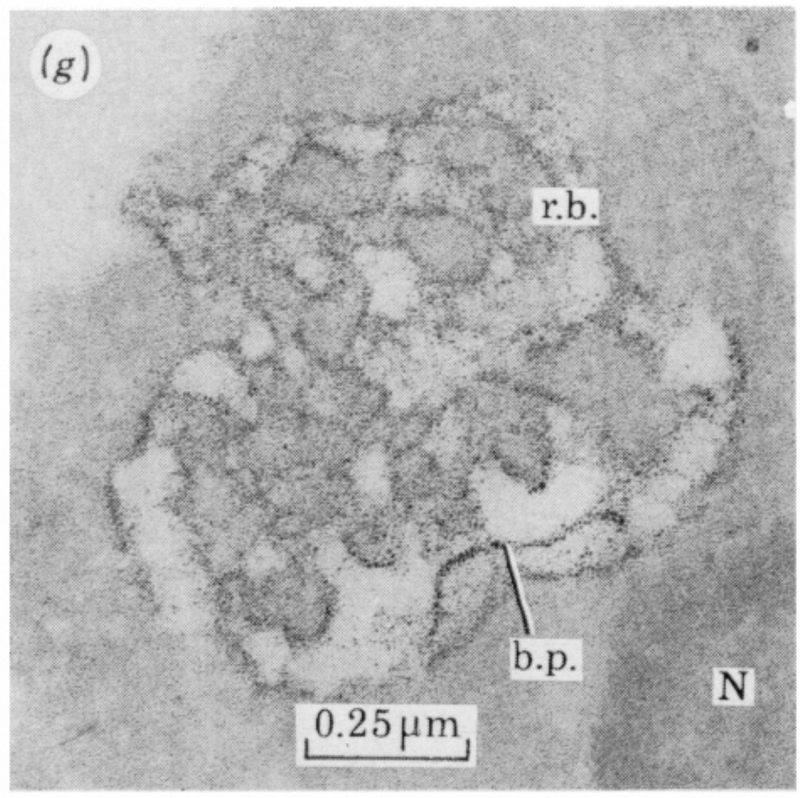

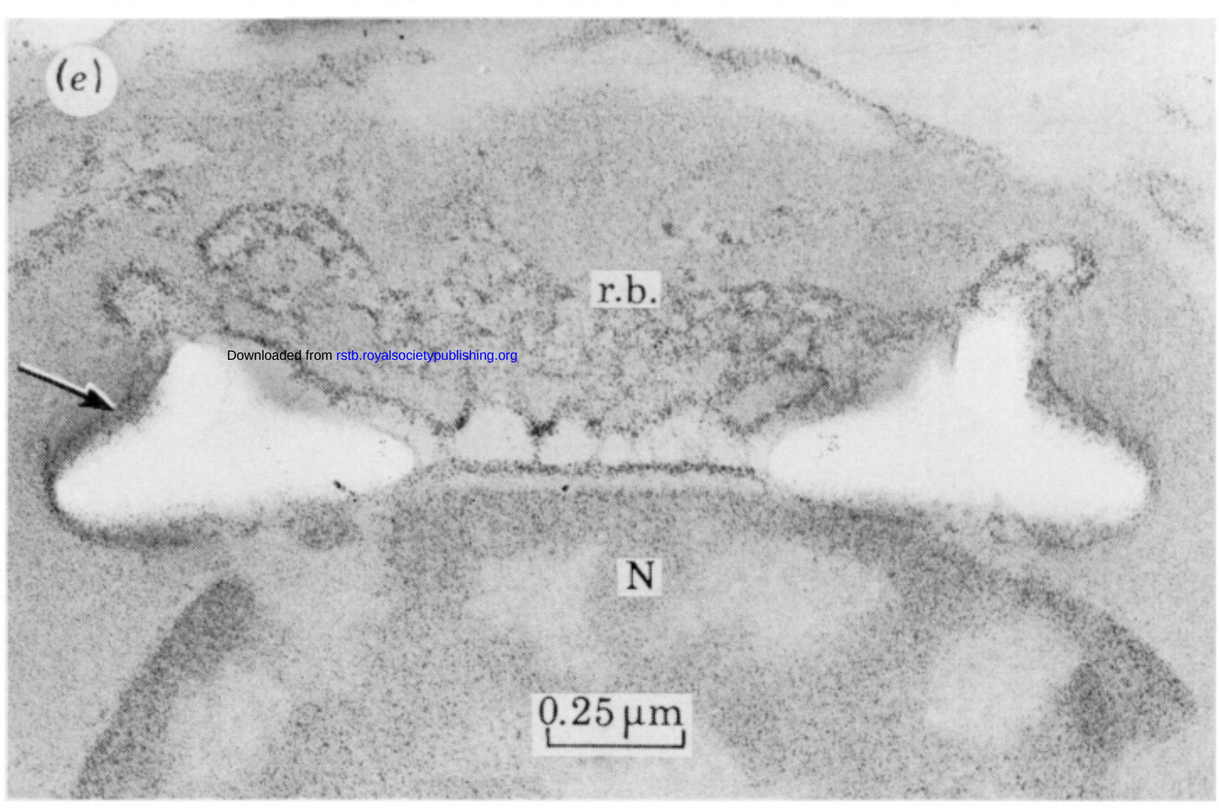

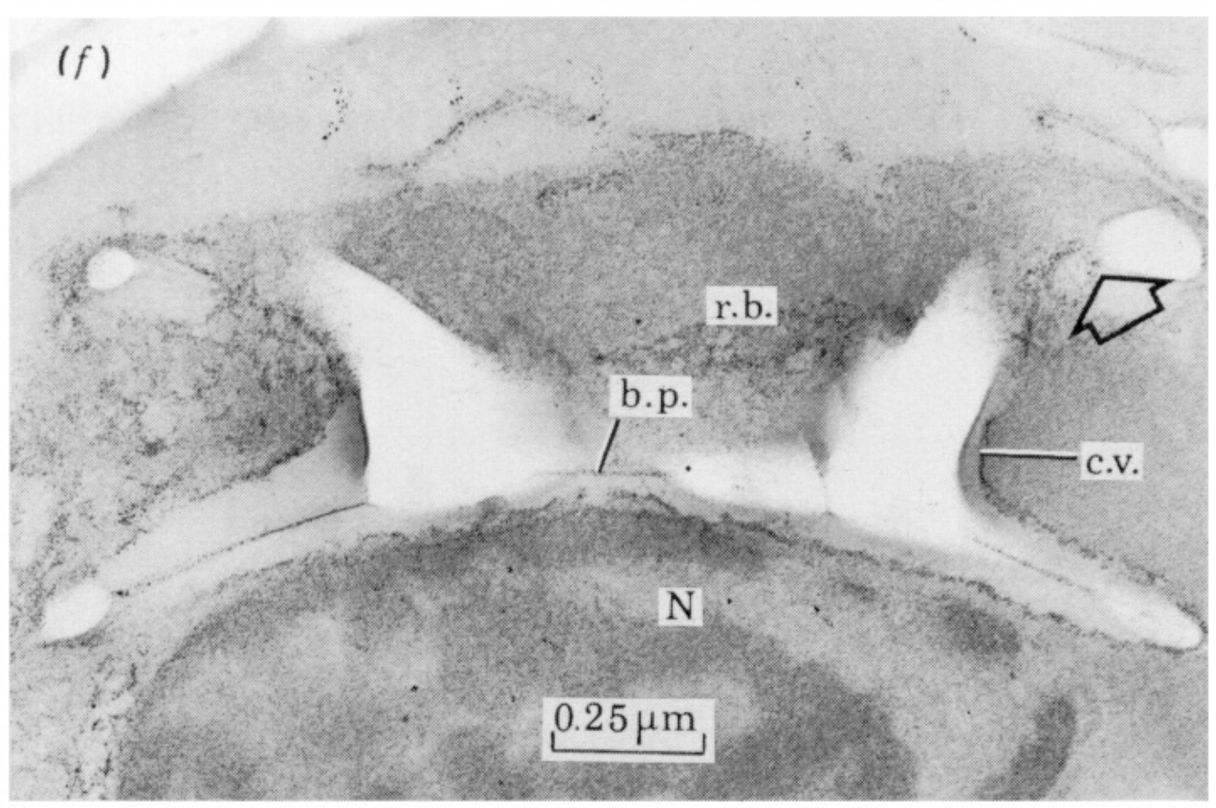

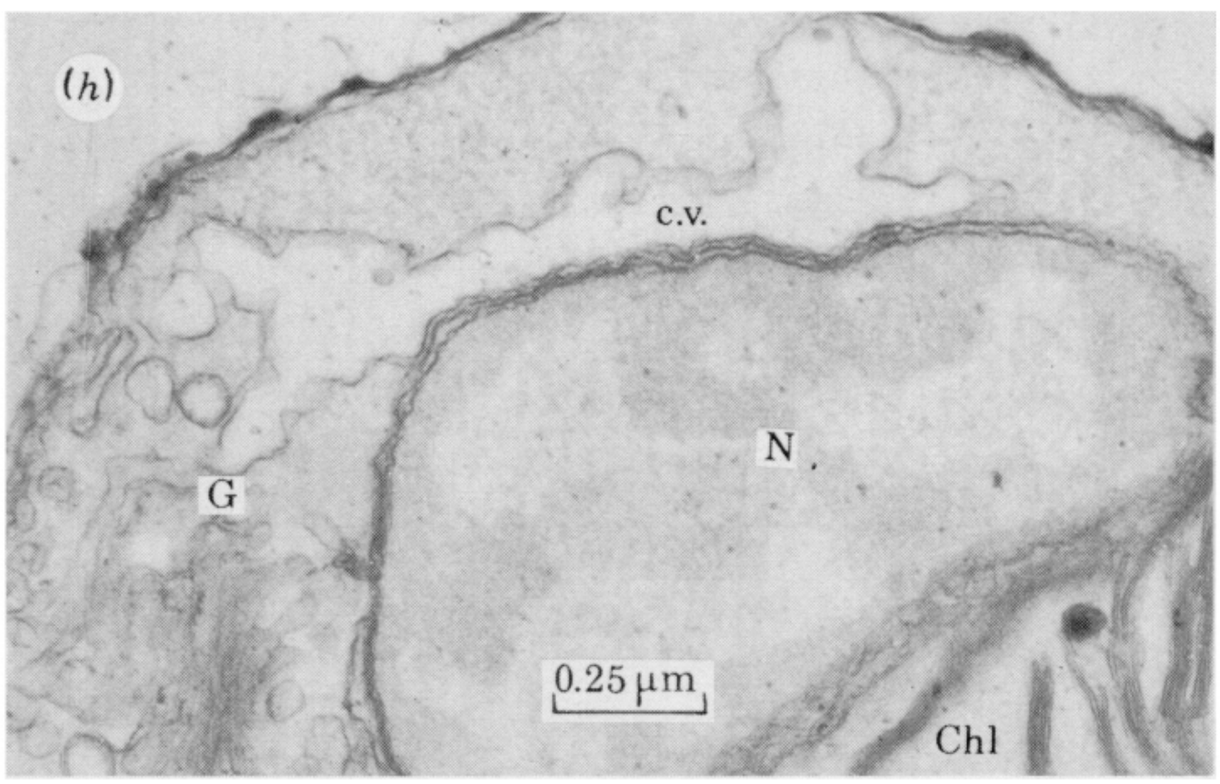

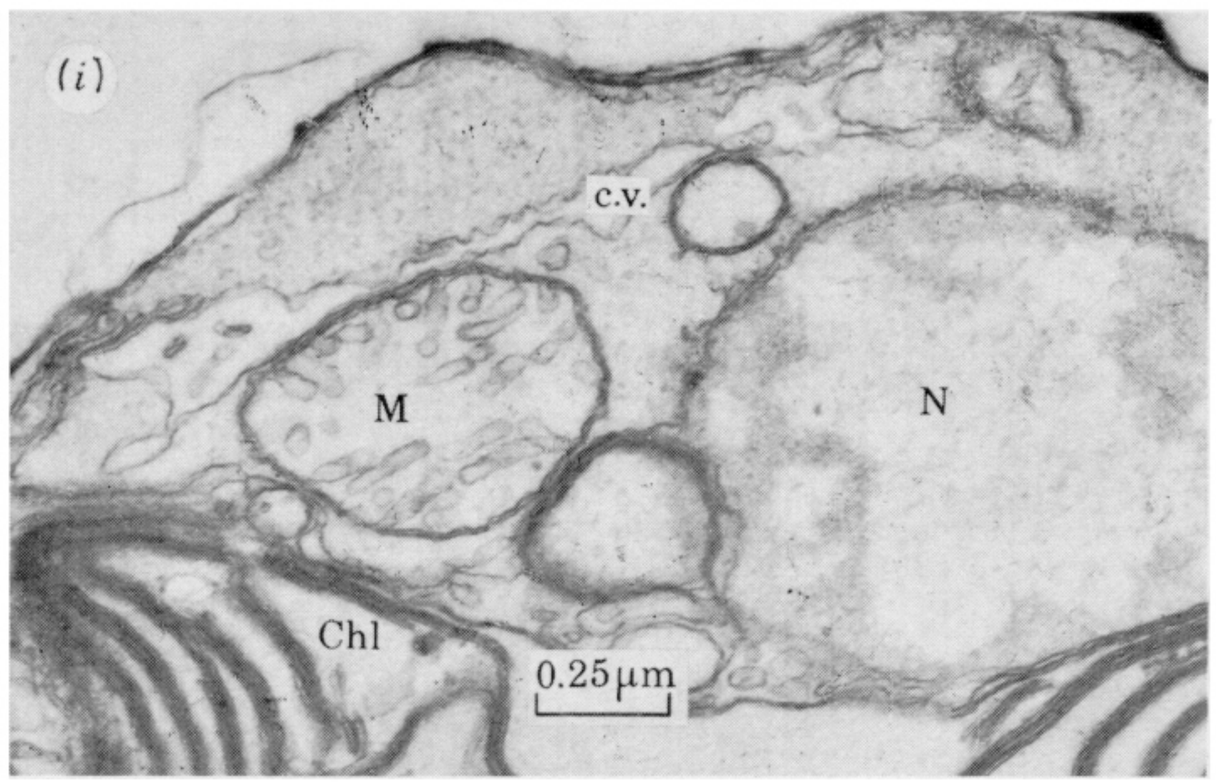

FIGURE 4. For description see opposite.