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MORPHOLOGICAL AND CULTURAL STUDIES OF A MOTILE STAGE IN THE LIFE CYCLE OF *DERMOCYSTIDIUM MARINUM* [1, 2]

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ABSTRACT

Dermocystidium marinum *hypnospores, obtained by exposure to thioglycollate (the Ray technique), were isolated from oyster tissue and placed in sea water under aerobic conditions. The cells differentiated into sporangia, and sporulation occurred by successive bipartition of the protoplast, resulting in the formation of motile cells within the hypnospore wall. The planonts escaped through one or two preformed discharge pores and tubes. All motile cells were biflagellated with both flagella attached laterally and subapically. The anterior flagellum had* Flimmern *and the posterior flagellum was a whiplash. The planont cell body was intermediate between reniform and pyriform. Sporulation morphology is discussed from light microscope studies. Infection of oyster organ and tissue explants was accomplished with motile cells from single sporangia and from populations of sporangia. Living oysters were infected with the hypnospore isolates, but it was not determined which cell type gave rise to the infections — hypnospores, prehypnospores, or planonts.*

INTRODUCTION

The literature on *Dermocystidium marinum* has been voluminous since the initial description by Mackin, Owen, and Collier (1950). Excellent reviews by Johnson and Sparrow (1961) and by Mackin (1962) summarize most of the literature.

The assay method of Ray (1952) has been of great value in determining the incidence and intensity of *D. marinum* infections, but, in addition, has raised the question of the role of the hypnospores in the life cycle. Hypnospores were found in heavily infected oysters and oyster explants in sea water (Ray, 1954a, 1954b; Mackin, 1962); therefore, it was suggested that the response to thioglycollate medium was normal but exaggerated (Mackin, 1962). Mackin and Boswell (1956) suggested that the hypnospores develop and release aplanospores which could act as infective elements if ingested by oysters and/or phagocytized by oyster amoebocytes. However, the hypnospores were never observed to produce aplanospores.

This study demonstrates that hypnospores develop into sporangia which, in turn, develop planonts. The term sporangium is used in its general sense to include asexual structures (Starr, 1955), because it was not demonstrated whether the planonts were isogametes or zoospores or either. A morphological description of the sporulation process is given and the motile cells are shown to be capable of infecting oyster explants.

METHODS

Dermocystidium marinum cells were induced to differentiate into hypnospores by the Ray technique (Ray, 1952). Infected oyster tissues were held for 48 hours in fluid thioglycollate medium (F. T. M.) then digested for 6-8 hours in 0.25 per cent trypsin (1:250; NBCo), prepared in Cameron's

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sea water (Cameron, 1950). The artifical sea water was equivalent to 32 ppt salinity and was adjusted to a pH of 7.8 to 7.9.

A magnetic stirrer was used to agitate the trypsin preparation. During digestion, temperatures rose no higher than 32°C. This procedure separated all tissues into individual cells or into small clumps of cells around 0.5 mm^3 or less (with the exception of gills and associated gill bars).

After trypsinization, the suspension was poured through six to eight thicknesses of cheesecloth to remove the larger clumps of cells and tissue debris. The suspension was then centrifuged at 340 x *g* for two minutes. Four washings were made in each of three conical, 15 ml centrifuge tubes. The fungus cells were resuspended each time with Cameron's sea water.

Following the washing procedure, the cells were placed in flasks with Cameron's sea water or natural sea water (28 to 32 ppt). Five-tenths of a mg/ml of penicillin "G" potassium and of streptomycin sulfate were added to retard bacterial growth. The incubation temperature was 30 ± 1°C and a shaker table was used to agitate the culture. Cell densities from several hundred to 50,000/ml were used.

The morphology of sporulating sporangia and of planonts is described from light microscope studies. Periodic observations of single sporangia undergoing sporulation and of planonts were made with an inverted microscope. The cells were contained in a chamber perfused with Cameron's sea water.

Whole and sectioned sporangia were stained in Heidenhain's haematoxylin, using the method outlined by Starr (1955), in acetocarmine (Humason, 1962), in Schiff's reagent, using the Feulgen method (Jensen, 1962), and in Weigert's iron haematoxylin (Humason, 1962). Whole planonts were stained, using the methods of Couch (1941). Lipid was detected, using a Sudan IV method (Humason, 1962).

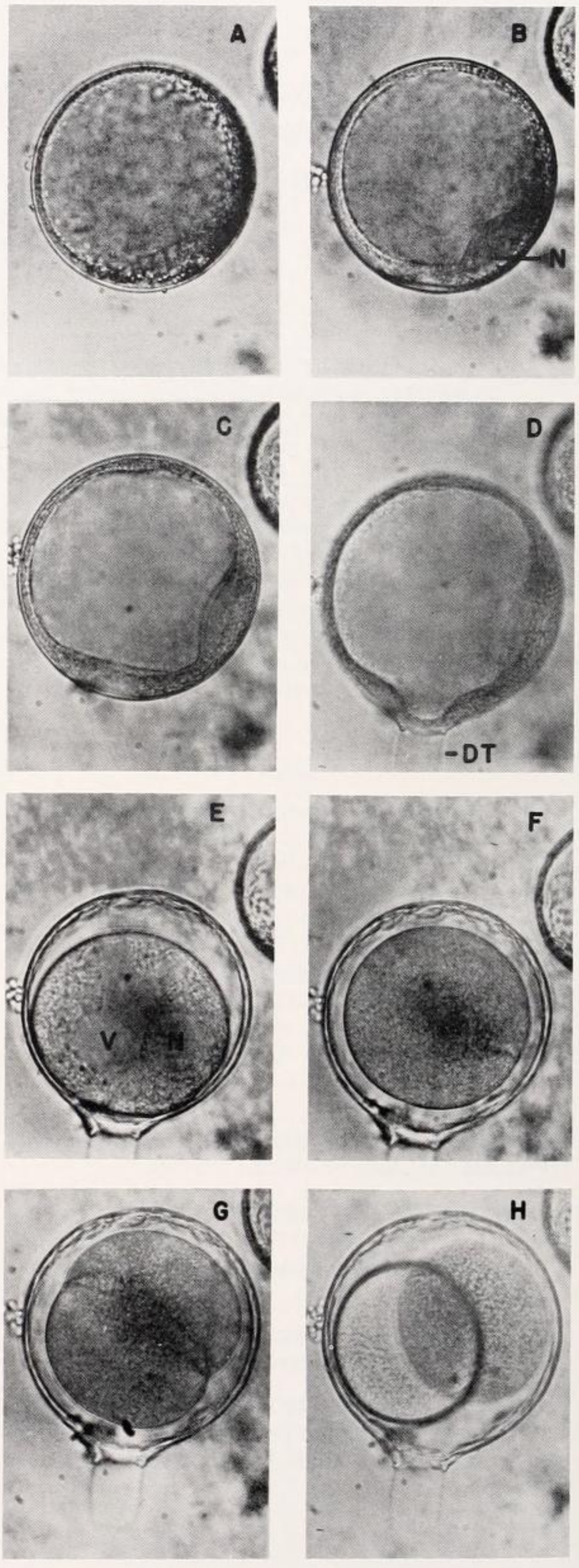


FIG. 1 *(A-D). Sporulation of a single sporangium in Cameron's sea water. All micrographs; 730X. Note enlargement of sporangium between* 1*B and* 1*C.* FIG. 1*A — plus 13 hours;* FIG. 1*B — plus 30 hours; FIG.* 1*C — plus 39 hours;* FIG. 1*D — plus 42 hours. Nucleus (N); discharge tube (DT). Light micrographs.*

FIG. 1 *(E-H). Sporulation of a single sporangium in Cameron's sea water. All micrographs; 730X.* FIG. 1*E — plus 42 hours, 40 minutes;* FIG. 1*F — 43 hours, 10 minutes;* FIG. 1*G — 43 hours, 20 minutes;* FIG. 1*H — 43 hours, 41 minutes. Nucleus (N); vacuole (V). Light micrographs.*

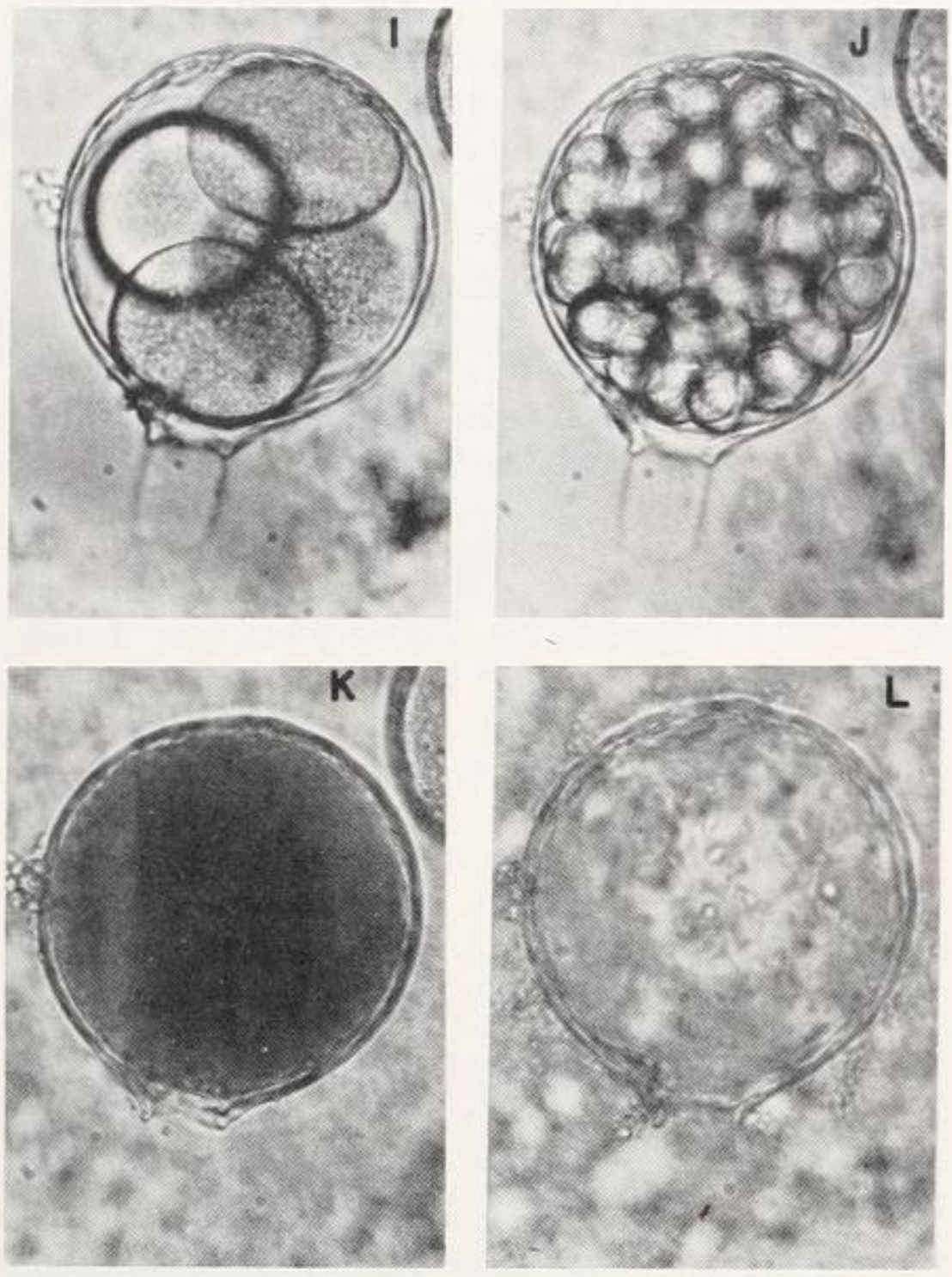


FIG. 1 *(I-L). Sporulation of a single sporangium in Cameron's sea water. All micrographs; 730X.* FIG. 1*I* — *plus 44 hours, 22 minutes;* FIG. 1*J* — *plus 46 hours, 35 minutes;* FIG. 1*K* — *plus 60 hours, 30 minutes;* FIG. 1*L* — *plus 92 hours. Light micrographs.*

Attempts were made to infect oyster explants and whole organs with planonts. Two methods were used. In the first method, explants and single sporangia were sealed in perfusion chambers and observed under an inverted microscope. Each chamber consisted of a glass ring, 3.5 mm high and 10 mm inside diameter, a microscope slide, a coverslip (22 mm square), and two, 0.8 mm (I.D.) glass capillary tubes, tapered at one end to 0.1 to 0.2 mm (I.D.). Two notches were scored in each glass ring so that the capillary tubes could be slipped between the ring and slide. The tubes were positioned across the ring from each other. All components, except the coverslip, were sealed together with epoxy resin (Durcopan ACM). The explant and sporangium were placed in a drop of Cameron's sea water on the coverslip and a small fragment of coverslip was used to sandwich the preparation against the main coverslip. The system was then inverted over the glass ring and sealed to the ring with paraffin. A reservoir of Cameron's sea water was used to perfuse the chamber at about 1 to 3 ml per hour.

In the other method, organ explants were placed in an experimental apparatus designed to allow only motile infective elements to reach the organs. Adductor muscle and heart explants were placed in a flask, containing Cameron's sea water, which was connected to another flask by means of side arms. The side arms were 5 to 10 mm above the bottom of the containers, and were 15 mm long and 4 mm inside diameter. A thick suspension of about 2 x 10^6 hypnospores was gently layered on the bottom of the flask not occupied by the explants. The preparations were maintained at room temperatures (22-25°C).

Individual living oysters were exposed to planonts in experimental systems, each of which consisted of a beaker, connected to a flask by means of side arms, 5 to 10 mm above the bottom of each container. An oyster was placed in each beaker and a suspension of hypnospores was added to each flask. Cameron's sea water was the medium and incubation temperatures were 22-25°C.

In this study observations were made using *D. marinum* cells from oysters of Apalachicola Bay, Florida, and Galveston Bay, Texas. No differences in morphology or infectivity were detected. Uninfected control oysters were obtained from upper Delaware Bay.

RESULTS

Morphology of Sporulation

Hypnospores were observed in sea water perfusion chambers as single cell isolates and in populations of hypnospores. The hypnospores used in this study stained blue, blue-black, or green in Lugol's iodine solution as reported by Ray (1954b).

The observed pattern of development is pictured in Figure 1 (A-L). The elapsed times for the series are given with each figure; but in the following discussion, elapsed times will not be given, because of the extreme variations in elapsed times which occur. In a population where 97 per cent sporulation occurs, cells may demonstrate first cleavage as soon as 24 hours after being placed in sea water, and others may not initiate cleavage until plus five days.

Prior to first cleavage of the protoplast large lipid droplets are either dissolved and utilized by the cell or are subdivided, because the cytoplasm assumes a fine granular appearance (Fig. 1A) rather than being masked by the droplets as in hypnospores recently isolated from F. T. M. (Fig. 2). Further subdivision and/or utilization of the lipid deposits allows one to clearly see the large, characteristic vacuole and the nucleus (Fig. 1B).

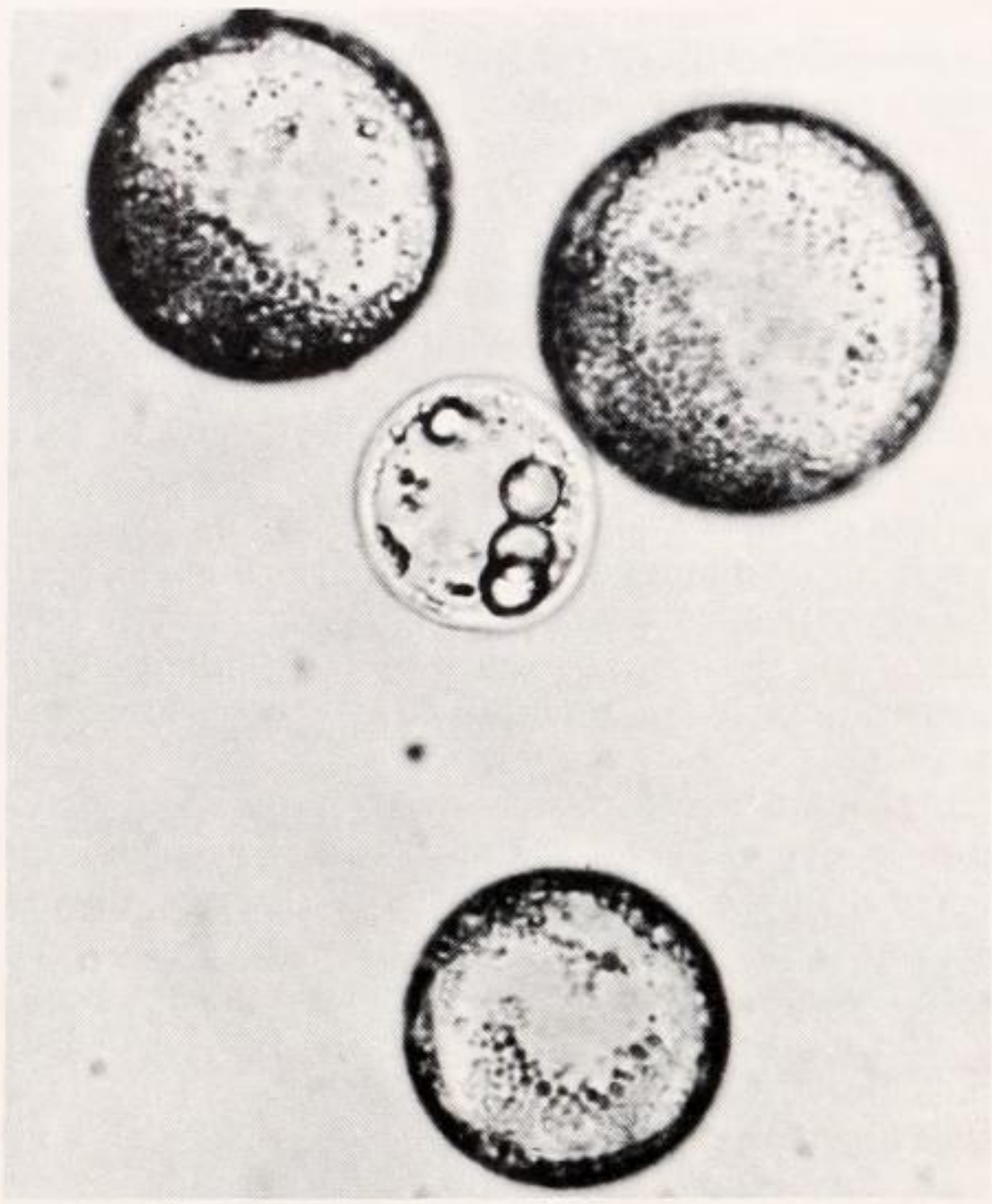


FIG. 2. *Hypnospores in sea water, showing concentrations of lipid droplets within the cytoplasm. The smallest cell has lysed and most of the lipid has coalesced into several large droplets. Light micrograph; 1,600X.*

A clear cytoplasmic area, immediately under the cell wall, appears early in development (Fig. 1B). The area becomes progressively larger (Figs. 1C; 3), a bulge appears in the wall overlying the area (Fig. 4A) and rupture occurs, thereby forming a discharge pore (Fig. 1D). One, or rarely two, pores may be formed. Sections indicate that the clear area represents a plug of material which gives rise to a discharge tube and also serves to block the pore area until planonts have differentiated. The plug is quite strong. Until the last few hours prior to planont discharge, the sporangia can be crushed without being ruptured.

The wall, surrounding the pore, is flared out and ragged in appearance (Fig. 4B), indicating that pore formation results from a forceful pressure from within. The bulge which forms prior to rupture also supports this idea (Fig. 4A). As can be seen in Figure 4B, discharge tubes do not stain blue in Lugol's iodine.

Sporulation stages, prior to first cleavage, show a startling change in the nucleus. Hypnospores, as isolated from fluid thioglycollate medium, have peripherally located, compact nuclei which are spherical or oval (Fig. 5, A-B). The longest axis is usually 3 to 6μ. A nucleus may have one or two lightly staining areas (Fig. 5A) or it may stain homogeneously dark in haematoxylin (Fig. 5B). It is not known what the light areas represent. If they represented endosomes or nucleoli, one would expect them to be more, rather than less, basophilic than the rest of the nucleus. Acetocarmine-stained preparations gave a similar picture. Feulgen preparations did not yield reproducible or interpretable results when used on whole cells. Sectioned cells were not subjected to the Feulgen procedure.

When hypnospores are placed in sea water and differentiate into sporangia, the nuclei enlarge greatly prior to first cleavage (Fig. 1A-E), and the chromatin is either dispersed or chromatin duplication occurs (Fig. 6). The striking increase in size indicates that the latter possibility exists. One would expect that nuclear division would be preceded by condensation rather than dispersal of chromatin.

Prior to the first bipartition, the protoplast contracts and forces much of the vacuolar fluid into the area between the cell wall and plasma membrane (Fig. 1E). The vacuole becomes irregular in outline and is subdivided. Upon completion of the contraction process the cytoplasm becomes dense, and the nucleus and vacuoles can no longer

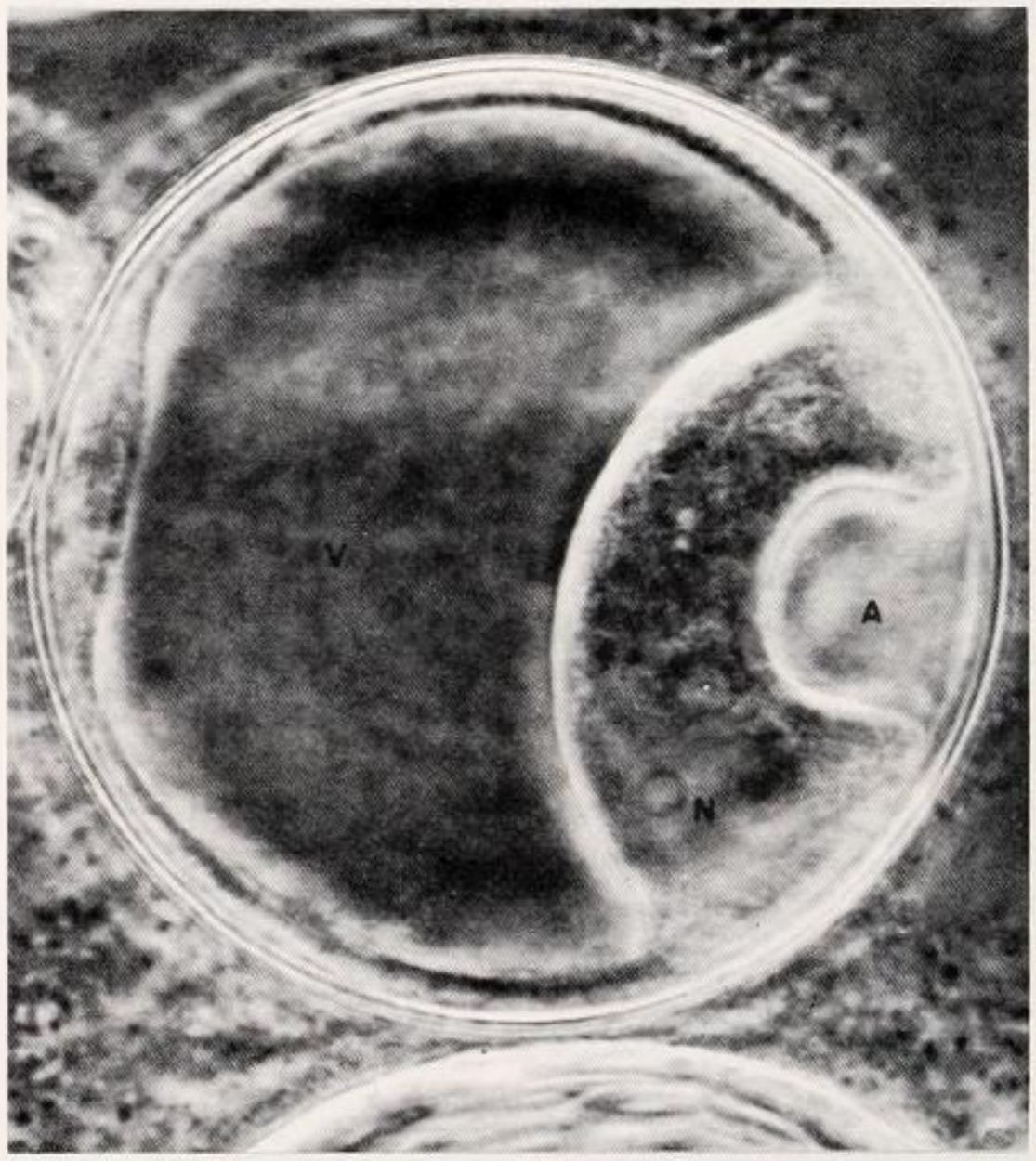
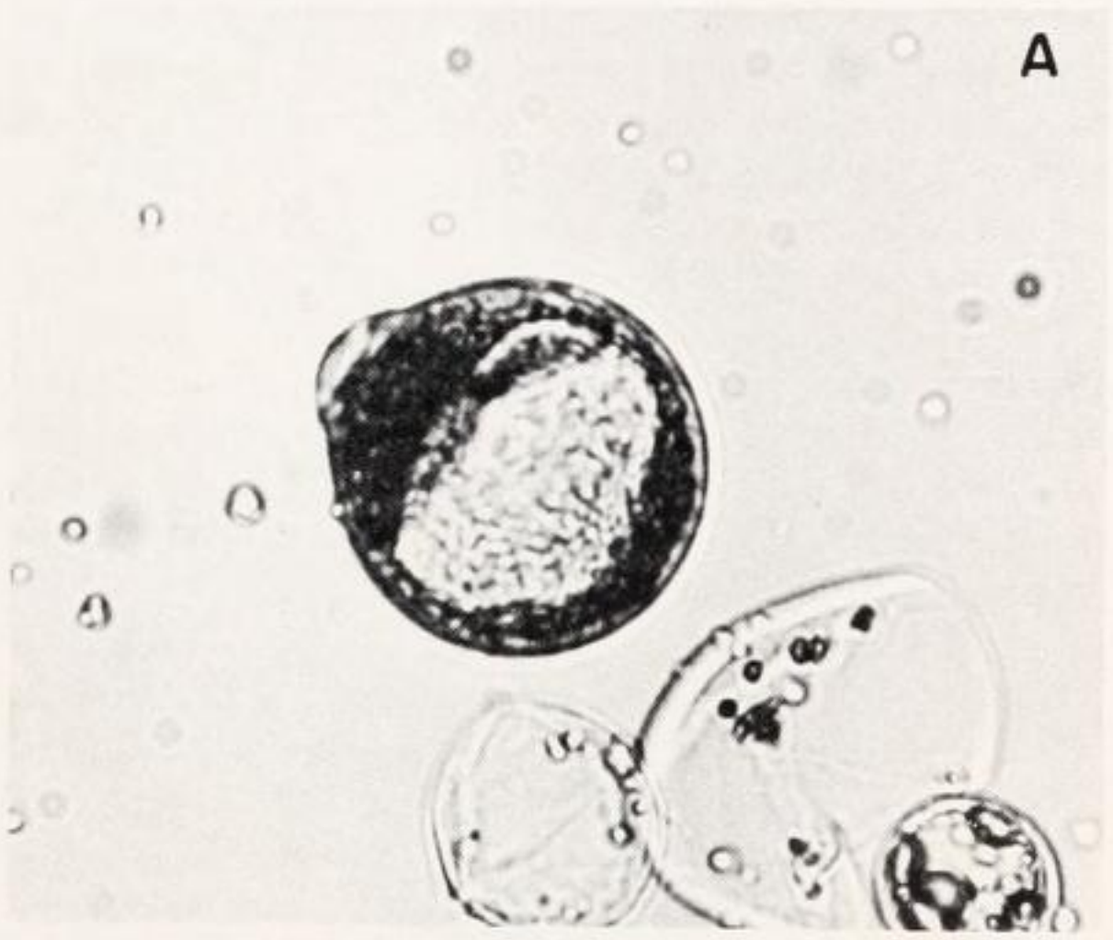


FIG. 3. *A living precleavage sporangium which had been in sea water 36 hours. Vacuole (V); nucleus (N); area responsible for discharge pore and tube formation (A). Light micrograph; 3,600X.*



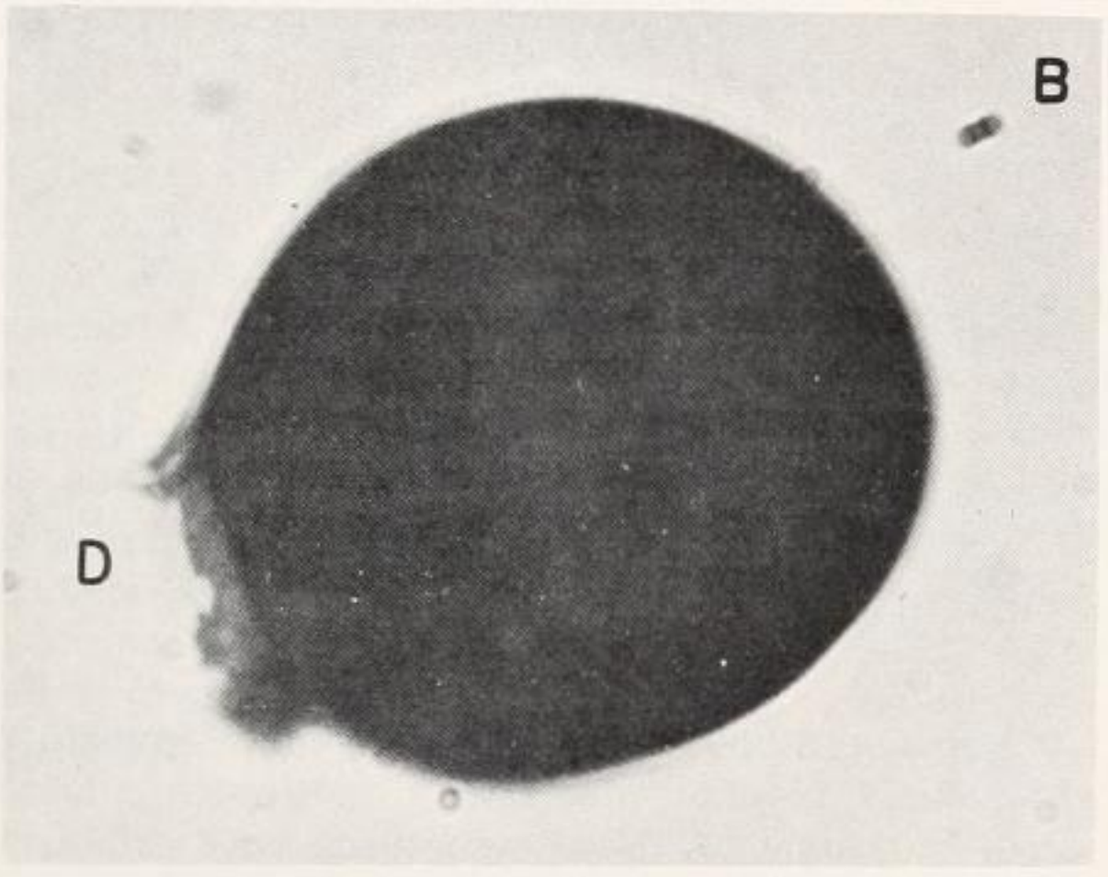


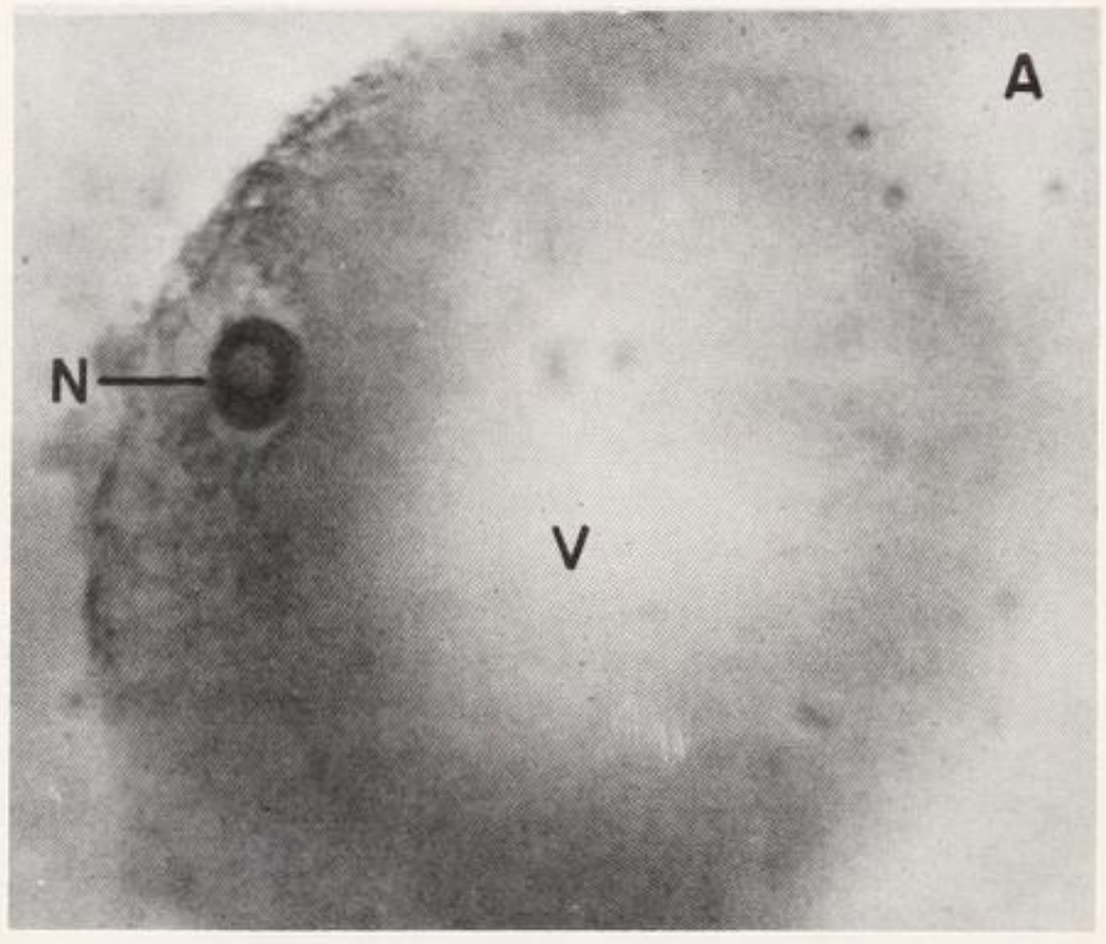
FIG. 4 *(A-B). Sporangia before and after discharge pore formation.* FIG. 4*A is a light micrograph of a living sporangium prior to pore formation.* FIG. 4*B is a light micrograph of a sporangium stained in Lugol's iodine. The ragged edge of the pore and the nonstaining discharge tube (D) can be seen.* FIG. 4*A — 1,200X; 4B — 1,650X.*

be seen (Fig. 1F). At this time the protoplast has drifted to the center of the region encompassed by the cell wall.

Cleavage of the protoplast is similar to the process observed in the first few divisions of some invertebrate eggs, such as in sea urchins. With each division, cleavage furrows divide the protoplast into equal halves (Fig. 1F-J). The first two bipartitions are synchronous. Subsequent divisions are slightly asynchronous; however, no two cleavages are more than about one division out of phase.

Mitotic figures were found in cells resulting from the first five cleavages (Fig. 7). Apparently spindle fibers are formed, but it was not ascertained whether the nuclear membrane disappeared during nuclear division, nor was it possible to count the number of chromosomes. The chromatin material appears to be subdivided with each division. In some early cleavage stages the nucleus can be seen in living cells which have just completed a cleavage (Fig. 8), thereby suggesting that the nuclear membrane is intact at that time.

As bipartition continues, the extracellular fluid within the cell wall is apparently reabsorbed by the cells (Fig. 1 I-K). About 1.5 to 2 days prior to release of planonts from the sporangia, the cells become motile. They move almost imperceptibly



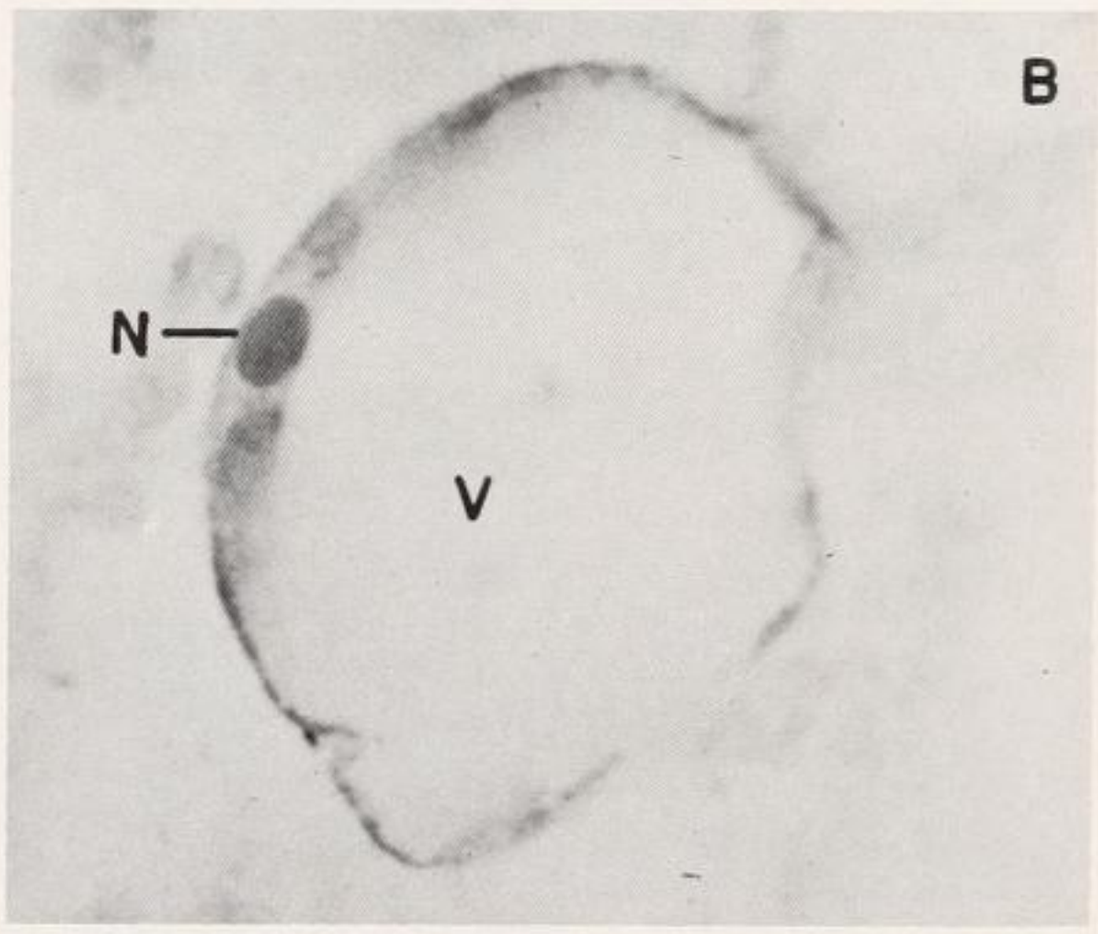


FIG. 5 *(A-B). Sectioned hypnospores, stained in Weigert's iron haematoxylin. Nucleus (N); vacuole (V). Light micrographs; 1,900X.*

at first, but the frequency of gyrations slowly increases over a period of about two days until the mass of planonts is undergoing rapid, random oscillations. At the peak of activity the plug apparently parts in the center and the planonts swim out singly and doubly. It is not unusual for a sporangium to be emptied of material, leaving no cell particulates or lysed planonts.

The mature planont is a biflagellated uninucleated cell with subapically and laterally attached flagella (Fig.9). The cell body is 2-3 μ by 4-6 μ and is intermediate between pyriform and reniform with a slightly invaginated area at the point of insertion of the flagella. The anterior flagellum propels the cell without direct aid from the posterior flagellum; therefore, on the recovery stroke the cell slows down, resulting in a jerky swimming motion. The posterior flagellum is about two-thirds as long as the anterior one (6-10 μ and 9-16 μ, respectively), and appears to act mainly as a rudder trailing behind the cell. The cell body does not change shape as do many fungal planonts.

The anterior flagellum has tinsels *(Flimmern)* along one side as shown by stained preparations (Fig. 10). Unless a mordant and stain are used to increase the apparent diameter of the tinsels, they are invisible in bright field or phase microscopy. The posterior flagellum is naked; i.e., a whiplash flagellum.

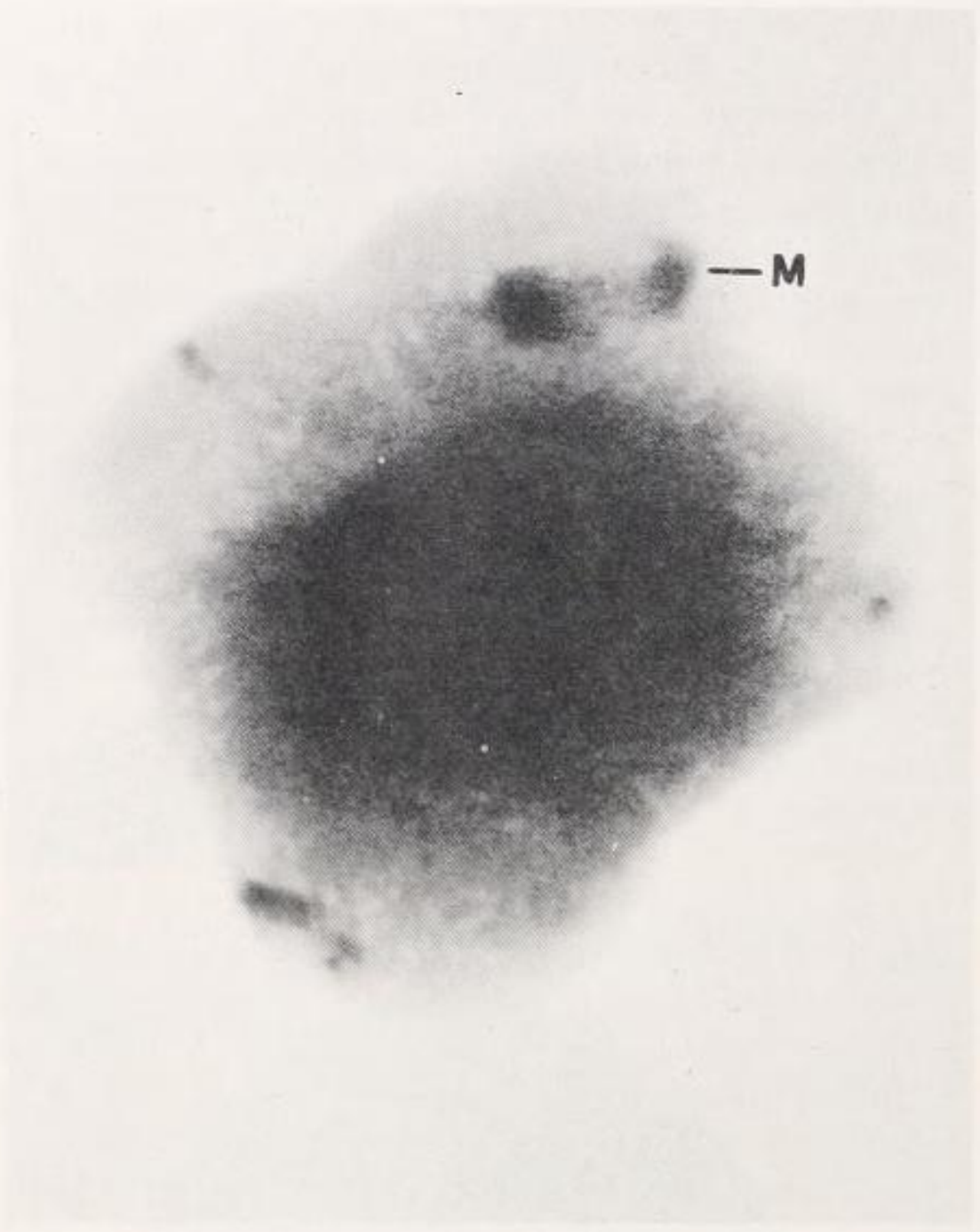


FIG. 7. *Sporangium in third cleavage. Mitotic figure (M) represents late anaphase. Stained in Heidenhain's haematoxylin. Light micrograph; 3,500X.*

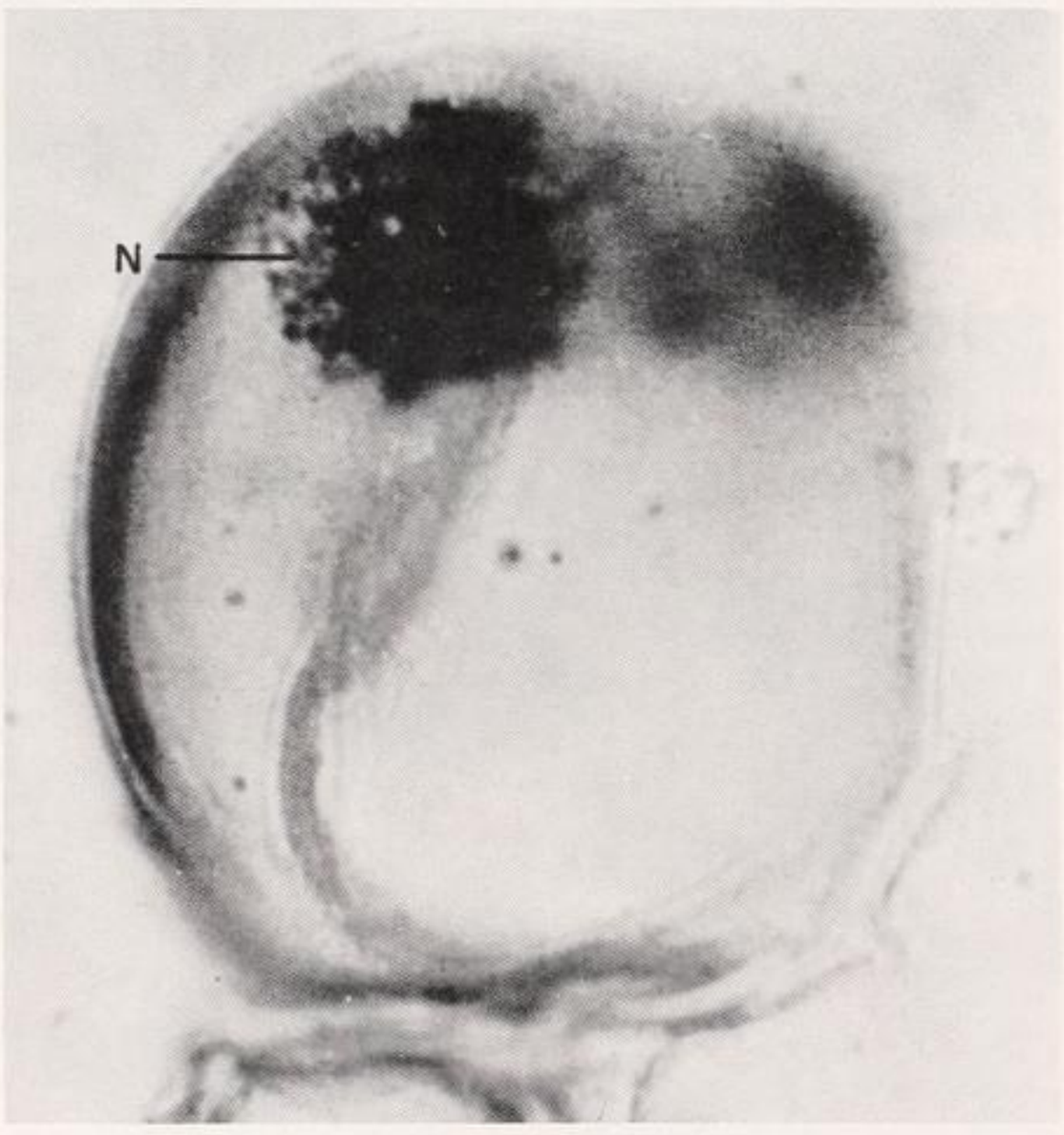


FIG. 6. *Precleavage sporangium in sea water 35 hours. Fixed in Schaudinn's fixative and stained in Heidenhain's haematoxylin. Nucleus (N). Light micrograph; 4,800X.*

Koch's methods (Koch, 1958) for revealing rhizoplasts, blepharoplasts, and nuclear caps were used, but none of these structures was detected. Little intracellular detail was seen. A large refringent body in the posterior end of the cell body, the nucleus, and two vacuoles in the anterior end were the only evident structures.

In about 0.1 per cent or less of the sporangia, a secondary sporangium with a normal cell wall was found within the primary cell. Both cells have the potential to sporulate. Several times motile planonts were released from both sporangia simultaneously, although it was more normal for the primary sporangium to release its planonts prior to first cleavage of the secondary cell. This double sporulation is probably the result of endogenous budding of the immature thallus which forms a cell within a cell. Endogenous budding has been previously reported by Mackin *et al.* (1950).

Infection Experiments

Oyster explants were successfully infected with the planonts using the methods described above. After 4 to 14 days of maintenance the explants in perfusion chambers were demonstrated to contain

infective cells by perfusing F. T. M. into the chamber and observing hypnospore formation *in situ*. Six out of 11 explants were successfully infected. The maximum number of hypnospores demonstrated in a single explant was four and they were always found separately, never in clusters; therefore, no reproduction occurred after the planont differentiated into a nonmotile cell.

Intermediate stages between the motile form and the hypnospore were not observed in a developmental sequence. Presumably the planont becomes an immature thallus and, when placed in thioglycollate, differentiates into a mature thallus (prehypnospore), then into a hypnospore.

The explants contained in the flask systems were infected in five of eight attempts, but in only two explants out of two experiments was there any indication that multiplication of the fungus had occurred. One cluster of 21 hypnospores and another cluster of four were found after culturing the explants in F. T. M.

Ray (1954b) demonstrated that after culturing infected oyster tissue in F. T. M., the tissue could be used to infect oysters by injecting the mass into the mantle cavity. He suggested that those cells which did not differentiate into hypnospores were responsible for the infections. In the present study, the remote possibility exists that nonmotile cells caused the infections; however, it is extremely unlikely that cells flowed passively through the connecting tubes to the explants — a distance of 90 mm. The fact that no hypnospores with discharge pores were found in the flasks with the explants is interpreted as strong evidence that the experimental procedures were effective in preventing passive flow of cells through the flask side arms.

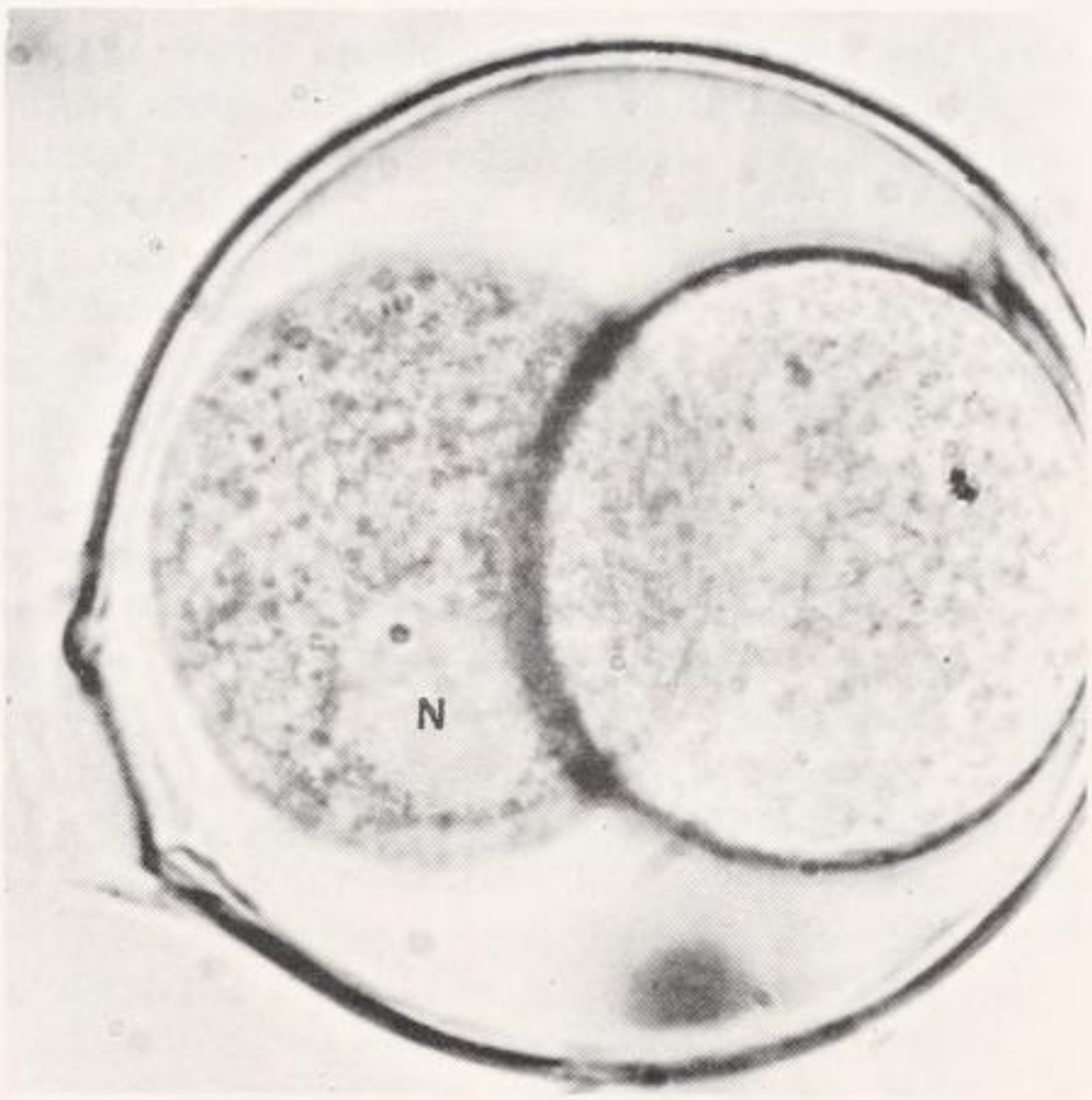


FIG. 8. *Living sporangium after completion of first cleavage. Nucleus (N). Light micrograph; 2,300X.*

Three oysters out of five were infected by using the beaker and flask method. One oyster was infected heavily enough to isolate the hypnospores after exposure to F. T. M. and to induce sporulation. Planonts which were morphologically typical *D. marinum* biflagellates were obtained; there-

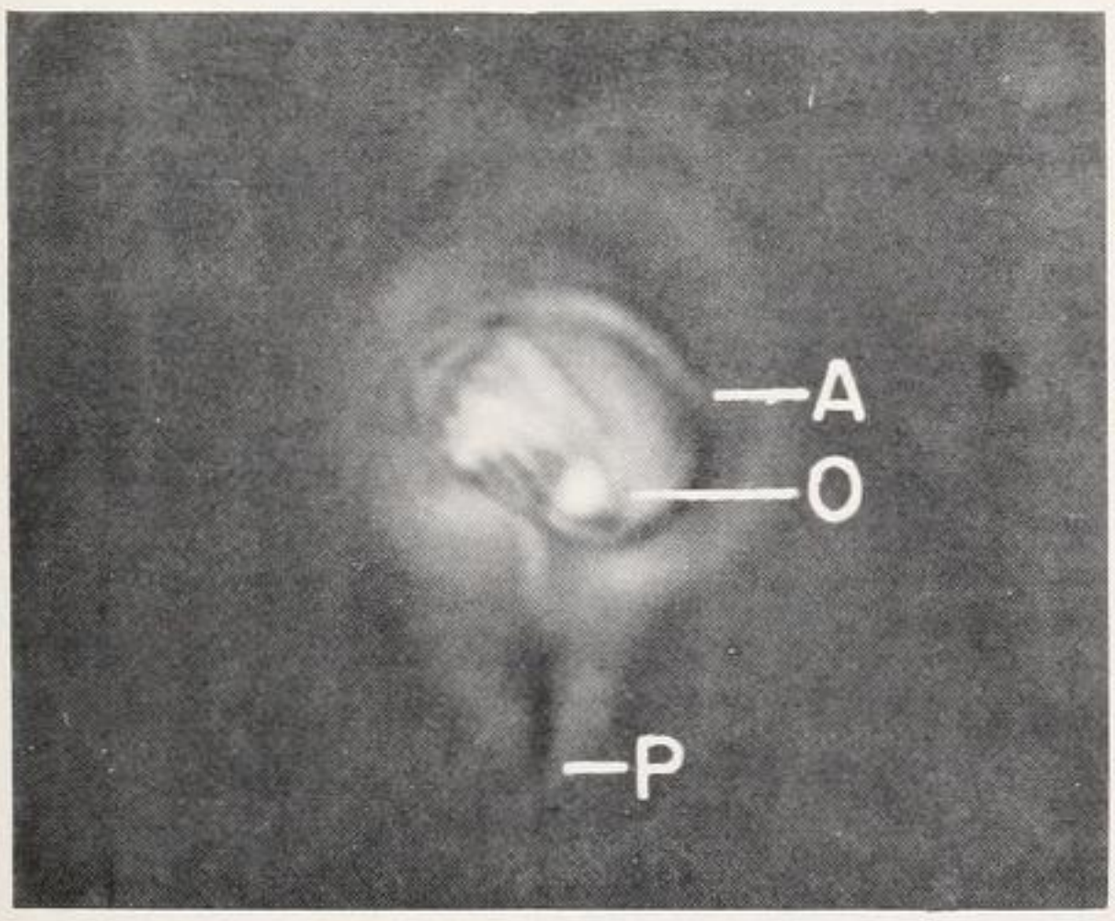


FIG. 9. *Planont fixed in 1 per cent Os04 fumes. Anterior flagellum (A); posterior flagellum (P); osmophilic body (O). Phase micrograph; 3,400X.*

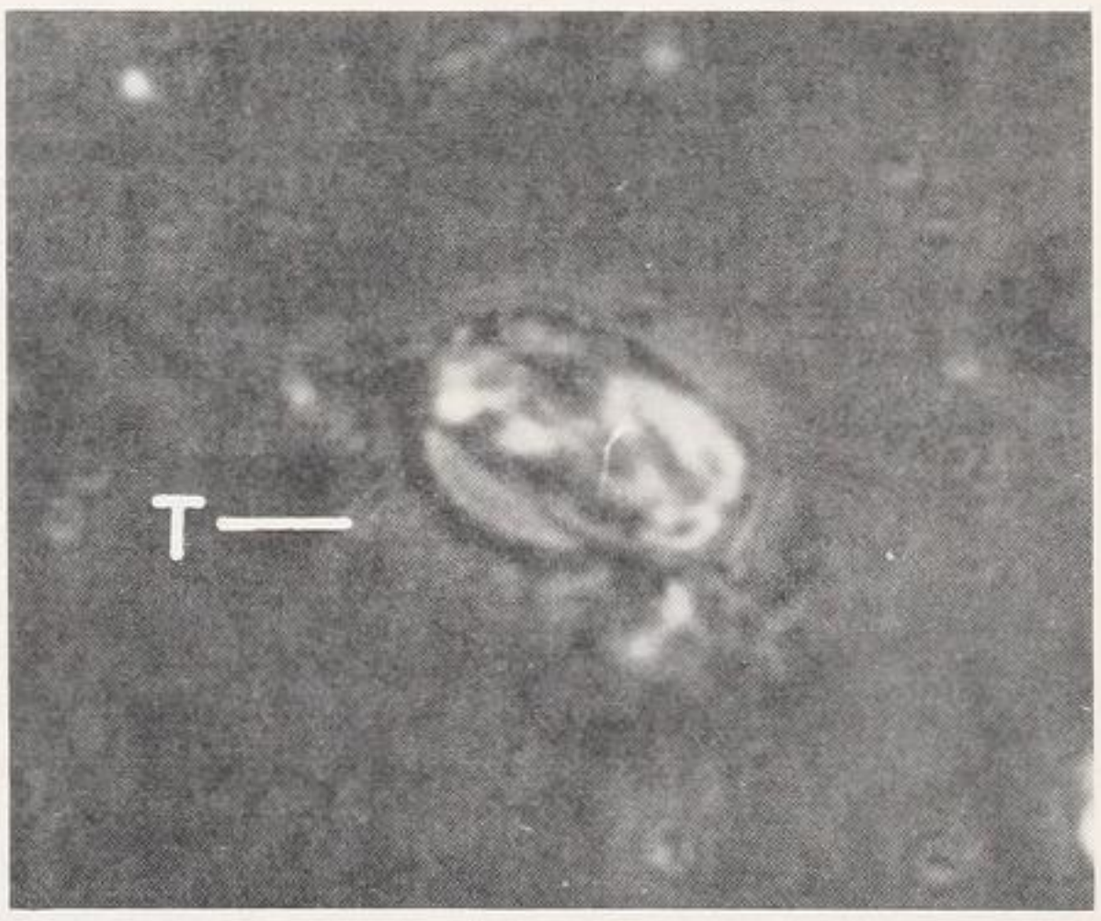


FIG. 10. *Planont, stained with mordant and crystal violet. Tinsels (T). Light micrograph; 4,000X.*

fore, the life cycle was completed under laboratory conditions, if one assumes that some of the prehypnospores, which differentiated into hypnospores in F. T. M., arose from planonts and not from prehypnospores. The authors know that prehypnospores were present in the experimentally infected oyster, because individual hypnospores were observed to form in F. T. M. slide preparations.

As was anticipated, hypnospores were drawn across the bridge between the flask and beaker in each experiment, probably by the ciliary action of the oysters. Empty hypnospores with discharge pores were found on the mantle surface of the infected oysters; therefore, it cannot be definitely stated that planonts were responsible for the infection. Nonmotile elements probably caused at least some of the infections.

DISCUSSION

The developmental stages which follow hypnospore formation have been demonstrated. Whether the planonts represent zoospores or isogametes has not been shown. Attempts to induce copulation of the planonts were inconclusive. Some *D. marinum* biflagellates were observed to be linked in rare instances, but the point of fusion was randomly located on the cell bodies. If copulation occurred, one would expect the copulation point to be a constant feature. It is believed that the "doublet" cells were examples of incomplete cell division.

The planonts can definitely infect oyster explants; therefore, it is reasonable to assume that living oysters are also infected by the biflagellates. The relative importance of the motile cells in transmission of infections under natural conditions is not certain. Ray (1954b) has demonstrated by injection of nonmotile cells into oysters that planonts are not the only infective elements. Further evidence concerning the role of planonts in transmission of infections will be discussed in a later paper.

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