BIOLOGY AND FINE STRUCTURE OF *EUGLYPHA ROTUNDA* (TESTACEA: PROTOZOA)

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BIOLOGY AND FINE STRUCTURE OF *EUGLYPHA ROTUNDA* (TESTACEA: PROTOZOA)

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SYNOPSIS

Euglypha rotunda a fresh-water, moss and soil-inhabiting testacean has been established in clonal culture with a doubling time of between 33 and 45 hours; it is capable of reproducing in monoxenic culture with the bacterium *Klebsiella aerogenes* (strain NCIB 8017).

Full descriptions are given of the non-crystalline siliceous shell, the vegetative stage and the process of reproduction by simple division.

A detailed account of cytoplasmic fine structure reveals certain features of special interest: subpellicular microtubules; two or three contractile vacuoles each with a surrounding spongiome; a single well-developed Golgi apparatus with associated smooth and coated vesicles, and reserve siliceous plates stacked nearby; a well-defined region of endoplasmic reticulum, with a high concentration of ribosomes, surrounding the nucleus; the presence of a polysaccharide cement or glue binding the siliceous plates of the shell; microfilaments in the pseudopodia; and the occurrence of cross-striated fibrils in the cyst membrane of encysted individuals.

The habit of individuals clumping together to form rosette groups with cytoplasmic connections between them is described; the alignment of masses of microfilaments in these regions to form stress areas or desmosome-like structures is noted.

INTRODUCTION

THE widely distributed, fresh-water, soil- and moss-inhabiting rhizopods belonging to the genus *Euglypha* have attracted little attention by cytologists and protozoologists since an early study of *E. alveolata* by Schewiakoff (1888). Most of the

work on these organisms has been concerned with faunal surveys of testate amoebae ; no study of the cytoplasmic ultrastructure has been reported previously.

The present account concerns the biology and fine structure of cultured specimens of *E. rotunda* Wailes, 1911, isolated from collections made in Battersea Park, London, England.

Previous work – biology

The first general study of the cytoplasm of *Euglypha* was made by Carter (1865) who compared the internal structures of several protozoa. He observed that in *Euglypha* the nucleus normally occupies a posterior position, that union between two individuals is common and that there appear to be two types of structure associated with the division – 'ovules' (globular, nucleated 'cells') between four and fifty in number, and 'spermatozoids' (granules) both of which are situated close to the nucleus. Several authors (Gruber, 1881 ; Blochmann, 1887 ; Schewiakoff, 1888 ; Penard, 1890 ; Popoff, 1912, and Wailes, 1915) have described some aspects of nuclear division or reproduction in *E. alveolata*. Schewiakoff (1888) described and illustrated reproduction and nuclear division in *E. alveolata*. Penard (1890) reported that at conjugation the apertural teeth of two individuals locked together, and suggested that the increase in cytoplasmic volume at division was the result of water uptake. Blochmann (1887) observed copulation resulting in the formation of one extra large daughter cell. The types of reproduction were summarized by Wailes (1915) who suggested that there are three alternative methods : (i) spores which become amoeboid individuals, (ii) budding and (iii) simple division into two animals.

Penard (1890) reported that the pseudopodia of *Euglypha* appear to be stronger than those of naked amoebae and are apparently characteristic for each species. He also considered that the small granules in the body are not excretion granules as previously suggested by Bütschli (1880). The perinuclear cytoplasm was observed to be darkened with Giemsa stain by Popoff (1912). A detailed study of the cytoplasmic inclusions of *E. alveolata* by Hall & Loefer (1930) showed that the number of refractile bodies in the granular zone is approximately inversely proportional to the number of reserve shell-plates ; the authors concluded from this that these refractile bodies might be the origin of the reserve plates.

Penard (1890) reported that at encystment the cytoplasm is contained behind a membrane within the shell and suggested that this is probably resorbed during excystment. He also described the unusual encystment of a large individual, in which numerous small, nucleated cysts could be seen. The same author (1902 ; 1940) described the encystment of *E. brachiata* (*armata*) ; in this species the cyst develops inside a smaller secondary shell which is formed within the parent shell. From a comparison of encystment in several testaceans, Thomas (1962) suggested that the large encysted test formed after copulation enabled the dissemination of the biotypes. In the most recent review of the biology of soil protozoa, Stout & Heal (1967) comment on the ecology and nutrition of some testaceans, including *Euglypha*.

In conclusion it appears that apart from a lot of information on the distribution of *Euglypha* we have a very limited understanding of any aspect of its biology.

Previous work – taxonomy

Several species of *Euglypha* were described by Penard (1890 ; 1902) before the first description of *E. rotunda* by Wailes (in Wailes & Penard, 1911). This species was considered to be distinct from *E. laevis* by the denticulate apertural-plates, the circular aperture and oval shape of the shell ; from *E. strigosa* by its size, unthickened apertural-plates and absence of spines ; from *E. ciliata* by its size and oval aperture and from *E. alveolata* var. *minor* by its shell-plates and oval shape as seen in section through the minor axis. It was recorded again by Wailes (1912) from the Shetland and Orkney Islands, who later (Wailes, 1915) redescribed the apertural-plates. In a review of the *British Freshwater Rhizopoda* fauna, Cash *et al.* (1915) described fourteen species of *Euglypha*, including *E. rotunda*, and recorded some additional localities. Some sizes of *E. rotunda* from soil in Italy were given by Grandori & Grandori (1934). The variation in shapes of apertural-plates was described by Decloitre (1950), who described the shell-plates and gave further data on size. In a later review of the genus, Decloitre (1962) lists descriptions for numerous specimens including *E. rotunda*. Recently, Decloitre (1964) reported upon the variation in size between specimens from Ceylon and Tasmania.

Two recent reports (Cambar *et al.*, 1964 and Mercier *et al.*, 1964) have described the surface ultrastructure of the shell of *E. strigosa* and a *Euglypha* sp., from metal and carbon-shadowed preparations.

The classification of the family Euglyphidae adopted here is that proposed by Loeblich & Tappan (1961) :

Class	*RHIZOPODEA*	Von Siebold, 1845
Subclass	*FILOSIA*	Leidy, 1879
Order	GROMIDA	Claparède & Lachmann, 1859
Superfamily	**EUGLYPHACEA**	Loeblich & Tappan, 1961
Family	**EUGLYPHIDAE**	Wallich, 1864 ;

test hyaline, symmetrical, elongate, composed of rounded siliceous scales, aperture rounded or elongate ; one nucleus.

MATERIAL AND METHODS

Euglypha rotunda was isolated from samples of sphagnum moss, weed and mud collected from ponds in Battersea Park, London, in November, 1967. Initially, rough cultures were obtained by allowing small portions of these samples to stand in a shallow layer of the culture liquid at room temperature, 18–20°C. Agnotobiotic cultures were kept in small plastic containers on a thin layer of agar agar (1 per cent in distilled water), and covered by a shallow layer of culture liquid. A sterilized wheat grain was added to the agar just before it set. The basal culture liquid was a 5 per cent (w/v) solution of soil extract in distilled water, to which

nutrient salts were added so that the final culture medium contained 100 mg/l^{-1} sodium nitrate and 15 mg/l^{-1} sodium dihydrogen orthophosphate.

Single active animals were isolated to produce clonal cultures. Of twenty such cultures started only four reproduced, three within four days and the other after fourteen days. One clone was used subsequently to produce the working cultures and this clone is now registered (No. 1520/1) and maintained at the Culture Centre of Algae and Protozoa, The Natural Environment Research Council, Cambridge, England. The animals which feed and reproduce readily were sub-cultured approximately every four or five weeks.

Cultures made available to Dr H. Netzel (Zoologischen Institut der Universität Tübingen) have been used to produce cine-photomicrographs of locomotion and reproduction (Netzel, 1971).

Optical microscopy

The animals were examined either live, or after fixation, by both phase-contrast and bright-field microscopy. Smears fixed in Schaudinn's fluid were stained with borax carmine and iron haemotoxylin. Clumps of animals for sectioning were fixed in Schaudinn's and embedded in agar (Harris, 1965), the sections being stained with either Mayer's haemalum, toluidine blue or Mallory's triple stain.

Scanning electron microscopy

Specimens removed from the cultures were either cleaned by transferring them directly from the culture vessels through several changes of triple-distilled water or, alternatively, first fixed in osmium textroxide or glutaraldehyde and then washed. They were subsequently pipetted with a minimum of water onto a cover-slip, which had been cleaned in acetone and polished with lint-free tissue, to which they adhere quite well on drying. Single specimens for use in the examination of individual siliceous shell-plates were cleaned by immersion in a slightly acidic solution of hydrogen peroxide (20 vol.) for approximately thirty minutes, prior to washing. Suitably clean animals were then placed individually onto small pieces of cleaned cover-slip. To this a drop of concentrated sulphuric acid was added, and evaporated by gentle heating. This liberates the plates from the organic cement material. The prepared cover-slips were then glued to a 'Stereoscan' specimen stub with 'Silver Dag', an electrically conductive paint, and coated evenly with 10–15 nm of gold in a coating-unit using the apparatus described by Harris *et al.* (1972). Specimens were examined in a Cambridge Stereoscan Mk II at either 15 or 20 kV, and the results recorded on Ilford HP3 35 mm film.

Transmission electron microscopy

The animals were fixed at room temperature for 15 minutes in 1 per cent glutaraldehyde in 0·05 M Sorensen's phosphate buffer with 0·015 M calcium chloride, followed by 10 minutes in 3 per cent glutaraldehyde in the same buffer, and finally post-fixed in 1 per cent osmium tetroxide in 0·1 M Sorensen's phosphate buffer. After rapid dehydration in ethanol the specimens were embedded in Epon 812.

Sections were cut with a Du Pont diamond knife on either a Porter–Blum or Reichert ultramicrotome, and stained with a saturated solution of alcoholic uranyl acetate and Reynold's lead citrate. Some animals for whole-mount examination were placed on Formvar-coated, gold grids in a drop of fresh water. When they had extended their pseudopodia, a Petri dish containing a few drops of 2 per cent osmium textroxide was inverted over the grid for 1–2 minutes. Osmium vapour fixes the animals and pseudopodia instantly. The excess liquid was removed and the grid allowed to dry. Sections mounted on gold grids were used for the detection of periodic acid-reactive carbohydrates, according to the silver methenamine technique of Rambourg (1967). The micrographs were obtained using an A.E.I. EM6B electron microscope operating at 60 kV and recorded on Ilford's ' special lantern contrasty ' plates.

DISTRIBUTION

E. rotunda is commonly found in damp and wet mosses, soil, forest litter and standing water. A full list of locality records and references was recorded by Bonnet & Thomas (1960) and Decloitre (1962 ; 1964).

The following list illustrates the geographically widespread distribution :

EUROPE :	Spitzbergen, England, Scotland, Ireland, France, Belgium, Germany, Hungary and Italy.
NORTH AMERICA :	Greenland, Canada and the United States of America.
SOUTH AMERICA :	Argentina, Colombia, Peru and Venezuela.
AFRICA :	Angola, Morocco and Madagascar.
ASIA :	Ceylon, Java, Sumatra and Tahiti.
AUSTRALASIA :	New Zealand and Tasmania.
ANTARCTICA :	South Georgia.

MORPHOLOGY AND VARIATION

The shell is small, varying in length from 34 to 56 μm and having a breadth of 14 to 24 μm (Pl. 1, fig. A) ; it is elliptical in shape through both the minor and major axes. The aperture is terminal and circular, having a diameter of 6 to 10 μm and is surrounded by between eight to fourteen, evenly spaced, apertural-plates (Pl. 1, fig. D ; Pl. 2, fig. B), although the most frequent number of plates is eight, nine or ten. Each apertural-plate is circular in shape, between 4·5 and 5·5 μm long, 3·6 and 4·5 μm wide, 0·59 and 0·82 μm thick (at its widest point) and carries a large median tooth, with either two or three smaller teeth situated on each side (Pl. 1, figs. C & D). The shell-plates range from 5·0 to 7·3 μm in length, 2·9 to 4·5 μm in width and 0·16 to 0·27 μm thick (Pl. 1, fig. B), and are arranged regularly in alternate longitudinal rows (Pl. 1, fig. A). Shell-plates overlap to a certain extent at the lateral margins and in the posterior region (Pl. 2, fig. C). At the posterior extremity (Pl. 2, fig. D) the shell appears to end abruptly.

An electron probe microanalysis of some shells showed that they had a high silica content and an almost negligible amount of calcium. No evidence of crystalline material was obtained from electron diffraction examination carried out on both sectioned material, and on shells cleaned of most tissue by sodium hypochlorite, separated ultrasonically and washed with distilled water. It is concluded that the shell-plates are composed of a high percentage of amorphous silica.

Variation in structure of the shell appears to be confined to the occasional formation of an extra large individual with more than the normal number of shell-plates. Such forms appear to result from conjugation of a pair of, or possibly more, mature specimens. The process is similar to that described by Blochmann (1887) and Penard (1902 ; 1940), in which it appears that the cytoplasm and plates for two daughter-cells are united to form a single shell. This possibly accounts for the specimens that have extra, unevenly spaced apertural-plates. Nevertheless, these animals appear to have a normal arrangement of shell-plates.

REPRODUCTION

Observations by optical microscopy indicate that the formation of a daughter-cell by means of simple division commences with cytoplasmic movement of the parent to produce a short, thick, pseudopodial trunk. As the apertural-plates of the daughter-cell emerge they are arranged around the outside of the pseudopodial trunk and lie with their teeth opposed to those of the parent. This is followed by rapid movement of the shell-plates from parent to daughter, where they become arranged in a regular pattern. The shell-plates are always added in sequence so that the plates at the terminal region are positioned last. When the plates are in their final position the daughter-cell rocks from side to side. At about this time the first signs of cytoplasmic transfer are seen ; globular vacuoles pass from parent to daughter-cell, and the nucleoles of the nucleus change in density and shape. When the shells are of equal size the granular material of the parent moves to the apertural region and the nucleus becomes indistinct. As the nucleus begins division it is no longer visible due to the rapid movement of granular material between parent and daughter. The plasmalemma and the contractile vacuoles are now visible in the daughter-cell. The rate of cytoplasmic movement then decreases and a nucleus appears in the posterior region of each cell. At the same time a granular band, slightly anterior to each nucleus, becomes evident. There is now a period of apparent inactivity before pseudopodia are extended and independent movement begins. The total time taken for such division is approximately sixty minutes although the shell itself is produced more rapidly, in approximately fifteen minutes.

In order to estimate the length of time required to double the population (doubling time), three identical cultures were established and maintained under similar conditions. Daily counts of the number of animals observed were recorded, and these have been used to produce growth curves (Text-fig. 1) from which the doubling time has been calculated to be between 1·4 to 1·9 days.

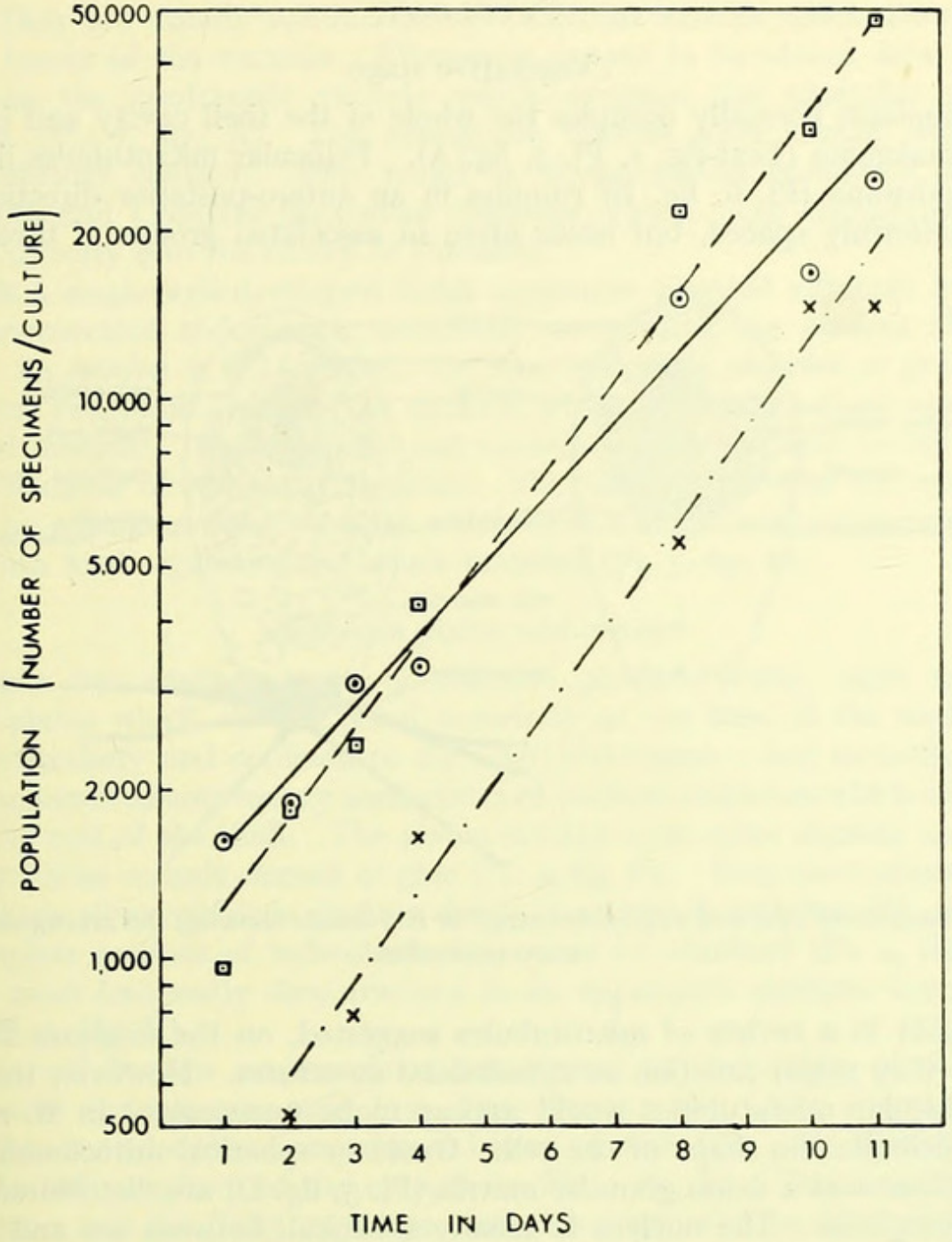

FIG. 1. Growth curves for *E. rotunda* in culture. The symbols represent three replicate cultures through which curves have been fitted by eye. The calculated doubling times are ⊡ – – ⊡ 1·9 days; ⊙——⊙ × 1·4 days and —·— × 1·85 days.

FOOD

Although the cultures in the laboratory were normally grown on a mixed bacterial flora, it is noted that successful cultures can be maintained using a single bacterium as the sole food source. Agar plates, streaked with *Klebsiella aerogenes* (strain NCIB 8017) and covered with the *Euglypha* culture medium, were incubated overnight at 25°C to establish the bacteria. The plates were then inoculated with washed *Euglypha* and incubated in the dark at 20°C. Sub-cultures were made at weekly intervals and under these conditions the *Euglypha* appeared to grow and reproduce well.

CYTOLOGY

Vegetative stage

The cytoplasm normally occupies the whole of the shell cavity and is enclosed by a plasmalemma (Text-fig. 2, Pl. 3, fig. A). Pellicular microtubules lie beneath the plasmalemma (Pl. 6, fig. B) running in an antero-posterior direction. They are not uniformly spaced, but occur often in associated groups of three or four.

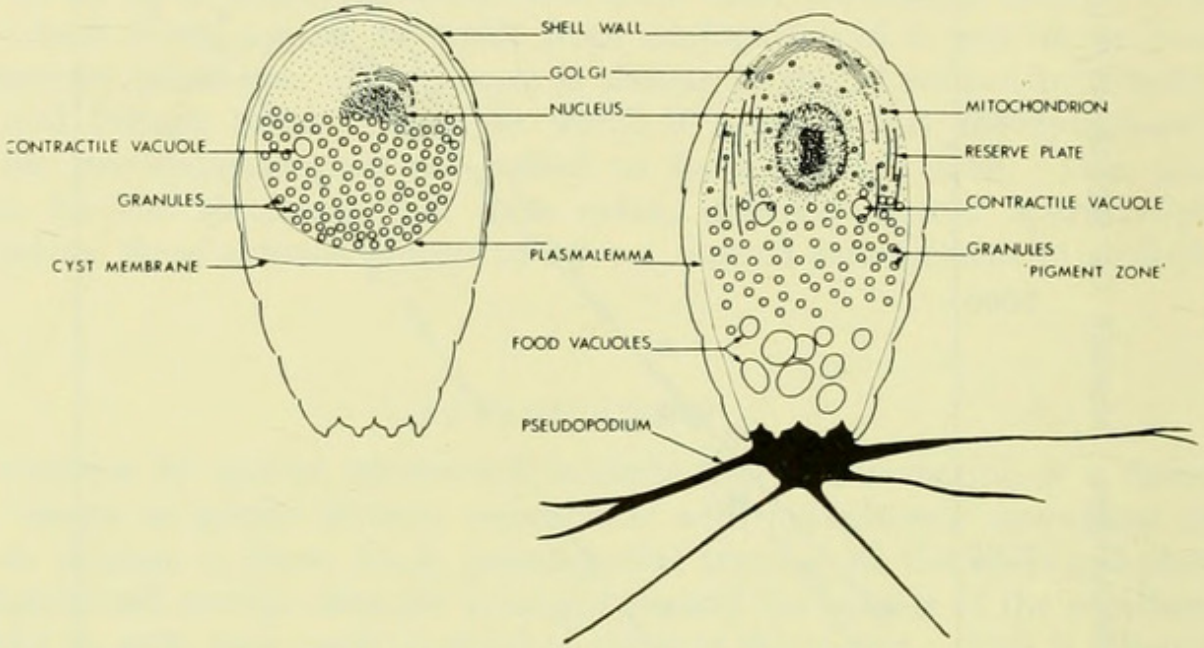

FIG. 2. Diagram of cyst and vegetative stage of *E. rotunda* showing the arrangement of the main organelles.

Porter (1966) in a review of microtubules suggested, on the evidence then available, that they might function as cytoskeletal structures. However, the presence of sub-pellicular microtubules would appear to be unnecessary in *E. rotunda* as the shell delimits the shape of the cell. Ovoid or spherical mitochondria having tubular cristae and a dense granular matrix (Pl. 3, fig. D) are distributed throughout the cytoplasm. The nucleus is usually spherical, between 4·2 and 5·9 μm in diameter, and enclosed within two tripartite membranes (Pl. 3, fig. C). The nuclear matrix is finely granular with small concentrations of chromatin scattered throughout. A prominent and densely stained nucleolus, of variable shape, occupies the centre of the nucleus. Cisternae of granular endoplasmic reticulum arise from the outer membrane of the nuclear envelope and occupy a large compact region around the nucleus (Pl. 3, fig. D). Due to the concentration of ribosomes, this region is more heavily stained than the remainder of the cytoplasm. A region of large vacuoles, usually containing electron-dense material, lies immediately anterior to the endoplasmic reticulum. This electron-dense material appears to be leached out in some preparations and the vacuoles appear empty (Pl. 3, figs. A & D). This region corresponds to the ' pigment zone ' of optical microscope descriptions of some earlier authors (Text-fig. 2). Two or three contractile vacuoles are present at the same level as the large vacuoles, but lie in the endoplasmic reticulum (Pl. 3,

fig. A). They are usually surrounded by numerous vesicles which are continuous with the lumen of the vacuole. Ribosomes appear to be absent from that area surrounding the contractile vacuole which contains the canicular system or spongiome (Pl. 7, fig. A). The spongiome tubules have similar internal and external fibrillar coats to those found in *Acanthamoeba castellanii* (Bowers & Korn, 1968) and *Crithidia fasciculata* (Brooker, 1971). The contractile vacuoles discharge directly into the cavity of the shell.

There is a single well-developed Golgi apparatus situated adjacent to the base of the concentrated endoplasmic reticulum surrounding the nucleus (Pl. 3, figs. A & D). In section it is U-shaped, the base lying in a channel or groove of the endoplasmic reticulum close to the nucleus, whilst the arms extend upwards into the lateral margins. Both smooth and coated vesicles are seen to be associated with the margins of the Golgi saccules. The anterior third of the cytoplasm is occupied by numerous food vacuoles which have a single unit membrane and may contain both food organisms and waste material (Pl. 3, fig. D).

Siliceous plates and cement

There are two distinct types of siliceous plates: firstly, eight to fourteen apertural-plates which are thickened anteriorly at the base of the median tooth, tapered posteriorly and confined to the apertural region; and secondly, approximately one hundred and twenty shell-plates of uniform thickness which are arranged to form the rest of the shell. The plates overlap each other slightly and are held together with an organic cement or glue (Pl. 4, fig. E). They are composed mainly of amorphous silica which is electron-dense in sectioned material (Pl. 4, figs. A & B). Complete sections of individual plates may be obtained (Pl. 4, figs. E & F) although more frequently they fracture in an apparently uniform manner (Pl. 4, fig. D; Pl. 5, fig. H).

Prior to cell-division reserve-plates are formed in the region of the granular vacuoles and endoplasmic reticulum, but close to the Golgi apparatus. Each reserve-plate is enclosed in a membrane-bound vacuole (Pl. 4, fig. F) and is usually situated at the periphery of the cytoplasm (Pl. 4, fig. B). The area between the reserve-plates and the Golgi apparatus contains many vesicles of both the smooth and coated type (Pl. 4, fig. C). Vesicles of a similar nature have been reported to be associated with the Golgi apparatus and scale-containing vesicles in the Haptophyceae – *Prymnesium* and *Chrysochromulina* (Manton, 1966; 1967a, b), and the coccolithophorids – *Coccolithus pelagicus* and *Cricosphaera carterae* (Manton & Leedale, 1969). Although we have no evidence that these vesicles discharge into the reserve-plate vacuoles, their close proximity suggests some correlation. As more plates are formed they become packed in ranks around the nucleus (Pl. 4, fig. A). The reserve apertural-plates are usually found in the most posterior position with their dorsal teeth facing inwards (Pl. 4, fig. D).

Sections of specimens fixed with glutaraldehyde, but without the inclusion of heavy metals in this or subsequent procedures, were examined, in an attempt to locate sites of silica deposition. Apart from the siliceous plates, however, no other electron-dense regions were observed (Pl. 4, figs. A & B).

Previous reports on the formation of siliceous structures in other organisms have dealt mainly with either sponge spicules (Travis *et al.*, 1967 ; Drum, 1968 ; Garrone, 1969 ; Fjerdingstad, 1970) or the diatom frustule (Desikachary & Dweltz, 1961 ; Drum & Pankratz, 1964 ; Lauritis *et al.*, 1968). Such structures are formed in similar membrane-bound vesicles to those described here for *E. rotunda*. The close association of smaller vesicles in *E. rotunda* which could coalesce with the silicon deposition vesicles are also observed by Lauritis *et al.* (1968) in the diatom *Nitzschia alba*. Fine structure studies have shown that sponge spicules in *Spongilla lacustris* and *Haliclona rosea* are formed initially on an axial thread of protein (Fjerdingstad, 1970 ; Garrone, 1969) and an analysis by Desikachary & Dweltz (1961) shows that a small amount of organic material is also present in the diatom frustule. Fjerdingstad (1970) suggests that the silica in the sponge spicule of *Spongilla lacustris* is arranged in parallel ' silica units ' of regular structure. Although we have observed similar ' units ' in *E. rotunda*, we consider that this is only the result of imperfect sectioning of hard material.

Despite many observations on the formation of siliceous structures in plants and animals very little is known of the source of the silicon or of silicon metabolism. The biochemical and physiological problems involved in silicification in organisms are virtually unexplored.

A minimum of organic cement or glue is present to hold the plates in position and maintain the specific shape. The cement is a fine fibrillar material (Pl. 4, fig. E), similar to that found in those membrane-bound vesicles that lie close to the periphery of the cytoplasm (Pl. 5, fig. B), mainly in the anterior body-region (Pl. 5, fig. H). Such vesicles have been found fused with the plasmalemma and discharging their contents into the shell-cavity (Pl. 5, fig. A). The contents of these vesicles stain strongly for polysaccharide (Pl. 5, fig. C), using the silver methenamine technique (Rambourg, 1967), as do the organic cement connections between the plates.

Variation in the composition of these vesicles is sometimes seen, in which the contents have a regular striated structure and occasionally there is a strongly electron-dense area in the polar regions (Pl. 5, figs. D & E). The striations or bands are 12 nm apart and are separated by a fibrillar region 25 nm wide.

Pseudopodia

The filose pseudopodia of *E. rotunda* are usually straight, do not appear to anastomose and radiate from the apertural region (Text-fig. 3 ; Pl. 3, fig. B). Over most of their length the pseudopodia are of uniform thickness, tapering at the extremities. They have a maximum length of 50 μm and a maximum thickness of 2 μm. The animal moves horizontally on glass, by the extension of unattached pseudopodia, which adhere at the tip and then shorten to exert traction. In this way the animal normally moves at approximately 2 μm per second. The pseudopodia are extended quickly and apparently at random, but are also capable of rapid withdrawal. In addition to horizontal movement the animal often moves with the shell held in an upright position above the pseudopodia. In this condition it appears that the tips of the pseudopodia act as feet, being retracted only

slightly before moving and reattachment. Viewed from above the shell progresses with an uneven rolling motion, rather than a smooth gliding action.

The fine structure of the pseudopodia appears to consist of ground-plasm, limited by a membrane, containing microfilaments. Microtubules are apparently absent, although sections through cytoplasmic extensions within the test do contain microtubules. This is consistent with the recent observations on *Difflugiella* sp. in which microtubules were numerous in pseudopodia within the mouth, but extended for only 1–2 μm into pseudopodia 20–30 μm long (Griffin, 1972).

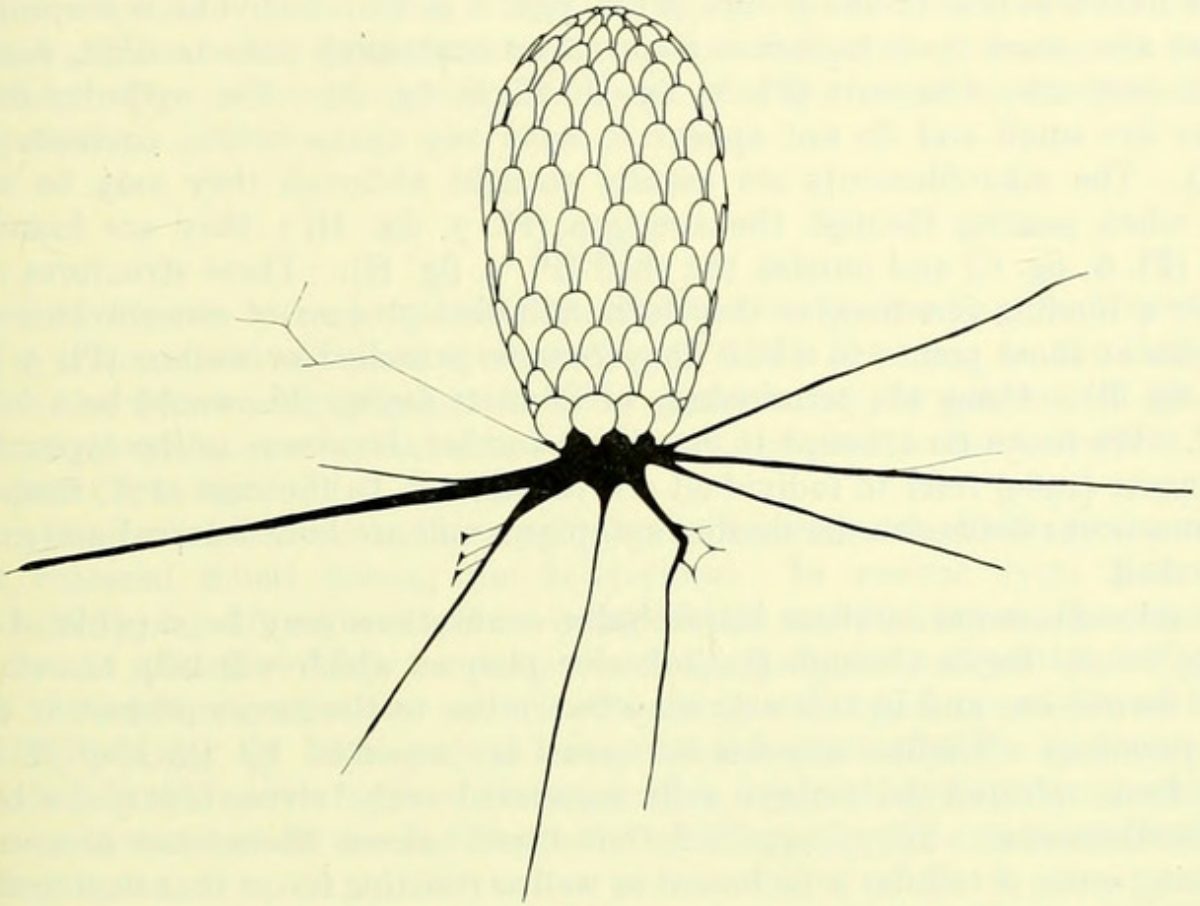

FIG. 3. Diagram of adult *E. rotunda* showing a typical arrangement of filose pseudopodia, drawn from life.

Reports of similar microfilaments have been described from slime moulds (McManus & Roth, 1965) and several amoebae, both naked (Daniels & Breyer, 1967; Pollard *et al.*, 1970; Pollard & Korn, 1971) and with shells (Wohlman & Allen, 1968; Moraczewski, 1970; Griffin, 1972). Microfilaments in the testate amoeba *Difflugia* have been demonstrated by Wohlman & Allen (1968) to be formed during pseudopod extension, and they suggest that these fibrils participate in forcible pseudopod retraction during cell locomotion. In the naked amoeba, *Amoeba proteus*, Pollard & Ito (1970) described thick and thin filaments, the latter being associated with an increase in viscosity, although both are required for movement. More recently they (Pollard & Korn, 1971) found that the thin filaments were indistinguishable from F-actins obtained from muscle (Huxley, 1963),

Acanthamoeba (Pollard *et al.*, 1970) and the slime mould *Physarum* (Nachmias *et al.*, 1970) ; it was concluded that these structures are involved in motility. Using the drug cytochalasin B, Wessels *et al.* (1971) demonstrated that microfilaments are the contractile machinery of non-muscle cells, and suggested that the evidence for this behaviour is overwhelming.

Rosette groups

Some two or three weeks after clonal cultures are established numerous animals appear linked in rosette-like groups (Pl. 2, figs. A & E). Individuals forming these rosettes are joined by cytoplasmic connections containing mitochondria, numerous vesicles and microfilaments (Pl. 2, fig. F, Pl. 6, fig. A). The majority of these vesicles are small and do not appear to have any characteristic contents (Pl. 6, fig. A). The microfilaments are usually straight although they may be seen to curve when passing through the aperture (Pl. 7, fig. H) ; they are found both inside (Pl. 6, fig. C) and outside the shell (Pl. 7, fig. H). These structures appear to have a binding function, as they form adhesion plaques of concentrated microfilaments at those points to which they become attached or anchor (Pl. 5 fig. H, Pl. 6, fig. B). Using the terminology of Bennett (1969) this would be a ' desmosome '. We make no attempt to classify it further, however, as the types defined by Bennett (1969) refer to individual cell junctions. In the case of *E. rotunda* the junctions occur between cells sharing cytoplasm and are both internal and external to the shell.

The microfilaments in these intracellular connections may be capable of transmitting tensile forces through the adhesive plaques which will help to retain the rosette formation, and in this way may be similar to the forces present at desmosome junctions. Similar attachment areas are reported by Buckley & Porter (1967) from cultured rat embryo cells associated with ' stress fibers ' (= bundles of microfilaments). They suggested that these ' stress fibers ' are concerned in stabilizing areas of cellular attachment as well as resisting forces that stretch the cell.

Certain morphological changes have been detected in the cytoplasmic contents of those animals in rosette formation. In the mitochondria the intracristate space becomes enlarged and tubular material appears (Pl. 5, figs. F & G). Similar structures have been reported previously from encysting *Acanthamoeba* by Vickerman (1960, 1962), Bowers & Korn (1969) and more recently from encysting *Bodo caudatus* by Brooker & Ogden (1972). Bowers & Korn (1969) suggest that these features are probably related to starvation rather than encystment. The exocytoic vacuoles are seen at this time to contain reserve-plates in addition to the normal undigested materials (Pl. 6, fig. D).

One unusual feature of these rosette-like groups is that although the evidence suggests either starvation or encystment, bacteria are still seen to be engulfed (Pl. 6, fig. A).

Testate amoeba in which individuals clump together and between which the cytoplasm is connected have been reported previously ; *Arcella vulgaris* (Reynolds, 1939), *Difflugia elegans* (Erth, 1965) and *Difflugiella* sp. (Griffin, 1972). Clumping

without cytoplasmic connections between individuals has also been reported in cultures of other protozoa. Band & Mohrlok (1969) found that the soil amoeba, *Mayorella palestinensis*, formed clumps alone and with another amoeba, *Hartmannella rhysodes*, although the latter species did not clump in pure culture.

Opinions differ over the factors which induce the formation of such groups in amoebae. Reynolds (1939) considers that this behaviour is related to nutritional factors, and observes that when such groups of *Arcella vulgaris* are transferred to fresh culture media or spring water they separate. Band & Mohrlok (1969) suggest that in *Mayorella palestinensis* it is temperature dependent and not associated with starvation and encystation. They found no extra- or intracellular changes on examination at the ultrastructural level, but showed that these cells became interlocked. Our observations would indicate that the cause is either starvation or encystation.

Cyst

Encysted animals occur in cultures usually four or five weeks after inoculation. The cyst is contained within a cyst-membrane which lies close to the shell wall, except in the anterior region where it forms a seal across the aperture of the shell. In mature cysts the membrane retracts so that it lies close to the mid-point region of the shell (Text-fig. 2, Pl. 7, fig. C).

The cyst-membrane consists of amorphous fibrillar material, similar to the organic cement material found joining the body-plates. In mature cysts it becomes striated (Pl. 7, fig. D) with striations being visible mainly at positions where the plates do not join or overlap, for example, at the posterior end of the shell (Pl. 7, fig. G). In addition, striations occur in the thickened region across the middle of the shell (Pl. 7, fig. E). An unusual feature of this portion of the cyst-membrane is that during the early stages of encystment, a layer of apparently unconnected membrane is enclosed in the fibrillar material (Pl. 7, fig. F) and is retained even in mature cysts.

The striated or banded appearance of the cyst-membrane (Pl. 7, fig. G) presumably reflects a fibrous protein composition. The fibrils are cross-striated, with a regular pattern being repeated every 40 nm (= axial period) and within each of these bands two alternating light and dark intraperiod bands of equal thickness are recognized. The limited detail from the available micrographs precludes further comment but it is noteworthy that cross-striated fibrils of the collagen type – not previously known to occur in protozoans – were reported recently in the rhizopod *Haliphysema* (Hedley & Wakefield, 1967).

During encystment the cytoplasmic volume is considerably reduced (Pl. 7, fig. C). There is a reduction in the number of mitochondria and they appear to be found only in the region of the endoplasmic reticulum. The matrix of the endoplasmic reticulum is reduced in size and appears dense compared with the remainder of the cytoplasmic matrix. The electron-dense vacuoles of the ' pigment zone ' are retained (Pl. 7, fig. C) but no reserve-plates are visible. As encystment proceeds the plasmalemma becomes progressively invaginated and the pellicular

microtubules are no longer found close to it (Pl. 7, fig. B). Thin strands of cytoplasm connect the young cyst to the cyst-membrane (Pl. 7, fig. G), while in older cysts, these appear to be confined only to that portion of the cyst nearest to the unattached cyst-membrane. No apparent changes in the Golgi apparatus or the nucleus have been observed.

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NOTE

Since this paper was submitted for publication a brief account of the production of a new daughter-shell by *E. rotunda* is reported by Netzel (1972).

These observations were based on the present authors' original material.

REFERENCES

Band, R. N. & Mohrlok, S. H. 1969. An analysis of clumping in the soil amoeba *Mayorella palestinensis*. *J. Protozool.* **16** : 35–44.

Bennett, H. S. 1969. The cell surface : components and configurations. In *Handbook of Molecular Cytology*, Vol. 15 : 1261–1293. Amsterdam & London.

Blochmann, F. 1887. Zur Kenntnis der Fortpflanzung von *Euglypha alveolata* Duj. *Morph. Jb.* **13** : 173–183.

Bonnet, L. & Thomas, R. 1960. Faune terrestre et d'eau douce des Pyrénées-Orientales. *Thécamoebiens du sol.* Hermann, Paris. 103 pp.

Bowers, B. & Korn, E. D. 1968. The fine structure of *Acanthamoeba castellanii*. I. The trophozoite. *J. Cell Biol.* **39** : 95–111.

Bowers, B. & Korn, E. D. 1969. The fine structure of *Acanthamoeba castellanii* (Neff Strain). II. Encystment. *J. Cell Biol.* **41** : 786–805.

Brooker, B. E. 1971. The fine structure of *Crithidia fasciculata* with special reference to the organelles involved in the ingestion and digestion of protein. *Z. Zellforsch.* **116** : 532–563.

Brooker, B. E. & Ogden, C. G. 1972. Encystment of *Bodo caudatus*. *Protoplasma* **74** : 397–409.

Buckley, I. K. & Porter, K. R. 1967. Cytoplasmic fibrils in living cultured cells. *Protoplasma* **64** : 349–380.

Bütschli, O. 1880. In Bronn's *Klassen und Ordnungen des Tierreichs*. I. *Protozoa* : 1–224. Leipzig & Heidelberg.

Cambar, R., Le Blanc, M., Mercier, M. & Thomas, R. 1964. Étude en microscopie électronique des microstructures superficielles de Protistes fossiles ou vivants. *C. r. Acad. Sci.* (Paris) **258** : 3554–3555.

Carter, H. J. 1865. On the fresh- and salt-water Rhizopoda of England and India. *Ann. Mag. nat. Hist.* (3) **15** : 277–293.

CASH, J., WAILES, G. H. & HOPKINSON, J. 1915. *The British Freshwater Rhizopoda and Heliozoa.* Vol. 3. *Rhizopoda.* The Ray Society London. 156 pp., 24 pls.

DANIELS, E. W. & BRYER, E. P. 1967. Ultrastructure of the giant amoeba *Pelomyxa palustris. J. Protozool.* **14** : 167–179.

DECLOITRE, L. 1950. Études sur les rhizopodes. *Feuille Nat.* **5** : 41–46.

DECLOITRE, L. 1962. Le genre *Euglypha* Dujardin. *Arch. Protistenk.* **106** : 51–100.

DECLOITRE, L. 1964. *Thécamoebiens de la XIIème Expédition Antarctique Française. Territoire des Terres Australes et Antarctique Françaises.* Expéditions Polaires Françaises (Missions Paul-Emile Victor). Publ. No. 259 : 1–47.

DESIKACHARY, T. V. & DWELTZ, N. E. 1961. The chemical composition of the diatom frustule. *Proc. Indian Acad. Sci.* **53 B** : 157–165.

DRUM, R. W. 1968. Electron microscopy of siliceous spicules from the freshwater sponge *Heteromyenia. J. Ultrastruct. Res.* **22** : 12–21.

DRUM, R. W. & PANKRATZ, H. S. 1964. Post mitotic fine structure of *Gomphonema parvulum. J. Ultrastruct. Res.* **10** : 217–223.

ERTH, M. 1965. Zur Kenntnis der Testaceenfauna der slowakischen Reisfelder. *Hydrobiologia* **26** : 13–20.

FJERDINGSTAD, E. J. 1970. Ultrastructure of the spicules of *Spongilla lacustris* (L.). *Symp. zool. Soc. Lond.* No. 25 : 125–133.

GARRONE, R. 1969. Collagène, spongine et squelette minéral chez l'éponge *Haliclona rosea* (O.S.) (Démosponge, Haplosclérides). *J. Microscopie* **8** : 581–598.

GRANDORI, R. & GRANDORI, L. 1934. Prime ricerche sui Protozoi dei terreni e delle acque dell'alta Montagna Alpina. *Boll. Lab. Zool. agr. Bachic R. Ist. sup. agr. Milano* **5** : 1–340.

GRIFFIN, J. L. 1972. Movement, fine structure, and fusion of pseudopods of an enclosed amoeba, *Difflugiella* sp. *J. Cell Sci.* **10** : 563–583.

GRUBER, A. 1881. Der Theilungsvorgang bei *Euglypha alveolata. Z. wiss. Zool.* **35** : 431–439.

HALL, R. P. & LOEFER, J. B. 1930. Studies on *Euglypha.* I. Cytoplasmic inclusions of *Euglypha alveolata. Arch. Protistenk.* **72** : 365–376.

HARRIS, R. H. 1965. Simple technique for embedding and supporting delicate biological specimens. *Nature, Lond.* **208** : 199.

HARRIS, R. H., MARTIN, B. S. & OGDEN, C. G. 1972. Notes on the preparation of natural history specimens for scanning electron microscopy. *Bull. Br. Mus. nat. Hist.* (*Zool.*) **24** : 223–228.

HEDLEY, R. H. & WAKEFIELD, J. ST. J. 1967. A collagen-like sheath in the arenaceous foraminifer *Haliphysema* (Protozoa). *Jl R. microsc. Soc.* **87** : 475–481.

HUXLEY, H. E. 1963. Electron microscope studies on the structure of natural and synthetic protein filaments from striated muscle. *J. molec. Biol.* **7** : 281–308.

LAURITIS, J. A., COOMBS, J. & VOLCANI, B. E. 1968. Studies on the biochemistry and fine structure of silica shell formation in diatoms. IV. Fine structure of the apochlorotic diatom *Nitzschia alba* Lewin and Lewin. *Arch. Mikrobiol.* **62** : 1–16.

LOEBLICH, A. R. & TAPPAN, H. 1961. Suprageneric classification of the Rhizopodea. *J. Paleontology* **35** : 245–330.

MANTON, I. 1966. Observations on scale production in *Prymnesium parvum. J. Cell Sci.* **1** : 375–379.

MANTON, I. 1967a. Further observations on the fine structure of *Chrysochromulina chiton* with special reference to the haptonema, ' peculiar ' Golgi structure and scale production. *J. Cell Sci.* **2** : 265–272.

MANTON, I. 1967b. Further observations on scale formation in *Chrysochromulina chiton. J. Cell Sci.* **2** : 411–418.

MANTON, I. & LEEDALE, G. F. 1969. Observations on the microanatomy of *Coccolithus pelagicus* and *Cricosphaera carterae,* with special reference to the origin and nature of coccoliths and scales. *J. mar. biol. Ass. U.K.* **49** : 1–16.

MCMANUS, M. A. & ROTH, L. E. 1965. Fibrillar differentiation in myxomycete plasmodia. *J. Cell Biol.* **25** : 305–318.

MERCIER, M., LE BLANC, M., THOMAS, R. & CAMBAR, R. 1964. Observations, en microscopie électronique, sur la constitution de la thèque de quelques Euglyphidae (Rhizopodes testacés). *C. r. Acad. Sci.* (Paris) **258** : 5967–5968.

MORACZEWSKI, J. 1970. Quelques observations sur l'ultrastructure du thecamoebien : *Arcella rotundata* Play. *Protistologica* **6** : 353–359.

NACHMIAS, V. T., KESSLER, D. & HUXLEY, H. E. 1970. Electron microscope observations on actomyosin and actin preparations from *Physarum polycephalum*, and on their interaction with heavy meromyosin subfragment I from muscle myosin. *J. molec. Biol.* **50** : 83–90.

NETZEL, H. 1971. *Euglypha rotunda* (Testacea) Bewegung und Fortpflanzung. Film E1642/1971. des Inst. Wiss. Film, Göttingen.

NETZEL, H. 1972. Morphogenese de Gahauses von *Euglypha rotunda* (Rhizopoda, Testacea). *Z. Zellforsch, mikrosk. Anat.* **135** : 63–69.

PENARD, E. 1890. Études sur les Rhizopodes d'eau douce. *Mem. Soc. Phys. Hist. nat. Genève* **31** : 1–230.

PENARD, E. 1902. *Faune Rhizopodique du Bassin du Léman.* Geneva. 700 pp.

PENARD, E. 1940. Protozoaires et psychologie. *Archs Sci. phys. nat.* **22** : 203–226.

POLLARD, T. D. & ITO, S. 1970. Cytoplasmic filaments of *Amoeba proteus*. I. The role of filaments in consistency changes and movement. *J. Cell Biol.* **46** : 267–289.

POLLARD, T. D. & KORN, E. D. 1971. Filaments of *Amoeba proteus*. II. Binding of heavy meromyosin by thin filaments in motile cytoplasmic extracts. *J. Cell Biol.* **48** : 216–219.

POLLARD, T. D., SHELTON, E., WEIHING, R. R. & KORN, E. D. 1970. Ultrastructural characterization of F-actin isolated from *Acanthamoeba castellanii* and identification of cytoplasmic filaments as F-actin by reaction with rabbit heavy meromyosin. *J. molec. Biol.* **50** : 91–97.

POPOFF, M. 1912. Über die geschlechtliche Fortpflanzung von *Euglypha alveolata* Duj. *Arch. Protistenk.* **25** : 8–26.

PORTER, K. R. 1966. Cytoplasmic microtubules and their functions. In *Principles of Biomolecular Organization.* Eds. Wolstenholme, G. E. W. and O'Connor, M., pp. 308–345. London.

RAMBOURG, A. 1967. An improved silver methenamine technique for the detection of periodic acid-reactive complex carbohydrates with the electron microscope. *J. Histochem. Cytochem.* **15** : 409–412.

REYNOLDS, B. D. 1939. Observations on the clumping of Arcellae into plasmodial clumps. *Proc. Va. Acad. Sci.* 1938–39 : 29.

SCHEWIAKOFF, W. 1888. Über die karyokinetische Kerntheilung der *Euglypha alveolata*. *Morph. Jb.* **13** : 193–258.

STOUT, J. D. & HEAL, O. W. 1967. Protozoa. In *Soil Biology*, Chap. 6, pp. 149–195. Academic Press, London & New York.

THOMAS, R. 1962. Kystes et enkystement chez les thécamoebiens. *Bull. Soc. zool. Fr.* **87** : 276–280.

TRAVIS, D. F., FRANÇOIS, C. J., BONAR, L. C. & GLIMCHER, M. J. 1967. Comparative studies of the organic matrices of invertebrate mineralized tissues. *J. Ultrastruct. Res.* **18** : 519–550.

VICKERMAN, K. 1960. Structural changes in mitochondria of *Acanthamoeba* at encystation. *Nature, Lond.* **188** : 248–249.

VICKERMAN, K. 1962. Patterns of cellular organisation in *Limax* amoebae. An electron microscope study. *Exp. Cell Res.* **26** : 497–519.

WAILES, G. H. 1912. Freshwater Rhizopoda from the Hebrides, Orkney and Shetland Islands and Western Scotland ; with description of a new species. *Scott. Nat.* 1912 : 59–65.

WAILES, G. H. 1915. Notes on the structure of tests of freshwater Rhizopoda. *Jl R. microsc. Soc.* **35** : 105–116.

WAILES, G. H. & PENARD, E. 1911. Clare Island Survey. Part 65. Rhizopoda. *Proc. R. Ir. Acad.* **31** : 64 pp., 6 pls.

WESSELLS, N. K., SPOONER, B. S., ASH, J. F., BRADLEY, M. O., LUDUENA, M. A., TAYLOR, E. L., WRENN, J. T. & YAMADA, K. M. 1971. Microfilaments in cellular and developmental processes. *Science* **171** : 135–143.

WOHLMAN, A. & ALLEN, R. D. 1968. Structural organization associated with pseudopod extension and contraction during cell locomotion in *Difflugia*. *J. Cell Sci.* **3** : 105–114.

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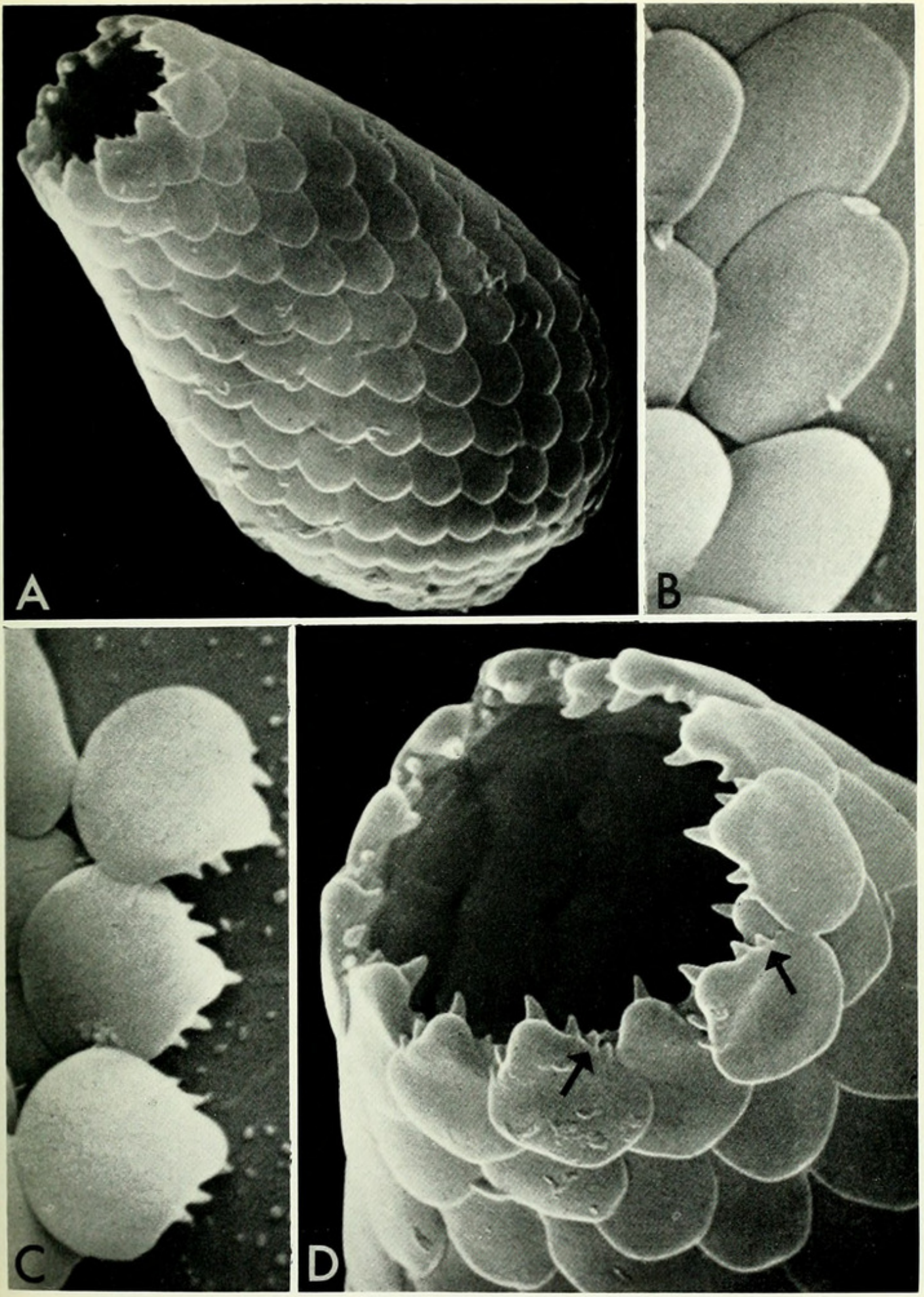
PLATE 1

A. Lateral view of *E. rotunda* showing the arrangement of shell-plates. × 2,300
B. Individual shell-plates. × 7,500
C. Lateral view of apertural-plates with two lateral teeth. × 7,500
D. Aperture of *E. rotunda* with fourteen apertural-plates ; note the thickened region associated with the median tooth and some plates (arrowed) with three lateral teeth. × 5,900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D

PLATE 2

A. Light micrograph of animals stained with eosin and Mayer's haemalum. × 250
B. Specimen with ten, evenly spaced apertural-plates. × 6,650
C. View of posterior region showing the arrangement of plates and lateral flattening of shell. × 2,850
D. Lateral view of posterior region showing the overlap of plates and blunt termination. × 4,280
E. Group of six animals in ' rosette ' formation. × 675
F. Cytoplasmic connections of ' rosette ' formation. × 5,000

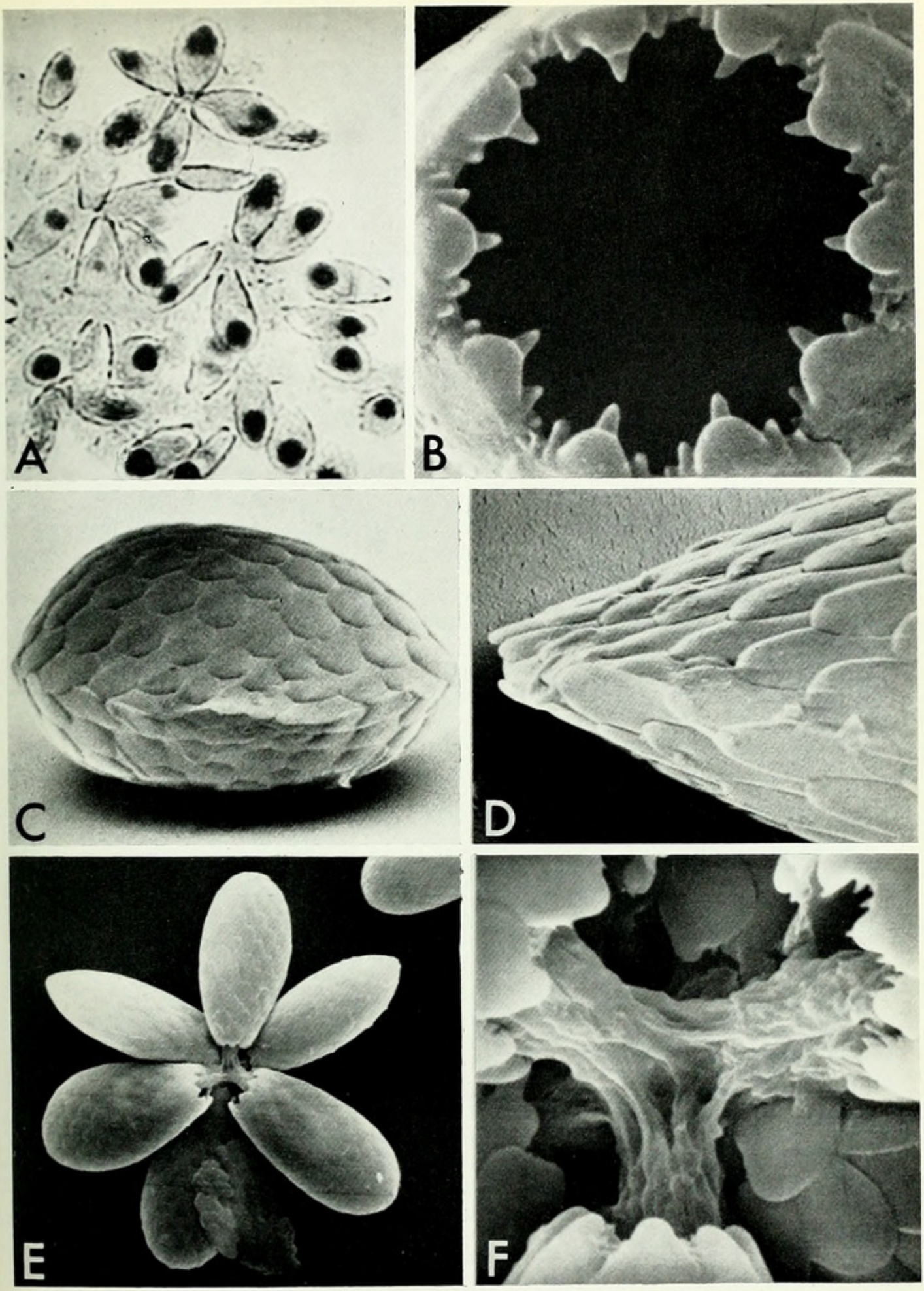
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PLATE 3

A. Longitudinal section showing the position of contractile vacuole (cv), Golgi apparatus (G) and endoplasmic reticulum (er) ; note the structureless appearance of the ' pigment zone '. × 5,760

B. Direct preparation of whole animal showing the arrangement of pseudopodia. × 1,400

C. Transverse section of normal nucleus, with prominent electron-dense central nucleolus. × 8,640

D. Longitudinal section of an unusually shaped nucleus (n) lying in the dense endoplasmic reticulum (er) ; Golgi apparatus (G). × 4,320

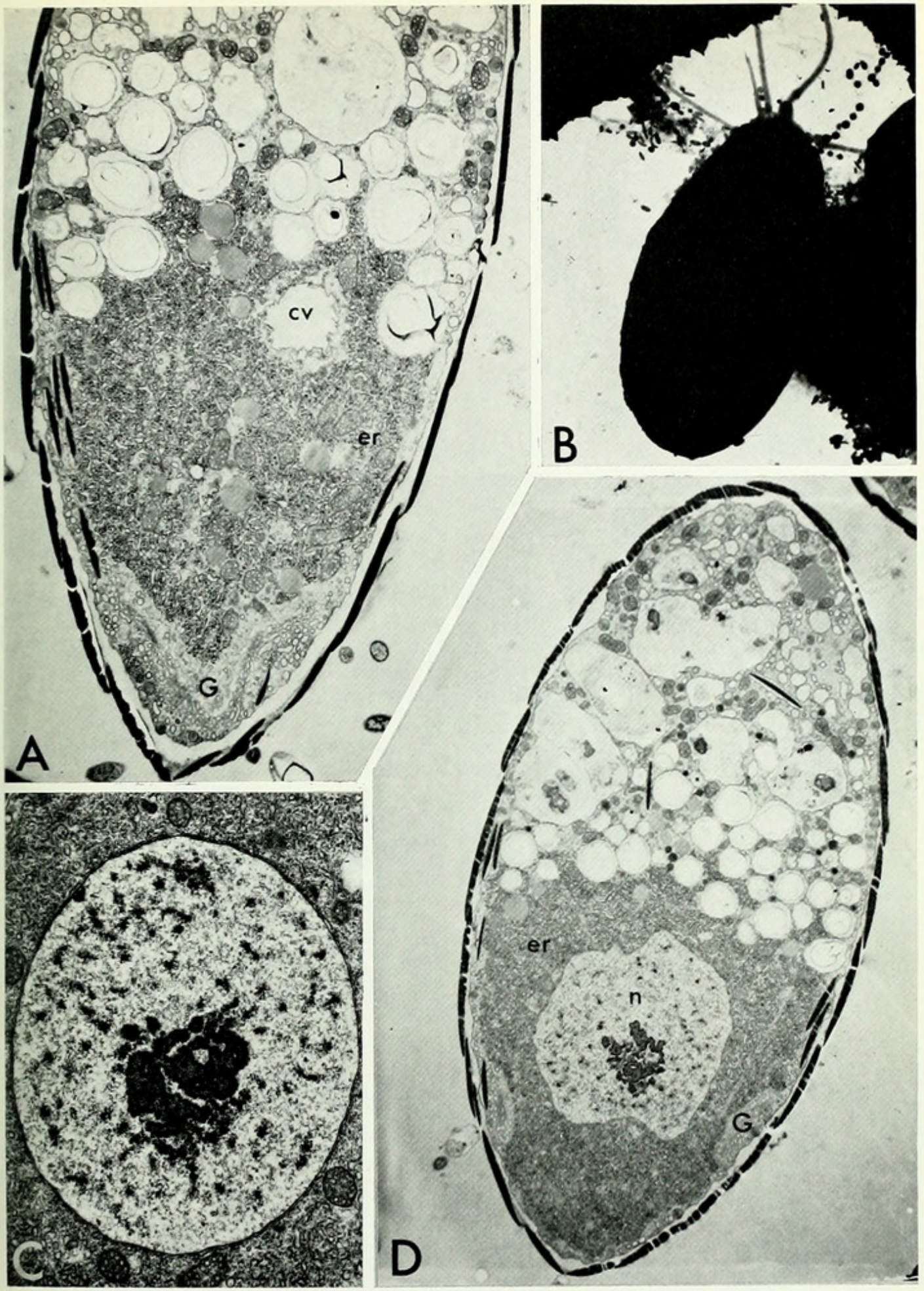
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PLATE 4

A. Glutaraldehyde fixed, unstained longitudinal section to illustrate the arrangements of siliceous plates in nuclear region ; note that the ' pigment zone ' does not contain electron-dense material. × 4,200

B. Glutaraldehyde fixed, unstained transverse section showing the peripheral arrangement of reserve plates. × 4,200

C. Section through part of Golgi apparatus, bottom left, showing proximity of coated vesicles (cves) and reserve shell-plate. × 44,400

D. Section showing a reserve apertural-plate in close proximity to the Golgi apparatus (G) ; note fracture lines apparently caused by sectioning procedures. × 15,570

E. Siliceous plate showing the associated amorphous (?) organic cement. × 28,350

F. Reserve shell-plate in membrane-bound vesicle. × 56,700

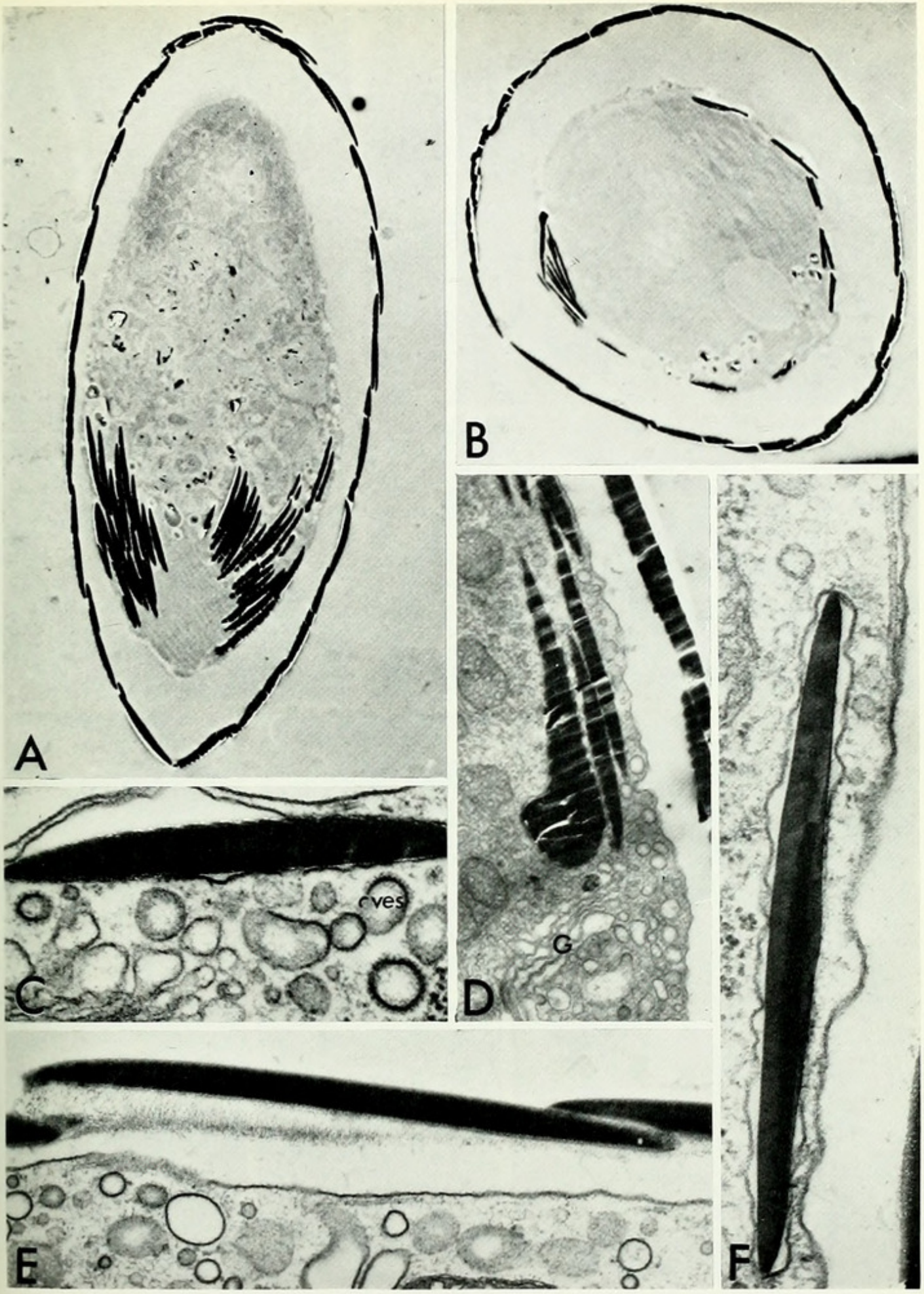
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PLATE 5

A. Vesicle containing organic cement discharging into shell cavity. × 37,800
B. Vesicles containing cement material lying adjacent to plasmalemma. × 56,700
C. Section stained with silver methenamine, showing positive reaction by organic cement vesicles for polysaccharide. × 7,780
D. Cement body showing regularly striated structure. × 60,400
E. Banded organic cement body with dense polar staining. × 56,700
F. & G. Examples of mitochondrial degeneration from ' rosette ' specimens. × 28,350
H. Section through apertural region of a specimen from a ' rosette ' group showing position of cement containing vesicles ; note the concentration of microfilaments (arrowed). × 5,760

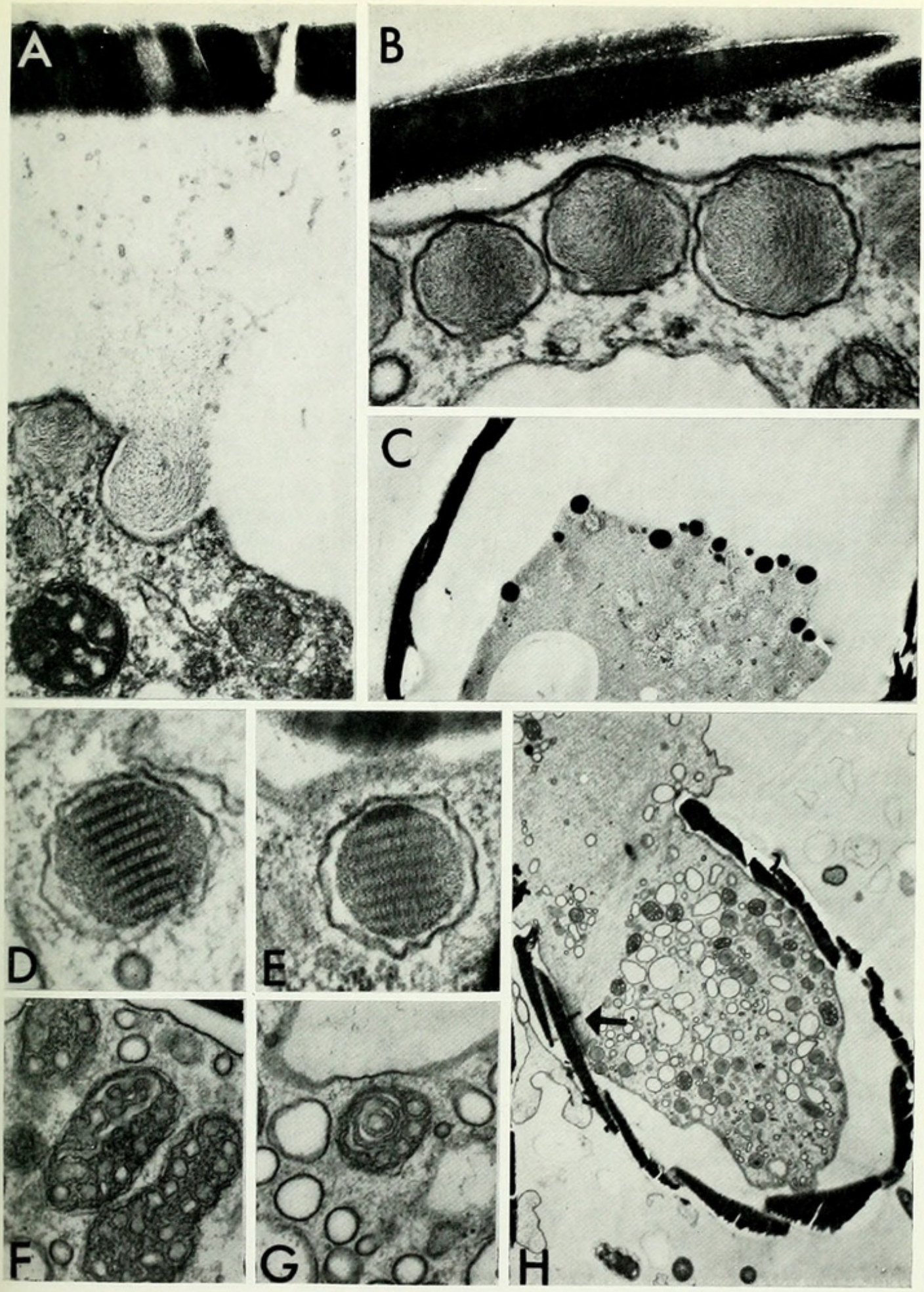
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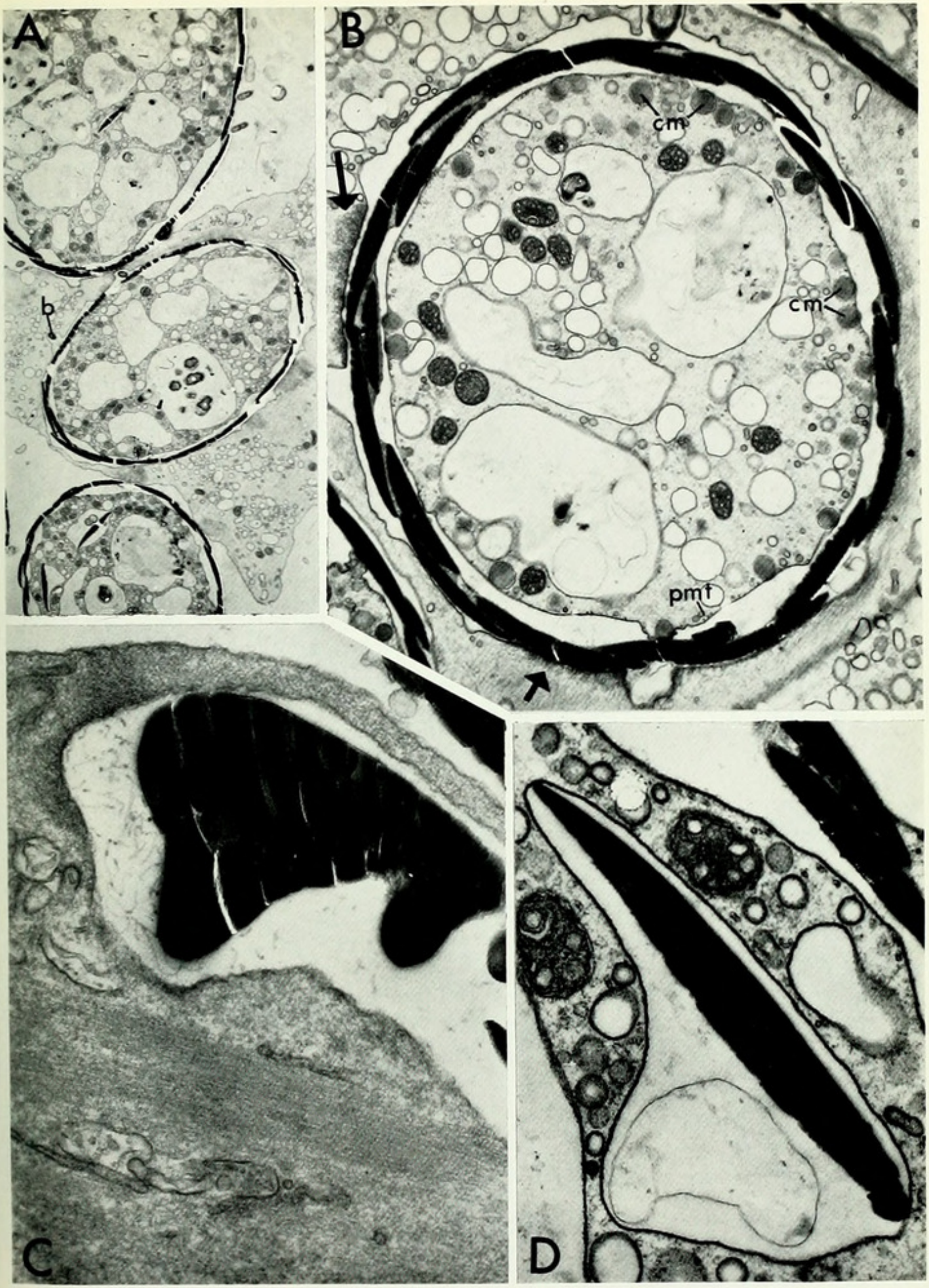
PLATE 6

Sections from specimens joined in ' rosette ' formation : A. Section through group of animals showing the extent to which cytoplasm protrudes beyond the shell ; note the bacteria (b) being engulfed. × 2,800. B. Transverse section showing the position of pellicular microtubules (pmt) and cement-containing bodies (cm) ; the arrows indicate concentrations of microfilaments. × 7,780. C. Section through apertural region showing microfilaments oriented in straight lines. × 30,090. D. Section of exocytoic vesicle containing siliceous shell. × 30,090.


A
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cm
cm
pmf
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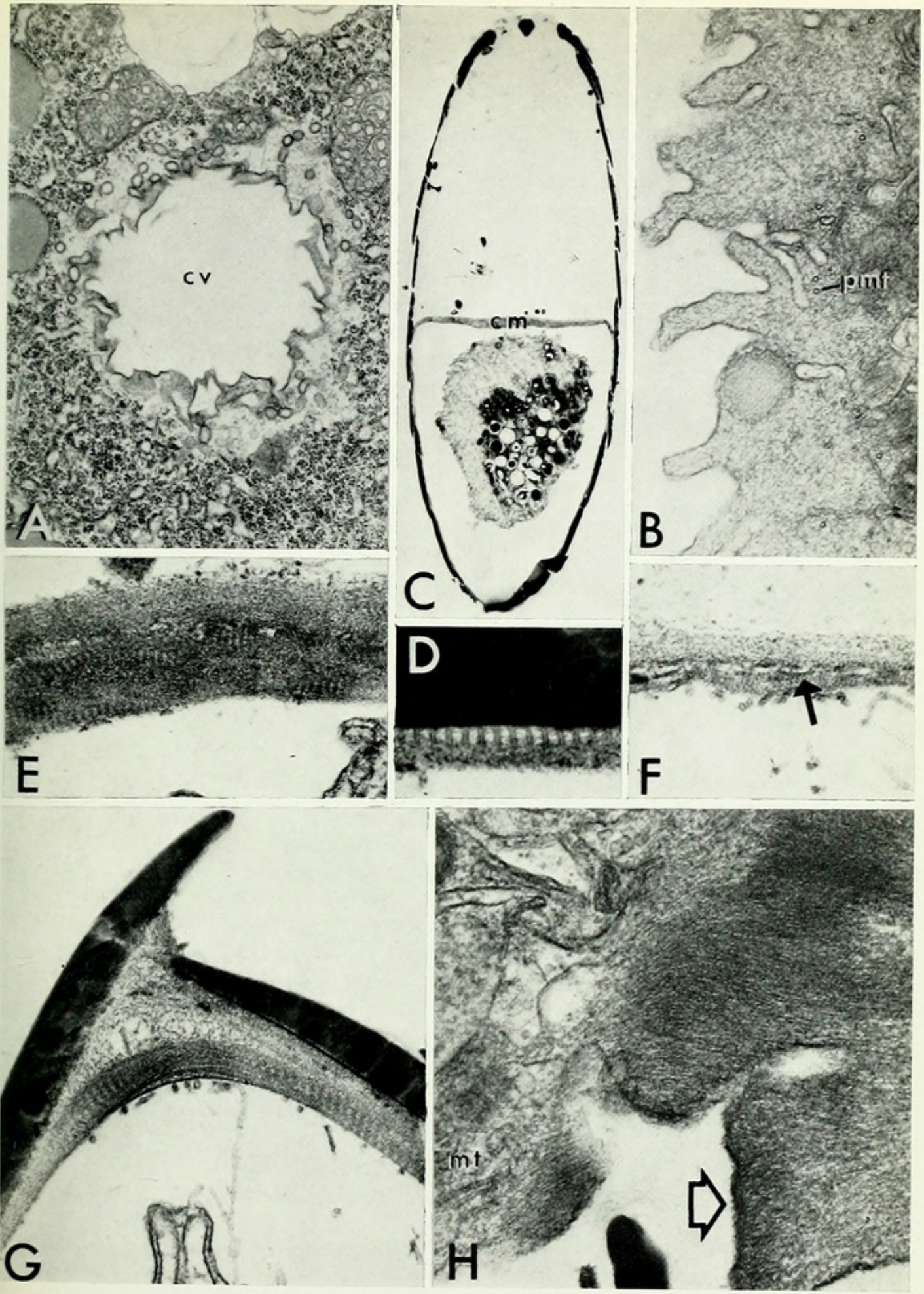
PLATE 7

A. Section through contractile vacuole (cv) and spongiome. × 18,900
B. Section of cyst plasmalemma showing invaginations and pellicular microtubules (pmt). × 29,700
C. Longitudinal section showing position of cyst membrane (cm) and cytoplasm. × 2,800
D. Striated cyst membrane on inside of shell-plate. × 55,200
E. Section through mature cyst membrane showing striated structure. × 30,090
F. Section through early cyst membrane showing unconnected membrane (arrowed). × 56,700
G. Junction of shell-plates at posterior of animal with unusually large amount of organic cement behind the plates and cyst membrane; note the striated cyst membrane. × 37,800
H. Microfilaments curving around apertural-plate; note the internal microtubules (mt) and concentration of microfilaments at the external surface of the plate (arrowed). × 37,800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