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Studies of Labyrinthula spp in Culture

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STUDIES OF LABYRINTHULA SPP. IN CULTURE

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ABSTRACT

Labyrinthula spp. were isolated from marine environments and maintained in culture. One isolate was shown to have a broad range of tolerance for salinity, temperature and pH. It was also shown that Labyrinthula spp. could be isolated from plants from almost all marine habitats. Studies of morphology showed that the isolates fell into two natural groups on the basis of cultural morphology and life cycle. Although nutritionally fastidious, these labyrinthulas could utilize several living substrates as food. In a study of the life cycles of the isolates, one was shown to have a motile stage which resembled that of the biflagellated Phycomycetes and the brown algae. A single motile cell was followed from the time of release from the sporangium to a final conversion to a typical labyrinthulid spindle cell. Another isolate of a separate grouping showed a tendency to form multinucleated cells which then cleaved to form new uninucleate spindle cells. The plasmodial cells of this group seemed to be resistant to aging but not to other adverse conditions. The phylogenetic implications of these features were considered.

An improved method for preparing Watson's bovine serum medium was developed.

LABYRINTHULA

INTRODUCTION

The family Labyrinthulidae is a group of poorly understood and rarely studied protistans. They received very little attention until Renn (1936) correlated Labyrinthula macrocystis with the wasting disease of the ecologically and economically important spermatophyte, Zostera marina. Although Renn did not use pure cultures, he presented strong evidence that Labyrinthula is the causative agent in eelgrass wasting disease. Others have shown that members of the genus Labyrinthula are parasitic, especially among the algae (Aschner, 1961; Chadefaud, 1956; Dangeard, 1932; DuBoscq, 1921; Hollande and Enjumet, 1955; Jepps, 1951; Johnson and Sparrow, 1961; Pokorny, 1967; Schmoller, 1960; Valkanov, 1929; Watson, 1951; Young, 1943; Zopf, 1892).

Recently, a renewed interest has arisen concerning a labyrinthulid of yet another economically important host, Crassostrea virginica, the American oyster. Mackin and Ray (1966) changed the name of the oyster pathogen Dermocystidium marinum to Labyrinthomyxa marina, placing it in the genus Labyrinthomyxa of the family Labyrinthulidae.

The genus Labyrinthula typically encompasses a group of uninucleate, single celled, spindle-shaped organisms traveling by a gliding motion within a reticulum of extracellular secreted tubes.

These tubes and the method of movement have been subjects of considerable interest and until just recently (Hohl, 1966; Schmoller, 1967), little information existed on their nature. Although the spindle cell form is a major part of the life cycle, other less recognizable forms exist. Some species form plasmodia and some form zoospores (Watson, 1957). Neither of these cellular stages possesses tubes or slimeways as some workers have termed them (Pokorny, 1967). However, in all known species, the slimeways exist for at least part of the life cycle.

Although many consider Labyrinthula a protozoan, placement of the Labyrinthulidae in any particular phylum is arbitrary at this stage, since it has been included variously in the Thallophyta, Protozoa, and Fungi. Studies of the various species and subspecies or varieties under controlled conditions will undoubtedly provide insights which will lead to a better understanding of the labyrinthulid systematics.

After examination of the existing literature, one is struck with the lack of concrete information about the different species involved. Much of the information is based upon studies of grossly contaminated cultures or from observations of non-living host material. In almost every instance the life history is only partially described. Watson (1951) made the first strides toward complete study of Labyrinthula by the development of a pure culture medium. Vishniac (1953) also developed a medium for pure cultures and proceeded to investigate the nutritional requirements of Labyrinthula. Watson's dissertation (1957) was a monumental step forward in the systematic investigation of

Labyrinthula. Information concerning the life histories and morphology of the species is still needed in order to correctly identify them. Two works, Johnson and Sparrow (1961) and Pokorny (1967), summarize the literature to the present.

Watson (1957) and Vishniac (1955a) made isolates which they named only by letters indicating a lack of knowledge about each (L. spp., LX, SELX, FPLX, WH-69, etc.). Schmoller (1960, 1966a) isolated other forms of Labyrinthula and gave them the names Labyrinthula coenocystis and Labyrinthomyxa pohlia. Others presently working on various species of Labyrinthula (Mackin and Ray, 1966; Pokorny, 1967; Watson, personal communication; Porter, 1967) have failed to name most of their isolates, thereby indicating that continued sampling is bringing out new isolates. In order to appreciate the systematics of this family, many isolates must be studied. If this is not done, the error resulting from incomplete studies of the group as a whole will confuse the literature for some time to come.

In an attempt to avoid bias due to incomplete sampling, the present author felt a need to establish a sampling program as large as time would allow. This program not only enabled isolation of several taxonomically distinguishable Labyrinthula spp., but also enabled a tabulation of hosts and produced a record of the availability of the organism on a year round basis.

Since the estuarine environment can exert many varied environmental stresses, experiments were set up to simulate some of

them. Other workers (Young, 1943; Vishniac, 1955a; Aschner, 1958) have done similar experiments on environmental tolerances. Aschner (1958) reported a species from fresh water and salinities above 35°/oo, and Young (1943) showed that L. macrocystis can tolerate fresh water. Vishniac (1955a) stated that her isolates are "obligately marine." Workers experimenting with temperature and pH ranges also reported a broad range of tolerances. The upper limit for temperature was near 35°C and the lower limit was undetermined below about 3°C (Young, 1943). Hydrogen ion concentrations from pH 4.5 to 8.2 were observed by Vishniac (1955a).

The environmental or cultural pH can be an important factor in spore germination (Sussman and Halvarsan, 1966) but would be important in the well buffered ocean only if the organism lived in a microhabitat which was not part of the buffered system. Since Labyrinthula is suspected of being an invasive organism, it may have to overcome some wider ranges of pH than are normally found in oceans and estuaries.

Watson (1957) and Hollande and Enjumet (1955) describe sporulation of Labyrinthula. Hollande and Enjumet (1955) have been able to follow the life cycle from spindle cell through zoospore formation to the spindle cell again. However, no single cell observations were mentioned, hence stages could have been overlooked. Watson (1957) was never able to determine the fate of his zoospores and the sorus he describes is somewhat different from that of L. algeriensis (Hollande

and Enjumet, 1955). Investigation of this aspect of Labyrinthula would be quite informative.

Several aspects of Labyrinthula need investigation, such as nutrition, host-specificity, physiological tolerances, motility, morphology, life histories, etc., but the major need is in morphology and life history. Knowledge of these features are basic to the understanding of the taxonomy and phylogeny of Labyrinthula. Therefore, the major portion of this work was directed toward investigation of life histories.

MATERIALS AND METHODS

Sampling in the Field

The primary aim of sampling was to determine the relative availability of Labyrinthula or similar organisms in nature. On this basis a program was set up whereby weekly samples of eelgrass, a known host of Labyrinthula spp. (Renn, 1936), were taken from the intertidal and subintertidal zone at VIMS; and periodic samples were taken from several suspected hosts along the eastern seaboard from Delaware Bay to Oregon Inlet when possible. Most of the latter sampling was confined to the York River (Fig. 1).

The majority of hosts mentioned in the literature are algae; but in an effort to eliminate sampling bias, samples were taken from plants and animals of many phyla available. Table 1 shows the organisms sampled and divides them into the groups described in Results.

TABLE 1

Organisms or substrates sampled for the presence of *Labyrinthula* spp.

Substrate	Presence (+) or absence (-) of *Labyrinthula*	Isolate No.	Group No.
Blue Green algae			
Lyngbya sp.	+	111a	I
Spiralina subsalsa	+	111b	I
Red algae			
Agardhiella tenera	+	113	I
Gracilaria foliifera	-	-	-
Hypnea musiformis	-	-	-
Callithamnion sp.	-	-	-
Ceramium strictum	-	-	-
C. rubrum	-	-	-
Dasya pedicellata	-	-	-
Grinellia americana	-	-	-
Polysiphonia denudata	+	114	I
Polysiphonia harveyi	-	-	-
Green algae			
Euglena sp.		-	
Spirogyra sp.	-	-	-
Enteromorphora linza	+	112	I
E. minima	+	L8	II
E. intestinalis	+	109, 110	I, II
Ulva lactuca	+	L9, 109a	I
Cladophora fascicularis	-	-	-
Bryopsis plumosa		-	
Miscellaneous algae in plankton		94	

(Continued)

Higher Plants			
Ruppia marina	+	68	I
Juncus roemerianus	+	27c	II
Typha (angustafolia?)	-	-	-
Sphagnum sp.	-	-	-
Ceratophyllum sp.	-	-	-
Distichlis sp.	+	27a	II
Spartina alterniflora	+	a(71, 39, 27) b(L1, L11, 44, 53, 64, 65, 67, 74, 82, 84, 89, 90, 107)	a=II b=I
Zostera marina		L1, L2, L4, 62, 77, 86	all=I
Several ditch weeds from North Carolina			
Pollen floating in York River (Pinus)		L12	
Animals			
Nephtys (Polycheate)			
Nereis (Polycheate)			
Leptogorgia (soft coral)			
Vollsella demissus (mussel)			
Nassarius (mud snail)			
Cucumaria pulcherrima (sea cucumber)			
Chelonibia testulenaria (barnacle)			
Crassostrea virginica (oyster)	+	C	I
Balanus eburneus (barnacle)	-	-	-
Molgula manhattensis (sea squirt)			
Ecteinascidia turbinata (sea squirt)			
Amphiodia atra (brittle star)			
Callinectes sapidus (blue crab)			
Eurypanopeus depressu (mud crab)	-	-	-
Mercenaria mercenaria	+	M	I

The weekly samples of Zostera marina at VIMS were taken from both floating and attached plants. In sampling, three to five petri dishes with a modified Fullers medium (Medium Number 1, page 11) were inoculated with pieces of 7 to 10 leaves of Zostera marina. The leaves involved were chosen randomly from a collection which included mostly necrotic or damaged leaves with a few healthy green ones. The inoculated plates were incubated at room temperature (20 to 24°C). Twelve to twenty-four hours after inoculation the material was examined macroscopically and with the light microscope (100X) for typical cells and colonies (Fig. 2). If no Labyrinthula were present, an additional 24 to 72 hours were allowed for incubation time. If no outgrowth was then noticed, the inoculum was removed in order that any cells beneath the leaves might be found. The total number involved relative to the number of leaves yielding Labyrinthula was recorded and note was taken of the isolate's affinity to any specific group. Isolations from other substrates employed similar techniques.

A secondary purpose of the sampling program was to try to isolate several species of labyrinthulid organisms. Any cells or colonies that seemed in the least different were transferred in a block of agar to a fresh plate. Precautions were taken to avoid all contaminating organisms except yeasts which were found to be useful as food organisms. After two or three subcultures, a monoxenic culture was established with yeast, Rhodotorula sp., as the standard food organism.

Isolation

The isolates were differentiated on the basis of original host or substrate, original environmental salinity, ability to sporulate, colonial morphology, cell size, and cell shape. Each isolate was given a number corresponding to the chronological order in which the isolates were obtained; therefore, the isolate numbers do not necessarily denote species or strain differences.

Medium Number 1

Oceanic seawater at desired dilution	1,000ml
Difco Agar	5 to 8mg
Glucose	1g
Gelatin Hydrolysate	1g
Yeast extract	0.1g
Liver extract	0.01g
Thiamine (HCl)[1]	0.2mg
Cholesterol[2]	5mg
Penicillin G	0.4g
Streptomycin (HCl)	0.4g

After the ingredients are mixed, they are slowly brought to a boil and immediately autoclaved for 10 minutes at 115.5°C. The medium is then quickly cooled to 40°C, the antibiotics are added, and the medium poured.

At least 0.5cm was poured in plates to retard evaporation effects. If cultures were to be stored for a period of more than two weeks, the

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[1] Supplemented occasionally by 0.001mg vitamin B_{12} and 0.001mg biotin, both added after autoclaving.

[2] Made up in 1:1000 ethanolic solution and added to hot medium after autoclaving.

petri dish was sealed with "Parafilm" to reduce evaporation. Transfers were made bimonthly. Uninoculated media were stored at 5°C.

One medium that was found to support monoxenic cultures nicely and also held them for long periods of time was C1:

Corn meal agar (Difco)	8g
Gelatin Hydrolysate	1.0g
Liver extract	.01g
Yeast extract	.1g
Seawater	1000ml
Cholesterol (in 1:1000 ethanolic solution)	5mg

For long periods of storage, screw cap tubes are used.

In field sampling it was found that heterotrophic diatoms and some fungi often hampered attempts to isolate Labyrinthula and at times competed successfully for food in the medium. The diatoms were eliminated by spreading a very thin layer of 0.003% germanium dioxide (Mackiernan, 1967) over the surface of the agar prior to inoculation. To eliminate the troublesome fungi, Mycostatin or Fungisone were used.

Axenic culture of Labyrinthula on Watson's Bovine serum (Watson and Ordal, 1957) was attempted several times with varying degrees of success. The following formulation seemed to be the best. Natural seawater and Difco agar (0.5 to 0.7%) are heated together until the agar has melted enough to make a clear solution. The mixture is then autoclaved for 10 minutes at 115.5°C and allowed to cool to 37°C, a temperature at which it is still a liquid. Difco TC Bovine (calf) Serum at room temperature is then mixed in a ratio of 1:9 with the seawater agar to make the final medium. This is then quickly poured in very thin

layers (4mm or thinner) in petri dishes. As a result, the medium cools and congeals quickly after addition of the serum, thus minimizing alterations in the serum which would be detrimental to the growth of the cultures. It was noted that pure cultures (axenic) could be maintained on this medium about 90% of the time, which was better than the 50% or less achieved with Watson's method.

Observations

Observations of living cells were usually made using an AO dark-medium phase microscope or Unitron inverted dark-medium phase scope. Preparations were made using agar media containing 0.3% agar with medium 1 or beef serum additives. The medium along with a small inoculum of Labyrinthula was held between two coverslips sealed with petroleum jelly. This assemblage was, in turn, mounted on a special metal ring which fits into the stage of the Unitron microscope. In this manner very thin preparations were prepared suitable for obtaining high resolution phase micrographs. These preparations survived up to 10 days depending on the care with which they were prepared. They were therefore very useful in making observations of cultural life cycles. Observations of colonial characteristics, such as the nature of the edge, were made with a 10x objective on a Zeiss binocular scope, an A.O. stereoscopic microscope, and with the naked eye.

Measurement of Growth

Measurements of growth were made with a Quebec Colony counter overlain with graph paper. The inoculum was centered and the number of square units covered by the circular outgrowth was measured. To insure uniformity the depth of the agar was constant and a standard inoculum was used. Although measurements were made only in two dimensions, penetration of the agar was noted in order to adjust readings where needed. Adjustment was made by increasing measurement by one-half the original figure when penetration occurred. Although it was found empirically accurate, adjustment is meant only to bias the final analysis in the proper direction.

Cultural Tolerances

Temperature experiments were conducted in incubators at 5, 10, 20 and 37°C and in a refrigerated centrifuge at 0°C. Constant temperature water baths were constructed to attain other temperatures. Baths were made by placing a water container heated by an aquarium heater in a 5°C or 20°C cold room. This combination was used to regulate the temperature of a glass vessel containing 3 to 5 small (60mm) plastic petri dishes. The water was circulated constantly by use of a magnetic mixer, and the entire device was covered with aluminum foil (Fig. 2). Temperature was monitored in the culture chamber and in the water and was found to vary no more than $\pm$ 0.5°C. It was found that use of this device eliminated the need for sealing petri dishes with "Parafilm" to avoid drying was was done in other incubators. Temperature

acclimated cultures were used as inocula and the salinity and pH remained near optimum (pH 8.1 and salinity = 23°/oo). The cultures were incubated at -20, -10, -5, 0, 5, 10, 15, 19, 20, 22, 26, 29, 30, 33, 34, 35, and 37°C. Growth was measured, as usual, by area of outgrowth.

Salinity tolerances were tested from 0 to 100°/oo at intervals of about 2°/oo. To obtain the wide range of salinities, a stock salt solution, made by adding "Seven Seas Salts" to York River seawater, was diluted with distilled water. To better simulate the natural complement of trace elements, pond water was used as the dilutant in the salinities 0 through 10°/oo. This procedure was later found unnecessary and oceanic seawater was used in most routine isolation and culture media.

Cultures used for inoculation were first grown at, or within 10°/oo to the salinity to be tested. This allowed the cultures to acclimate to that salinity and reduced the initial shock of the transfer.

The pH was tested at intervals of about 0.5 of a unit from 4.0 to 9.7. Temperature and salinity were kept near optimal conditions (Temperature = 22°C, salinity = 23°/oo), and microscopic examinations were made to determine any abnormalities.

In this work the pH of the medium was adjusted just before the sterile medium was cooled, thus avoiding precipitates and destruction of the agar as was encountered by Vishniac (1955a).

General

Sections of Z. marina were stained with safranin-fast green (Jensen, 1962) or Harris' hematoxylin-eosin (Johansen, 1940) to show the presence of Labyrinthula. Zoospores were fixed 30-45 seconds in 1% osmium tetraoxide fumes, and were shadowed at 18° with platinum-palladium to show the flagellum detail. The shadowed material was observed and photographed on a Hitachi HU-11B electron microscope. Nuclear stains were made using a Chrom-alum stain (Perkins, personal communication). The procedure is outlined in Appendix A.

RESULTS

Sampling in Field

During the 12-month period from September 1966 through September 1967, 215 plates were inoculated with pieces of Z. marina leaves. Of these, 210 were positive for Labyrinthula. The negative plates were scattered through the sampling period and showed no significant grouping.

Occasionally, it was possible to test the ability to isolate Labyrinthula from healthy leaves as opposed to necrotic ones. In almost every instance, Labyrinthula was isolated from necrotic leaves; but the green unspotted leaves were free of Labyrinthula sp. However, green healthy leaves did occasionally (2 out of 30 times) yield Labyrinthula. In every case the Z. marina from beds near VIMS yielded only one kind of Labyrinthula (Type L1). Washed leaves had no lower incidence of Labyrinthula than unwashed leaves. That is, 92 out of 115 fronds were positive in the washed group; and 97 out of 111 were positive in the unwashed group. A Chi-square test showed these values to be essentially equal. Leaves were washed in 0.1% chlorox in sterile seawater. Figure 3 shows Labyrinthula growing from a sample on isolation medium, and Figure 3 shows the protist in Z. marina tissues.

During the summer of 1966 several animals and plants of the

Chesapeake Bay area were sampled for the presence of Labyrinthula, but only Z. marina samples were positive. This could have been due to the high concentration of agar used early in the study, because it was later evident that a medium containing less than the 1% agar supports better Labyrinthula growth. The type of Labyrinthula in Z. marina was evidently capable of overcoming this limitation. This was corrected by September of 1966. Since that change was made, Labyrinthula has been isolated from several substrates (Table 1).

Most of the samples were taken along the York River and its tributaries where salinities range from about 0.5°/oo to 26°/oo. Other samples were taken from higher salinities along the Atlantic coast from Delaware Bay to Oregon Inlet, North Carolina (Fig. 1). Samples were taken from fresh water ponds and swamps in tidewater Virginia, as well as from estuarine and oceanic environments. Substrates from nearly all aquatic habitats above 3.5°/oo salinity harbored Labyrinthula spp. The protist was not found in water below 3.5°/oo.

Sampling provided several hundred separate isolates of Labyrinthula. Of these, over 25 selected on the basis of host, salinity of habitat, colonial morphology and growth characteristics, were set aside for later study. Over one half were from S. alterniflora and the rest were Z. marina and several algae. A complete list of substrates is found in Table 1. One isolate was obtained from a water sample containing only planktonic organisms. In this case the isolate could have developed from a zoospore in the plankton or from an infected plankter.

Some of these isolates were then used in studies of physiology, life cycles and systematics.

Physiological Tolerances

In attempts to find new stages in the life history of Labyrinthula, various physiological stresses were applied to one isolate (L1). In addition several other isolates were tested over a lesser series of salinities, namely 6, 15, 23, and 30°/oo. The choice of L1 for the major physiological studies was unfortunate since it produced only somatic cells and not zoospores. The salinity ranges of several isolates are shown in Table 2. The following results apply to isolate L1 only.

TABLE 2

Labyrinthula spp. isolates showing their salinity range and preference. Based on tests using salinities of 6, 7, 15, 23 and 35°/oo.

Isolate Number	Salinity Range	Preferred Salinity
L1	7-35	30
L2, L4, L9	23-35	23-30
L8	15-30	23
L11	7-35	30
27	15-23	?
39	15-35	?
44	7-35	7-35
62	15-30	30
64	15-30	15
65	7-23?	7
67	15-35	30
74	15?	15
77	15-30	23
82, 86	23-35	35
84	23-35	23
89	6-30	30
90	7-15	6
94	7-30	?
109, 110, 111a, 111b 113	23-30	30

Salinity

The tests showed survival in a range of 4.0°/oo to 51.0°/oo with maximum growth at 25.5°/oo in acclimated cultures. A broad peak growth was noted from 13.5°/oo to 42.0°/oo. Slight growth, which could only be detected at 100X magnification, occurred from 4°/oo to 7.5°/oo and from 46.5°/oo to 51.0°/oo (Fig. 4).

The only notable feature observed was the lack of visible slime tubes in the culture at the extreme salinities (4.0°/oo - 9.0°/oo and 46.5°/oo - 51.0°/oo). Penetration of the agar was most noticeable at optimal salinities.

pH

The range of pH tolerances was from pH 5.2 to pH 8.8 with a maximum amount of growth occurring at pH 6.5 (Fig. 5). No cellular forms other than the normal spindle cells appeared.

Temperature

Since I was able to isolate Labyrinthula throughout the 2 to 30°C temperature range observed on the collecting trips, experiments were set up to determine its entire temperature range in cultures.

Optimal growth occurred at 20 and 25°C with little difference in growth up to 30°C, after which a rapid decline was evident (Fig. 6). The lowest temperature at which growth occurred was 0°C ($\pm$ 0.5°C). The cultures kept at temperatures lower than 0°C were killed. Although no growth could be observed above 30°C, cultures could be kept at temperatures up to 33°C for 48 hours and then resume growth when

returned to room temperature (24°C - 25°C). A temperature of 35°C for over 6 hours killed the cultures. Those cultures kept at 29°C and above often formed sorus-like aggregations of brown cells. Isolate L1 never formed zoospores or showed the division features leading to sporangial development.

Water Content of the Medium

Throughout the experiments, it was evident that the occurrence of optimal growth was dependent on the availability of a moist substrate. The media used in the above experiments contained 0.8% agar. Later it was found that isolates L8, 27, 39 required a higher relative water content and the agar content was reduced to 0.6% for all cultures. Layering water over the medium was not found to be particularly useful.

Summary of General Morphological Features

The general morphology of six isolates was recorded in an attempt to differentiate them. Although the isolates were not definitely separated into species, Groups I and II were formed. Group I was composed of those isolates which formed sporangia and no plasmodia. Group II consisted of those which formed plasmodia but not sporangia. Appendix B contains a tabular description of the six isolates showing those features which were quantitated. Figure 10a-n show the general morphological features of a few isolates, and Figures 11 and 12 show diagramatic life cycles for each group.

The common characteristic which relates all labyrinthulas is the possession of the tube structures through which they glide. This is

present in all forms during the active somatic portion of the life cycle. During this phase the bulk of the cells are also spindle shaped, but here the similarity ends.

Group I is characterized by isolates L1, L11, 44 and 67. The cells are more spindle shaped, the colony has a fine appearance, and the growth rate is generally fast. Group II is characterized by isolates L8 and 39 which never sporulate but form plasmodial cells readily. The cells are more rounded than those in Group I, and the colony takes on a coarse appearance. Unlike Group I, the cells of Group II form swellings on their slimeways in their early development; and the growth rate is very slow (Figs. 11 and 12).

Life Cycle

Several isolates of Labyrinthula maintained by the author have formed sori and zoospores. Of these isolates, all belong to Group I. The life cycles of two separate isolates (67 and L11), which may be the same species, have been observed. In each isolate one cell was followed during differentiation from zoospore to spindle cell stage. Spindle cells were observed forming sori which, in turn, gave rise to zoospores. Each cycle is similar to that described by Hollande and Enjumet (1955).

In isolates 67 and L11, sorus formation is preceded by aggregation of colorless spindle cells, which make up a typical somatic colony. As aggregation occurs, the cells assume a slight orange color.

The cells within the aggregate cease movement, and the containing walls of the "slime tube" seem to become more rigid. The final form is a complex winding, branching network of tubes which covers as much as 7 square mm on the surface of the agar or, in some cases, which is below the agar surface. The spindle cells enlarge primarily in breadth becoming almost spherical and then by successive bipartition break down into eight daughter cells (Figs. 7a-b). These are still contained within the membrane delimiting the mother cell. While contained in this sporangium, one can see that each daughter cell has a bright red chromatic body consisting of three or, less often, four juxtaposed spherules. This body may be comparable to the "stigma" of Hollande and Enjumet (1955). Flagella then become evident. Daughter cells can be seen moving about while still encased in the mother cell membrane and sorus wall. Forty-eight to 72 hours after initial aggregation, zoospores begin to break through the sorus wall in several places. Due to differences in the rate of aggregation, some sections of the sorus sporulate later than others thereby extending sporulation for 1 to 2 days.

When examined closely, it is found that the zoospores are pyriform and mid-laterally biflagellate with the posteriorly directed whiplash type flagellum about one-half the length of the anteriorly directed tinsel flagellum. Isolated zoospores may swim about for 12 to 150 hours before settling down and losing their flagella. The tinsel-type flagellum is lost first. It first becomes sticky enough to entangle itself and then slowly fades from view. This occurs immediately, or

within 1 hour after settling down. The whiplash flagellum slowly shortens and disappears in about 36 hours. Whether this flagellum is withdrawn or dissolves cannot yet be determined. After the first flagellum is lost, the cell tends to become round and then enlarge longitudinally. The pointed anterior end of the zoospore is maintained throughout the differentiation process. In about 48 hours the cell becomes a small (7μ), roughly spindle-shaped cell and begins formation of the "slime" tube network. The cell may divide once before moving away from the place where the zoospore settled. Chromatic bodies in the young zoospores were not followed and their fate is unknown. Figures 6c-f show the development of a single cell from zoospore to spindle.

Zoospores are fairly consistent in size: 5μ long x 3μ wide with the anterior flagellum 14-15μ long and the posterior flagellum 7-9μ long. Electron microscopy revealed two apparent rows of thick mastigonemes or tinsels (1.3 to 1.6μ long) along opposite sides of the anterior flagellum. The posterior flagellum was naked and ended in a thinner whiplash (Fig. 8). The planonts move by an undulation of the anterior flagellum and a jerking of the posterior flagellum. Together these produce a spiraling, forward movement.

Isolate (44) showed another sorus type and slightly different zoospore, but the life cycle of this form was not followed. The sori consist of an unbounded hump of cells, possibly similar to Watson's "humps of sporangial cells" (Watson, 1957). The color is identical to

that of the previously described isolate. Sori form by the simple massing of spindle cells and do not show a delimiting sheath of the same tensile strength as isolate 67. The cells undergo three divisions to yield eight cells which then become biflagellated as in the previous type. The isolate has not three but four spherical bodies linked together to form the chromatic body in most daughter cells. Whether or not these spherical bodies are truly linked could not be determined under the light microscope. The zoospores are 4.9μ long by 2.7μ wide at the widest point and are roughly pyriform. The anterior flagella is about 13μ long and the other is 5μ long. It is presumed that the life cycle is similar to the previous isolate.

Even though an intensive effort was made to determine the conditions necessary for sporulation, only very tentative statements can be made. Sporulation was maintained or induced by transplanting presporangia or densely growing cultures to fresh medium by using a culture temperature of 22-24°C, low agar content, and the three vitamins in Medium Number 1. Sporulation occurred only in cultures using yeast as food.

It was found that isolate 67 required cholesterol for growth of its vegetative phase, but sporangia could be formed without it. Sporangial development was not enhanced by lack of cholesterol.

Isolate L1 never formed a true sporangium or zoospores, but on several occasions formed structures having the same morphology as the sorus of isolates L11 and 67. The only difference was that the spindle

cells did not divide into eight cells which became zoospore precursors. The color of the "sporangia or presporangia" was dark brown. In old cultures on yeast one sees aggregates different from the above brown aggregates. The former are spherical and possess a brown pigment. These will remain as such for long periods of time and when transferred to new medium, give rise to a robust growth of Labyrinthula. The dense pigment in these aggregates obscures cytological detail. Whether or not this represents a cyst is not clear.

Those isolates not forming zoospores and which have plasmodia are members of Group II. The isolates of this group have a different mode of cultural existence from those of Group I. Isolates L8, 27, and 39 are of Group II. In general, they require a more liquid medium for growth. The first stages of growth show typical spindle cells which are not quite as pointed on either end as the isolates which produce sporangia. The rows of cells in the colony seem to branch at a less acute angle and slime trails are not discernable under low power (100X). As the culture grows older, several cells line the outside of the slime tubes giving the colony a more coarse appearance. The outer cells enlarge as the colony ages, and they undergo several karyokineses to form plasmodial cells. The plasmodia lose the typical spindle shape and look somewhat amoeboid, although amoeboid movement was not noted. The slime substance is present for some time but disappears after 2 to 3 days. These cells sometimes form clumps pale yellow in color (isolates L8 and 39) containing cells ranging in size from 3μ to

77u or larger, and having various shapes ranging from spindle to amoeboid-like cells. Many dividing plasmodial and spindle cells are seen. Cultures of this type have been stored for over 9 to 10 months in agar (Medium C1) at room temperature without losing their ability to initiate new colonies. The new colonies at first form spindle cells in slimeways and then later form plasmodia. It is therefore assumed that the plasmodia divide to form new spindle cells (Fig. 9). Although the plasmodial stage seems to be resistant, no cyst wall was noted. However, it was shown that spindle cells of an old colony would not initiate new growth, but plasmodial cells would. This indicates that plasmodia are more resistant to adverse conditions than spindle cells.

Sexual Reproduction

The question of sexuality in Labyrinthula spp. has been ignored in the literature, but in this study attempts were made to determine whether or not sexual reproduction occurred. When planonts were produced, no fusion was seen even if zoospores of two isolates were mixed, thus indicating that the planonts were indeed zoospores. In a further attempt to demonstrate sexuality, several isolates were grown on the same petri dish so that they could intermingle. No evidence of plasmogamy or karyogamy were observed in this case either. Plasmodial morphology did not indicate the presence of karyogamy, but numerous observations on this point were not undertaken.

Although the above should indicate the lack of sexual activity in Labyrinthula spp., electron micrographs of immature sori show

presumptive synaptonemal complexes which indicate meiosis. Further study is needed to verify this point.

Nutrition

When Labyrinthula (isolate L1) was first isolated, it was accompanied by a pink yeast, Rhodotorula sp. Later attempts to grow Labyrinthula without the yeast or some other food were not successful. The Rhodotorula however did support a vigorous growth. Since the use of a food organism seemed to facilitate growth, the practice was continued throughout the entire experimental period. My modification of Watson's 10% bovine serum medium would support axenic culture about 90% of the time but was not used because of the extreme variability in growth which was obtained.

Several extracts of Z. marina, yeast, and beef were used as nutrients but only one gave acceptable results. A culture of Rhodotorula was autoclaved for 10 minutes at 115°C on medium number 1. When recongealed, this supported a vigorous growth of Labyrinthula type L1, but the poor optical qualities of the medium interfered with microscopic observations. The medium was tested several times for the presence of viable Rhodotorula cells but none were present. Several serial subcultures sustained growth for over 9 months and no contaminations occurred. Thus, this was considered a pure (axenic) culture.

Several additives were made to the basic medium number 1 in an attempt to attain pure cultures. The following is a list of those

additives.

Heat extract of Z. marina, filtered
Homogenate of Z. marina, filtered
Sonification extract of Rhodotorula sp.
Sonification extract of Saccharomyces sp.
Monosodium Glutamate
Beef extract
Yeast hydrolysate
Peptone
Coconut milk
Pine pollen (especially for zoospores)

The above were also used to substitute for various components of medium number 1. In no case could growth be maintained beyond the second transfer in axenic cultures.

It was noted that growth was obtained whenever any contamination occurred. Even the smallest population of bacteria could support Labyrinthula. However, some molds and diatoms had a detrimental effect.

Other material was presented to the Labyrinthula to see what types of foods it would use. Virtually everything tested appeared to be utilized by Labyrinthula. The protist survived and grew using the following substrates: excised oyster tissue (heart), Z. marina fronds, whole human blood, human epidermis, bread mold mycelia (Rhizopus sp.), several natural yeasts, Thraustochytrium sp., the common bacterium E. coli and several green algae (Enteromorpha spp. and Ulva lactuca). Of these a few are of especial interest; namely, Z. marina and Crassostrea virginica, the oyster. The former, because of the involvement with wasting disease and the latter because of the stated relation of Labyrinthula to the oyster pathogen Labyrinthomyxa marina

(Mackin and Ray, 1967) (formerly Dermocystidium marinum, Mackin, Owen, and Collier, 1950). Labyrinthula type L1 invaded excised tissues of both Z. marina and C. virginica.

Z. marina leaves were taken from healthy plants, washed in Chlorox solution (see Methods), and placed on agar plates. Experimental plates were inoculated with Labyrinthula type L1 (originally from Z. marina). The controls remained healthy and uninfected, but the experimental leaves had intercellular Labyrinthula and darkened tissue. The C. virginica tissue, which was exposed to Labyrinthula, was a heart explant in organ culture. Following exposure, the tissue ceased to pulse and was decomposed by Labyrinthula. Control tissues remained pulsing. In all cases vegetative spindle cells were the infective agents. This preliminary evidence led to an experimental attempt to infect living whole oysters. Small holes about 2mm in diameter were drilled in the upper valve just over the mantle, and through this a very dense inoculum of Labyrinthula was introduced into the mantle cavity. The oysters were kept out of water for 20 hours to prevent expulsion of the inoculum. At the end of 14 days in seawater at 22°C, the oysters were sacrificed and sectioned. No typical Labyrinthula cells could be found. Observations of squashed tissues under the phase microscope were also negative.

An attempt was made to grow Z. marina in axenic culture in order to prove its susceptability to Labyrinthula infection and subsequent wasting. Primary axenic cultures proved too difficult to handle and

beyond the scope of the present research, but the medium and methods are present in the Appendix.

Slimeway Structure and Dynamics

The character of the slime tubes is a challenging subject for investigation, but it is given only brief mention in the existing literature. Some isolates have well-developed tubes easily seen using 100X magnification, while others, though they have a similar network, cannot be detected at this magnification. Furthermore, during the development of these tubes, some isolates show distinct swellings which seem to pulsate in the first stages of formation, while the others rarely show similar structures. Figure 13 shows several forms of slime tubes.

Some isolates have tubes which are made of several parallel secretions joined together to form a very wide tube. Often cells traveling in such a system will bypass the main tube and take a smaller tube and then come back to the larger at some point further along the main tube. Sometimes a naked group of cells in a fresh preparation can be seen forming a complex branching network of fine secretions around its entire periphery (Fig. 11a). Later, one avenue will broaden with the passage of the cells.

Watson (1957) notes that, except in one isolate, motion in the tubes is unidirectional. My observations are contrary to this. While the net movement is always away from the point of inoculation, individuals or entire masses of cells have been seen moving opposite to

the main flow. While attempting to observe nuclear divisions in living cells, 5 cells were observed moving back and forth in a blind branch of a tube. They changed direction more or less in unison several times in one hour. While it is not a common occurrence, reversal has been seen in nearly all cultures at one time or another.

In isolates L8 and 39, and more rarely in the others, cells are often noticed outside the tubes. The method of escape, if it is truly escape, is not known; but in L8 and 39 escaped stationary cells are often found bordering the tubes in which other spindle cells are moving. These outer cells in isolates L8 and 39 were often plasmodial precursors.

The formation of the tubes can be observed easily with the phase contrast microscope. The cells, which may be stationary, seem to forcefully extrude out a filament or filaments. These may or may not mass together forming a wider structure. Also, they may or may not branch. The total length of the filament may be as much as 300u. As the cells finally begin to move through it, the substance spreads and then as the cell passes the tube closes behind as though the cell were a zipper mechanism. The slimeway, therefore, must be a very elastic substance. Initial passage through the tube may change its width. Whether its composition is changed is unknown.

DISCUSSION

Sampling in the Field

This study was based entirely on material collected in the field. Labyrinthula was first isolated from eelgrass and that isolate (L1) was used for further experiments on physiological tolerances. Since this isolate appeared consistently in the eelgrass samples and was shown in cells of sectioned Z. marina, it was evident that this was most likely the form described as L. macrocystis by Renn (1936) and Young (1943). This fact made the isolate an obvious choice for further experiments.

As can be seen from the data presented, Labyrinthula seems to be very capable of living in a wide range of habitats. Only freshwater sampling grounds were negative. The temperature and pH ranges could only rarely be exceeded in nature. Even though eelgrass wasting has been described as a summer phenomenon (Renn, 1936), the good growth of Labyrinthula sp. at temperatures near freezing indicates its possible ability to become more ecologically important in the winter months than in summer. During this study, Labyrinthula sp. was isolated from detritus and wind-rows of beached Z. marina and algae thus indicating that it is not an obligate parasite. One form which occurs on Spartina is isolated from the brown outer parts of the submerged stem and is

almost definitely a saprophyte.

New substrates have been discovered, but this is not surprising. Most of these are plants but it is significant to note that oysters (Perkins, personal communication) and clams (Chanley, personal communication) were found to contain unidentified Labyrinthula spp. In the light of the studies relating Labyrinthomyxa to oyster mortality (Mackin and Ray, 1966), this would seem to deserve much more attention. A program of careful investigation of a large number of possible hosts might also reveal some new life cycles and thus the taxonomic affinities of Labyrinthula.

Once a species of Labyrinthula has been isolated it can usually be maintained in monoxenic culture, but research is needed to determine whether all possible isolates of Labyrinthula will respond to this method of culture. It is conceivable that some may be more nutritionally fastidious. Some may do better on a yeast than on a bacterium or vice versa. Some may need an undefined component in the medium such as serum. Because of a noted difference in growth on different media, observations were carried out on medium number 1.

The exact way in which Labyrinthula acquires its food is also poorly understood. It does not phagocytize or photosynthesize. Cultures in which diatoms or yeast had been used as food contain a great number of empty tests which show no apparent points of entry, thereby showing an apparent ability to leach nutrients from the host cells.

When Labyrinthula is maintained in culture with some organism

as a food, there is always the possibility that the kind of food will in some way effect the biology of the Labyrinthula. For example, Schmoller (personal communication), who uses Klebsiella sp. bacteria as a food, has described L. coenocystis from that medium. I have similar isolates (L8 and 39) which are maintained on serum agar and in yeast culture. Until further study is made using my isolate and his food, they cannot be described as separate entities even though they differ in some respects such as color and wall formation. Standardization such as this will be essential to future studies on Labyrinthula. The first major step in this direction should be a vigorous follow-up to Vishniac's (1955a) nutritional study.

Food

The utilization of several foods while in culture indicates that any host-specificity in Labyrinthula may be incidental. However, the isolate from Z. marina would not infect oysters indicating some selectivity on the part of Labyrinthula or resistance on the part of the oyster. The case for host specificity is not a strong one. The Pokorny (1967) literature review reveals that L. macrocystis, L. vitellina and L. minuta have been found in several algae and some spermatophytes. The remaining species have been described only briefly and no range of hosts can be determined.

Since Labyrinthula can utilize these several diverse foods, one must assume that there is a common factor, or factors, provided by each. Possibly one of the key intermediates such as Co-enzyme A or

pyruvic acid may be supplied. How this factor can get to the limits of an actively growing colony is uncertain. Aschner and Kogan (1959) and Porter (1967) have shown that food organisms such as yeast attracted Labyrinthula. Aschner and Kogan gave evidence that the slimeways may be a significant factor in facilitating food detection.

Physiological Tolerances

As can be seen from the results, Labyrinthula has a broad range of physiological tolerances. However, the challenge it meets in nature may be quite different from the ambient environment of the ocean or estuary. Since this organism is closely associated with its host or substrate, it may be limited not only by the environment but by that of its host. Labyrinthula isolated from different hosts are immediately considered different, not because of any outward indications of host-specificity but simply to facilitate handling. It was recognized that some substrate preference could occur.

If one concedes that the salinities and temperature of the environment will be limiting, it is obvious that Labyrinthula has particularly good survival ability in temperate to Arctic climates. Salinity should present no problem in any of the eelgrass communities. Eelgrass (Z. marina) is not normally found below a salinity of 15°/oo. Papers by Meyers et al. (1965) and Orpurt et al. (1964) indicate that Thalassia (turtle grass), the southern counterpart of Z. marina, was also infected with Labyrinthula thus extending the range of Labyrinthula to warmer climates.

Both in this study and Watson's (1951) study, the need for a moist medium was noted. This could be due to its need for a fluid medium to transport nutrients or the need for a surface on which to form its slimeways. Since Labyrinthula often penetrates the agar, a harder (less moist) agar would inhibit this direction of growth. It is also possible that the lack of proper moisture inhibits the formation and maintenance of the slimeways.

Life History

The life history of protistan organisms is an important part of their taxonomy, but few life histories have been elucidated. Sparrow (1958) relates that the zoospore is an important taxonomic feature of phycomycete life histories; therefore, this feature was studied in detail in the present work.

Labyrinthula zoospores have been reported for L. vitellina, L. minuta (Watson, 1957) and for L. algeriensis (Hollande and Enjumet, 1955). Watson's species could not be induced to sporulate on a regular basis and little was said about them. No development of the zoospore was observed. L. algeriensis, described by Hollande and Enjumet, released 4 zoospores from each sporangium (not eight as in my isolate). The zoospores subsequently developed into new vegetative cells. Although this life cycle generally follows with the one I have described, it must be criticized for the lack of single cell observations. Also, it is not known whether L. algeriensis can be induced to produce zoospores with some regularity. The isolates I have maintained do not

decidedly respond to any of my means for achieving sporulation. They do spontaneously form zoospores 50% of the time when the medium is manipulated as described below.

The conditions necessary for bringing forth all the phases of the life cycle are not well understood. Nearly all of the observed cycles occurred in monoxenic cultures although plasmodium formation was often noted in axenic cultures. The host in the monoxenic cultures was consistently a yeast; and although the composition of the basal medium varied somewhat, no changes in cultural forms were noted. The author feels that the production of zoospores may well be attributed in part to the presence of yeasts in the cultures. However, some other parameters are certainly at work since some cultures containing the yeast do not sporulate. Watson (1957) states that a temperature of 13°C to 15°C was necessary for sporulation but the present author has observed that optimal sporulation occurred from 21°C through 25°C (room temperature). Basic nutrients such as glucose, gelatin hydrolysate, etc. do not seem to play a part in that sporangia form both in seawater with yeast present and in enriched agar media plus yeast. Exposure to the air is not detrimental, as is also noted by Watson (1957), but the great majority of sori appear on the mucoid edge of the yeast colonies or below the surface of the agar. Moisture may therefore have some importance in sporulation. For example, when a developing sorus with a few yeast cells was placed in a tube of sterile seawater, the presporangial cells ramified until the entire surface of the

tube below the water surface was laced with a typical sorus. Four days after initiation of this culture the zoospores were released and swimming about. The presporangial body of type L1 was also formed on seawater culture of this kind, but again it did not progress to sporulation.

The yeast may provide a minute quantity of by-product needed for initiation of sporulation. Whatever the case, each strain of Labyrinthula reacts differently in its presence.

Even though a complete cycle in the life history of Labyrinthula has been followed, one must bear in mind that the conditions under which it occurred were far from natural. Indeed, there is much evidence to indicate that the present techniques for culturing Labyrinthula are, at most, marginal. Watson (1951) states that his serum medium often did not work, though it was superior to those media used by Vishniac (1955a). Mackin (personal communication) reveals that fresh serum is the only reliable form for supporting growth. In the present work it has been found that the serum medium is very sensitive to improper handling and that reproducibility of results is not good when using it. The fact that living hosts support more vigorous growth indicates the need for a common but somewhat labile substrate. If cultural conditions are borderline, one can reasonably assume that anomolies could occur in the cultures.

If Labyrinthula is assumed to be like many other lower fungi, protozoa, or alga, a resistant or cyst stage would be predicted somewhere in the life cycle, possibly after zoospore release as in

Saprolegniales. Other stages might also come to light, most important of which might be some sexual stages.

From the above statements it follows that little is known of the true nutritional or physiological needs of Labyrinthula. Vishniac (1955a, 1955b) has made some preliminary contribution to the question of nutrition but did not follow any corresponding life histories. Elucidation of true life cycles is to be found only in exhaustive study of nutritional and physiological parameters of the organism.

Morphology

In the absence of adequate taxonomic criteria, at present, it is nearly impossible to classify each isolate as to genus and species. It must suffice to say that they are members of the Labyrinthuidae, and that they are different from one another in the respects described in the Appendix.

For taxonomic reasons much work needs to be done on several factors, such as, nuclear morphology, planes of division, structure of the tubes, and the size of cells. The nuclear features reported above are hard to interpret, but harder yet are those concerning division planes. Because the cells divide slowly, are in almost constant motion, and are densely granulated, it is difficult to follow a single cell through the process of division. Figure 10(a-e) shows a chrom-alum stain of nuclear features. The literature cites cases of shifting planes of division (Johnson and Sparrow, 1961) in several different "species." This information, while difficult to work with and interpret, may be of

significant value.

The structure and morphogenesis of the tubes is receiving a bit more emphasis now by investigation with the electron microscope (Hohl, 1966; Perkins', personal communication; Pokorny, 1967), but the physical and chemical process of formation is still an enigma. More simple, perhaps, would be an investigation on speciation by length-width ratio of individual cells. The width factor seems more consistent in my study and observations lead me to believe this would be of significant taxonomic value. This is based on the observation that some cultures could be separated on the basis of cell "plumpness." A statistical treatment of this subject is warranted. To get a better understanding of this, look back to the length-width ratio (L/W) of each isolate.

As was mentioned above, the Group II isolates have a coarse appearing colonial morphology. This is one of the few definite statements possible concerning colonial morphology. Yet, it is evident that each isolate could be separated on the basis of three features of the colonial morphology; 1) the colony edge, 2) the colony density or coarseness, and 3) the extension and vigor of the colony in a stated time period. Unfortunately, there is no concrete basis on which to verbalize these subtle variations. Therefore, photographs or detailed illustrations must suffice. Furthermore, it must be determined whether populations exhibiting these differences are specific in stature.

Although it is not likely to completely solve taxonomic problems,

a wealth of information lies in the study of ultrastructure. The only ultrastructure revealed in this study is the tinsel on the anterior flagellum of the zoospore, but this alone may have far reaching phylogenetic implications.

Tubes of slimeways have long been a subject of debate. Some claim that they are pseudopodial (Zopf, 1892). Some say they are tracks over which the cells glide (Young, 1943) and others claim that they are tubular in nature (Watson, 1951). Hohl (1966) and Perkins (personal communication) have shown by the electron microscope that they are indeed tubular structures. Photographs made by Perkins show a limiting double unit membrane not shown by Hohl (Fig. 11). How this structure is formed and whether it has phylogenetic significance, both remain unknown.

Taxonomy

In the foregoing statements the labyrinthulas have been referred to by number only. This was done primarily because I feel there is insufficient data to accurately describe and differentiate each species. A few characteristically well described species such as L. minuta would be easy to key, but the overwhelming number of isolates described by letter makes this nearly impossible (Pokorny, 1967; Johnson and Sparrow, 1961; and Watson, 1957). It is my belief that it would be highly artificial and possibly detrimental to include these in already existing groups. However, it would also be unrealistic to give them new species names without further investigation.

My isolates have been divided into Groups I and II, and I suggest that there may also exist two additional groups in the family Labyrinthulidae, Groups III and IV. In this manner Group I includes those labyrinthulas which produce sori of the type described. Group II does not form such sori, but the spores are produced directly from the division of the plasmodium. Group III would include those forms similar to L. minuta which has a unique form of division which produces tetrads. Since this form also forms plasmodia, it may be part of Group II. Group IV would then include those forms such as Labyrinthomyxa pohlia described by Schmoller (1965, 1966a). These are unique in that they have true amoebae in their life cycles.

It is interesting to note that this family has affinities to several groups, especially if each group is taken individually. Group I has affinities to both the Xanthophyceae and/or Phaeophyta and the Phycomycetes by virtue of its biflagellated zoospore stage. Xanthophyceae and Phycomycetes have biflagellate zoospores with an anteriorly directed tinsel flagellum and a posteriorly directed naked whiplash (Smith, 1955 and Sparrow, 1958, 1960). Group II, because of the plasmodium, may be related to the Plasmodiophorales and Group IV may be related to the Acrasiales (cellular slime molds) on the basis of amoebae encystment. Solely on the possession of a truly amoeboid form of movement, Group IV could be related to the Rhizopodea. Pokorny (1967) and Watson (1957) have treated the subject of systematics quite well.

Based on the possession of a phycomycete-like set of flagella, I must place Labyrinthula in the fungi. It may belong somewhere along the Phycomycetes line or it may be a primitive group derived directly from the Xanthophyceae or Phaeophyta. The red chromatic body is very closely associated with the flagella and could, as mentioned by Hollande and Enjumet (1955), be a stigma or eye spot. Since they also have said a leucoplast is present, that may further support the hypothesis. If the labyrinthulas are truly a line of non-photosynthetic algae, they might be considered a link between the algae and fungi.

To further clarify the affinities of the isolates, the following table relates the numbered isolates to some previously described forms.

TABLE 3

Previously described species of Labyrinthula which most closely resemble the present isolates.

Isolate	Similar to	References
L1	L. macrocystis or Watson LX	Watson, 1957; Johnson and Sparrow, 1961
L8 & 39	L. coenocystis or L. vitellina	Schmoller, 1960; Watson, 1957
L11 & 67	L. algeriensis	Hollande and Enjumet, 1955
44	L. vitellina	Watson, 1957

The following is a list of the genera I feel are members of the Labyrinthulidae:

	References
Labyrinthula spp.	Watson, 1957
Labyrinthomyxa spp.	Schmoller, 1966
Labyrinthularhiza spp.	Chaudfaud, 1956
Pyrrhosorus marina	Juel, 1901
Pseudoplasmodium aurantiacum	Molisch, 1926

Phagomyxa (Karling, 1944) has possibly been described as Labyrinthula but is a member of the Plasmodiophorales. Schmoller (1965) relates Vahlkampfia to Labyrinthula, but further evidence is needed on this point. The relation of Chlamydomyxa spp. to Labyrinthula has been suggested, but a careful examination of the literature reveals no good reason for relating them (Archer, 1875; Geddes, 1882; Lankester, 1896; Penard, 1904). Chlamydomyxa is probably more closely related to a form such as Leptomyxa (Hall, 1953; Kudo, 1966).

SUMMARY

1. From several hundred individual isolations, six isolates were put aside for observations of nutrition, life cycle and comparative morphology. All of the isolates were maintained in monoxenic cultures.

2. Experiments determined the physiological tolerances of one isolate, type L1. The tolerances are as follows:

A. A salinity optimum of 22 to 30°/oo in a range of 4 to 51°/oo.

B. A pH optimum possibly near 6.5 with a range of 5.2 through 8.8.

C. A temperature optimum of 19°C to 25°C with good growth 0°C to 33°C. Twenty-four hours at 35°C killed the vegetative cells.

3. Sori from several isolates were studied. Preliminary results indicate that sorus morphology may be distinctive for each species.

4. Zoospores from sporangia were shown to give rise to new vegetative colonies. They have been divided into two strains or species on the basis of the morphology of a chromatic body and the morphology of the colony in sporulation.

5. Stages which are resistant to aging and adverse conditions were noted. Although no true cysts were identified, their presence was proposed.

6. Two new spermatophyte substrates for Labyrinthula were discovered. Juncus sp. is the major substrate, and another grass (Distichlis sp.) served as a substrate for Labyrinthula in two instances. Several attempts were made to isolate Labyrinthula from other material in many environments but only Z. marina, Spartina sp., Juncus sp. Distichlis sp., and a few algae were positive. Spartina sp. seems to be the major substrate.

7. Several attempts to axenically culture Labyrinthula met with only partial success. Pure culture was achieved in two media, bovine seawater agar and a seawater agar containing a heat extract of yeast. Most cultures were monoxenic and the author feels this may be partly responsible for the success in eliciting zoospores.

8. Labyrinthula strains have been grouped by host, cell shape, and size, slime-way morphology, morphology of the zoospores, morphology of the sporangia and colonial type. No species designations were made since the available taxonomic criteria are insufficient.

9. Labyrinthula was isolated from Z. marina in weekly samples for a full year, thereby showing the ease in isolation and non-seasonal occurrence of the protist.

10. The life cycles for two groups are discussed.

11. Relation of Labyrinthula to the Phycomycetes, on the basis of zoospore morphology, is postulated.

12. Slimeway morphology and formation is described.

13. Use of Z. marina and C. virginica as foods is demonstrated in culture conditions, but not in whole living hosts.

The features of this thesis which are of the utmost importance are found in the description of zoospore formation. Since zoosporulation is an important step in the life cycle, it deserves even further study.

Another important factor included in this thesis is the pronouncement of a refined technique for preparing Watson's bovine serum medium. The new technique gives a 90% chance of attaining pure Labyrinthula culture.

APPENDIX

APPENDIX A

Gomori-Conger Staining Procedure Used to Demonstrate Nuclear Detail in Labyrinthula (Perkins, personal communication)

1. Fix in 3:1 100% ethyl alcohol--acetic acid for 15 min. (prepare fresh).

2. Hydrolyze in 1N HCl for 10 minutes at 60°C.

3. Rinse in tap water.

4. Stain 15 to 30 minutes at 60°C in Gomori's chrom-alum stain:
   a. 1.5g chromium potassium sulfate in 50ml distilled water.
   b. 0.5 hematoxylin in 50ml distilled water.
   c. Mix.
   d. Add 2ml 5% potassium dichromate plus 2ml 5% H_2SO_4. Ripen two days. Expires in 7 days.

5. Rinse in tap water.

6. Destain up to 1 hour in 45% acetic acid using two changes of acetic acid. (Use low power to observe destaining.)

7. Place in two changes of 95% ethyl alcohol, 1 minute each.

8. Absolute alcohol 1 minute (add a pinch of fast green here to stain cytoplasm lightly).

9. Xylene 5 minutes.

10. Mount.

11. Chromatin is black, rest of cell is colorless. Staining fades in few months.

APPENDIX B

General Characteristics of Selected Labyrinthula Isolates

Growth rates for the following isolates are represented on a comparative basis, four pluses being the highest rate and one plus being the lowest rate.

L1 (Group I)

Length	12.6μ, Range 5.0-20.0μ
Width	4.4μ, Range 2.5- 7.5μ
L/W	2.86

Nucleus 1.5-3.5μ
Endosome 0.5μ central
Plane of division - diagonal - longitudinal

Tubes
- Size - average - variable
- Swelling - none

Reproduction - other than binary fission
- Plasmodium - none
- Sporangia + Presporangia
- Zoospores - none
- Flagella - none

Edge of Colony - Tight

Growth rate - Fastest (++++)

Host - Zostera marina

Salinity Optimum - 30°/oo

Cytoplasm - granular

Color - white

Colony dense with edge showing trails several cells wide. Forming brown resting cells or presporangia which give rise to vigorous growth when transplanted. Dense band of cells on leading edge of colony.

L11 (Group I)

Length 10.1μ, Range 7.0-19.6μ
Width 3.0μ, Range 2.0- 4.9μ
L/W 3.36

Nucleus 1.9μ
Endosome 0.6μ central
Plane of division - longitudinal

Tubes
Size - varied
Swellings - not seen

Reproduction - other than binary fission
Plasmodia - none
Sporangia + yellow-orange
Zoospores + 5μ long x 3μ wide
Flagella - length not determined

Edge of Colony - homogeneous

Growth Rate - average to slow (++)

Host - Spartina alterniflora

Salinity Optimum - 7-35°/oo

Cytoplasm - granular, but granules not very dense

Color - white

Colony with powdery appearance; no definite radiate appearance seen in other isolates. No channels in a particular direction. Slimeways rarely have more than 1-3 cells abreast. Entire colony evenly spaced in 3-dimensional network. Sensitive to overcrowding by yeast.

44 (Group I)

Length 11.3μ, Range 7.0-15.0μ
Width 4.2μ, Range 3.0- 5.0μ
L/W 2.69

Nucleus 2.5μ
Endosome 0.75μ central
Plane of division - longitudinal

Tubes
Size - varied thin
Swellings - none

Reproduction - other than binary fission
Plasmodium - none
Sporangia + orange-hump-mass
Zoospores + 2.7 x 4.9μ (4 spheres)
Flagella + 5μ whiplash - 13μ tinsel

Edge of Colony - Rim of white - tight but not massed. Older colony of cells with dense foci of cells in several places.

Growth rate - fast (+++)

Host - Spartina alterniflora

Salinity Optimum - 7-35°/oo

Cytoplasm - granular

Color - white

Fast growing with noticeable dense band of cells on the leading edge of colony. Resembles L1 in this aspect, but never forms brown cells as in L1.

67 (Group I)

Length 10.0μ, Range 7.0-20.0μ
Width 4.1μ, Range 2.5- 5.0μ
L/W 2.43

Nucleus 2.0μ
Endosome 0.5μ central
Plane of division - longitudinal

Tubes
Size - varied - thin
Swellings - none

Reproduction - other than binary fission
Plasmodium - none
Sporangia + orange labyrinth
Zoospores + 3 x 5μ (3 spheres)
Flagella + 8μ whiplash - 14.5 tinsel

Edge of Colony - tight - but not massed

Growth Rate - fast (+++)

Host - Spartina alterniflora

Salinity Optimum - 15-35°/oo

Cytoplasm - granular

Color - white

Edge of colony has ragged appearance as if composed of several minute bushes. Band of L1 and 44 not so noticeable.

L8 (Group II)

Length	11.5μ, Range 6.0-17.0μ
Width	6.0μ, Range 3.0- 9.8μ
L/W	1.91

Nucleus 2μ (variable?)
Endosome 1μ central
Plane of division - diagonal

Tubes
Size - coarse - fine at first
Swellings - in early development

Reproduction - other than binary fission

Plasmodium	+ many nuclei after 2-3 days culture
Sporangia	- none
Zoospores	- none
Flagella	- none

Edge of Colony - Lobate - mostly surface

Growth Rate - slow (+)

Host - Enteromorpha minima

Salinity Optimum - 23°/oo

Cytoplasm - granular or fine (two sizes of granules in one cell)

Color - white

Colony initially forms spindles in tubes, later forms masses of plasmodia. Tubes, initially formed, end in blunt club-like structures after 2-3 days. Spindle development continues longer in liquid culture.

39 (Group II)

Length	10.9μ, Range 3.0-23.0μ
Width	6.4μ, Range 2.0-10.0μ
L/W	1.70

Nucleus 1.0-1.7μ (often little off center)
Endosome 0.25-0.30μ central
Plane of division - diagonal

Tubes
Size - thin at first to wide in old
Swellings - in early development

Reproduction - other than binary fission

Plasmodia	+ after 2-3 days culture
Sporangia	- none
Zoospores	- none
Flagella	- none

Edge of Colony - massing - coarse

Growth rate - slower than L8 - slow (+)

Host - Spartina alterniflora

Salinity Optimum - 15-35°/oo

Cytoplasm - varied fine

Color - white

Generally colony appears similar to L8 in most respects except that growth is slower. Cells seem plumper than L8.

APPENDIX C

Formula for Growing Axenic Z. marina

In an attempt to grow Z. marina for inoculation with Labyrinthula, preliminary cultures of Z. marina, free of other organisms, were grown in test tubes using a formula devised by Franklyn D. Ott. This medium proved quite adequate and its preparation is given below.

In order to free the culture of contaminating organisms, the whole plant was first washed vigorously in filtered seawater. Then the plant was stripped of its outer leaves, one at a time, taking care not to contaminate the inner ones with debris from the others. The root section was broken away just below the meristematic area. When a small leaf primordium about 1cm long was left, the entire piece was washed in sterile seawater with 0.1% Chlorox, then in two additional washes with 0.04g/100ml of Streptomycin and Penicillin added. This was then transferred to a culture tube and incubated at 20°C with constant illumination. Good growth began immediately and fronds 5cm long were attained before the experiment had to be terminated for lack of time.

It is suggested that these fronds should have been transferred to larger vessels with sterile sand in the bottom to accommodate the roots. This should allow growth of a larger plant if desired.

OTT'S MEDIUM

This medium when prepared according to the schedule given below produces a clear, precipitate-free medium:

I. To 4000ml of filtered seawater, add the following, and boil (or sterilize):

(1) 80ml of 1% $NaNO_3$

(2) 1.6ml of tris (Hydroxymethyl) amino-methane, (Tris Buffer), stock solution at the concentration of 250 mg/ml and adjusted to pH 7.3 with HCl. Use only with aeration of cultures. The buffer is toxic to some algae.

II. To 50ml of distilled water, add the following, and boil (or sterilize):

(1) 4ml each of micronutrient solutions Nos. 1, 2, 3, and 4. (See A below for their preparation.)

(2) 4ml of FeEDTA, 1ml = 1mg of Fe, (EDTA = Ethylene - diaminetetraacetic acid); see B below for its preparation.

III. To 50ml of distilled water, add the following, and boil (or sterilize):

(1) 10ml of 1% sodium glycerophosphate

IV. When (I) above has cooled, add II and III above, and 4ml of the 3-vitamin stock solution; see C below for its preparation.

A. Micronutrients: These have been modified from those presented by Deason and Bold (1960) in that the $CuSO_4$ is omitted in micronutrient solution No. 2, and the designations of the stocks have been changed.

		g/l
(1)	$FeSO_4.7H_2O$	4.98
	$ZnSO_4.7H_2O$	8.82
	$MnCl_2.4H_2O$	1.44
(2)	MoO_3	0.71
	$Co(NO_3)_2.6H_2O$	0.49

(3) EDTA 50.0

KOH 31.0

(4) H_3BO_3 . . . . . 11.42

B. FeEDTA: This is prepared by the method of Provasoli (1962) but at 10 times the concentration thereof.

To 500ml of distilled water add, and dissolve:

$Fe(NH_4)_2(SO_4)_2.6H_2O$ . . 3.51g

Na_2EDTA. 3.30g

C. 3-Vitamin Stock Solution: The following is modified from Guillard (1961).

To 100ml of distilled water add:

Biotin. 0.1mg

B_{12} . . . 0.1mg

Thiamin. HCl 20.0mg

This stock solution is dispensed in 4ml lots in screw capped test tubes, and sterilized. It then should be kept frozen until used.

APPENDIX LITERATURE

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FIGURES

Fig. 1. - Area sampled for Labyrinthula spp. The primary sampling area is shown in the inset of the York River. Hatching indicates areas sampled.

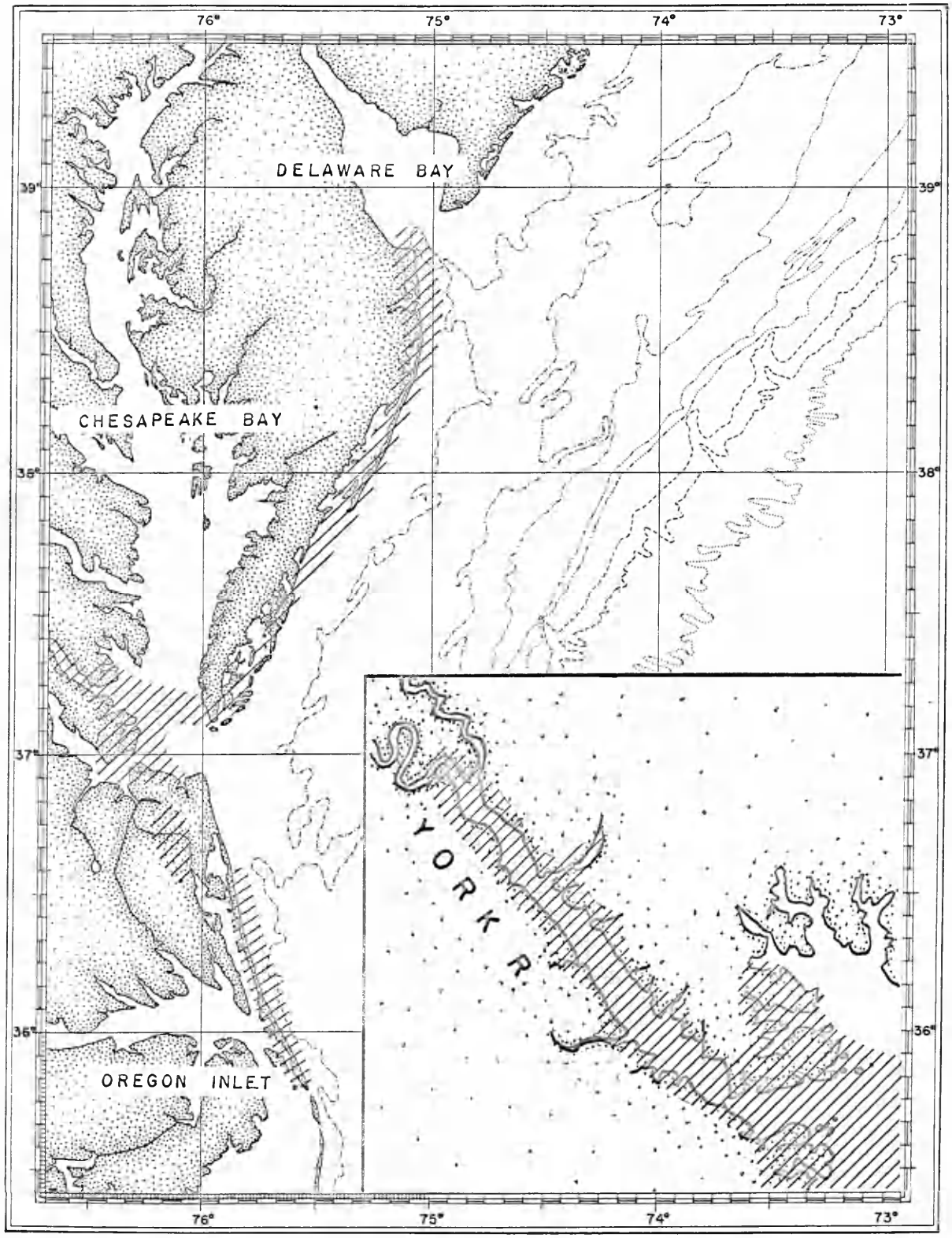
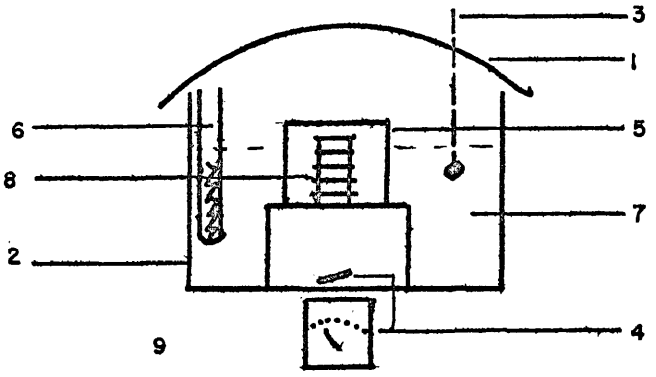

76°
75°
74°
73°
39°
38°
37°
36°
DELAWARE BAY
CHESAPEAKE BAY
OREGON INLET
YORK R.

Fig. 2. - Design of the device used to control temperatures. A list of components is included.

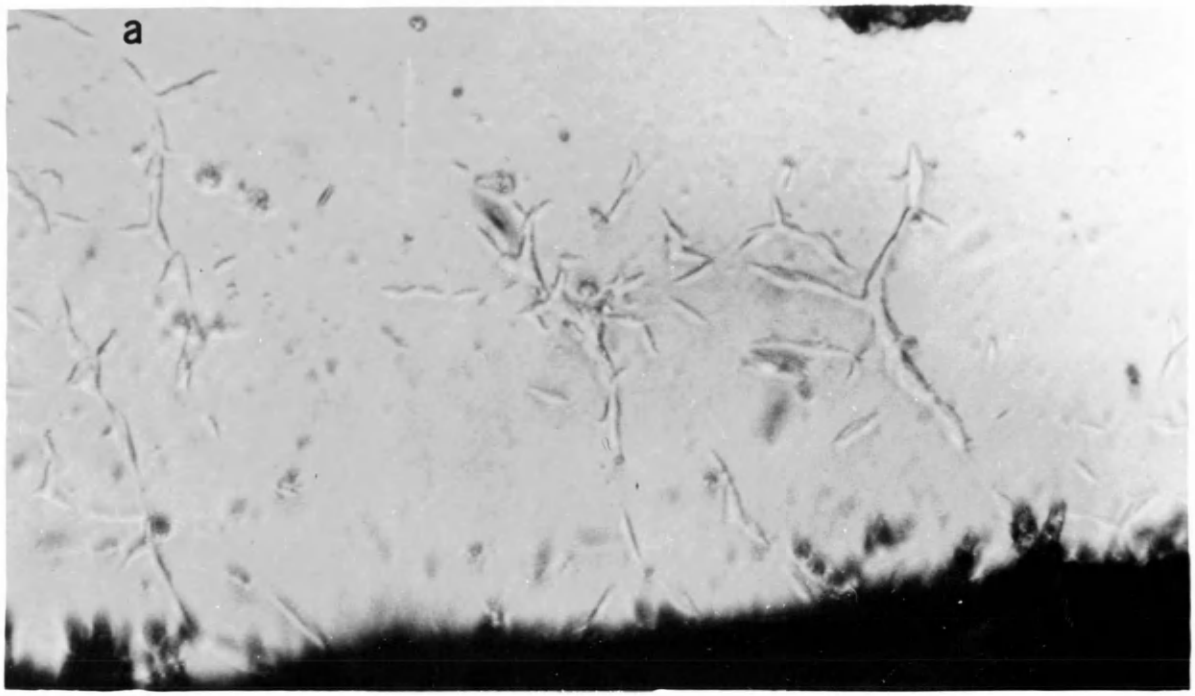


1. Aluminum foil
2. Plastic wash basin
3. Thermometer
4. Magnetic mixer
5. Glass container
6. 100W aquarium heater
7. Water
8. Petri dishes and cultures
9. The entire device is in a constant temperature room 5°C or 20°C.

Fig. 3. - Labyrinthula associated with Zostera marina.

a) Labyrinthula growing out from Z. marina on an isolation plate. 365X.

b) Labyrinthula in a mesophyll cell of Z. marina. Section was stained with Safranin O and Fast Green. 2530X.


a


b

Fig. 4. - Growth of Labyrinthula (L1) in square units (9 sq. mm/unit) with respect to salinity. Salinities are given in parts per thousand (°/oo).

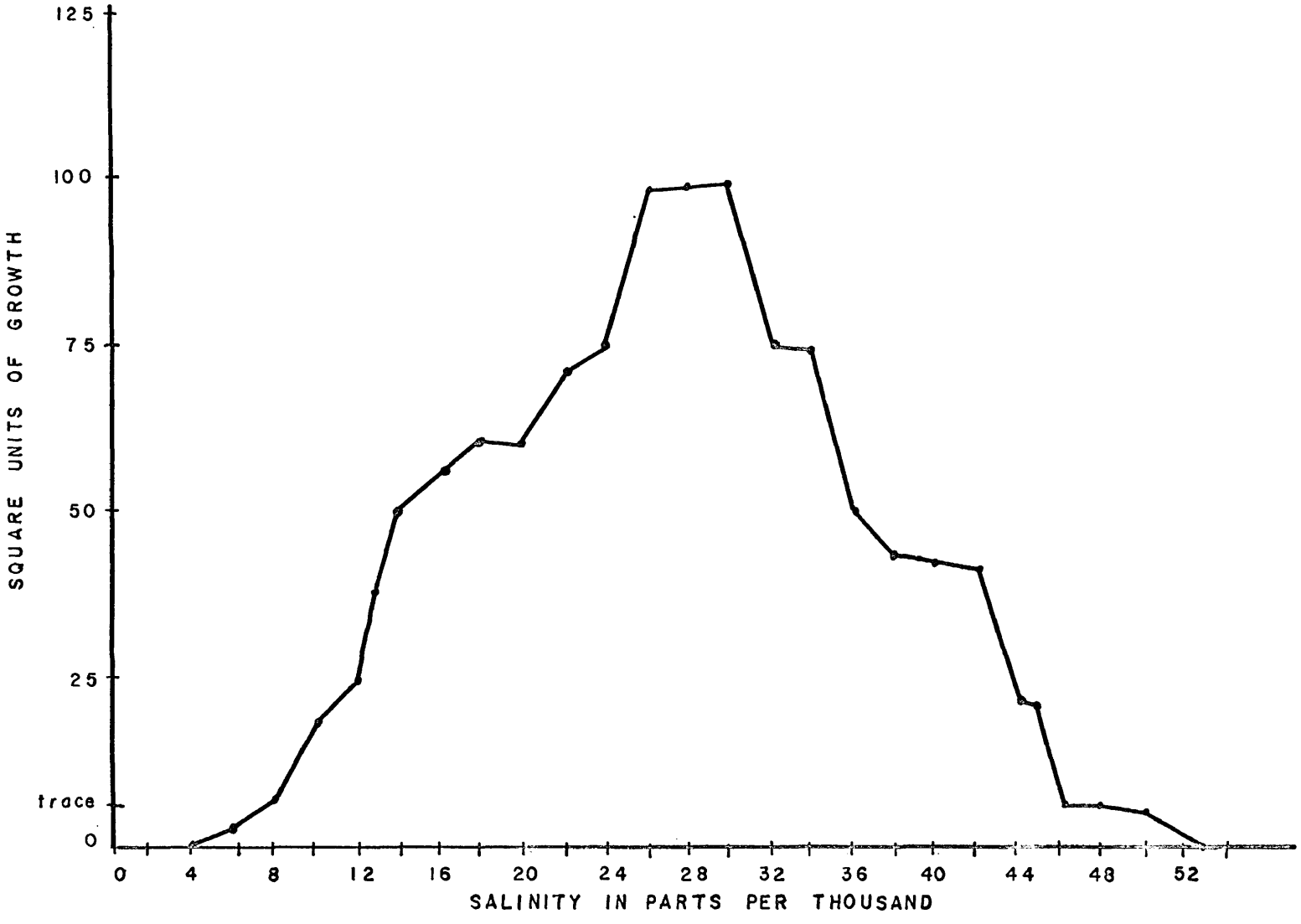

125
100
75
50
25
trace
0
SQUARE UNITS OF GROWTH
0
4
8
12
16
20
24
28
32
36
40
44
48
52
SALINITY IN PARTS PER THOUSAND

Fig. 5. - Growth response of isolate L1 with respect to temperature. The measurements were obtained using 10 day old cultures.

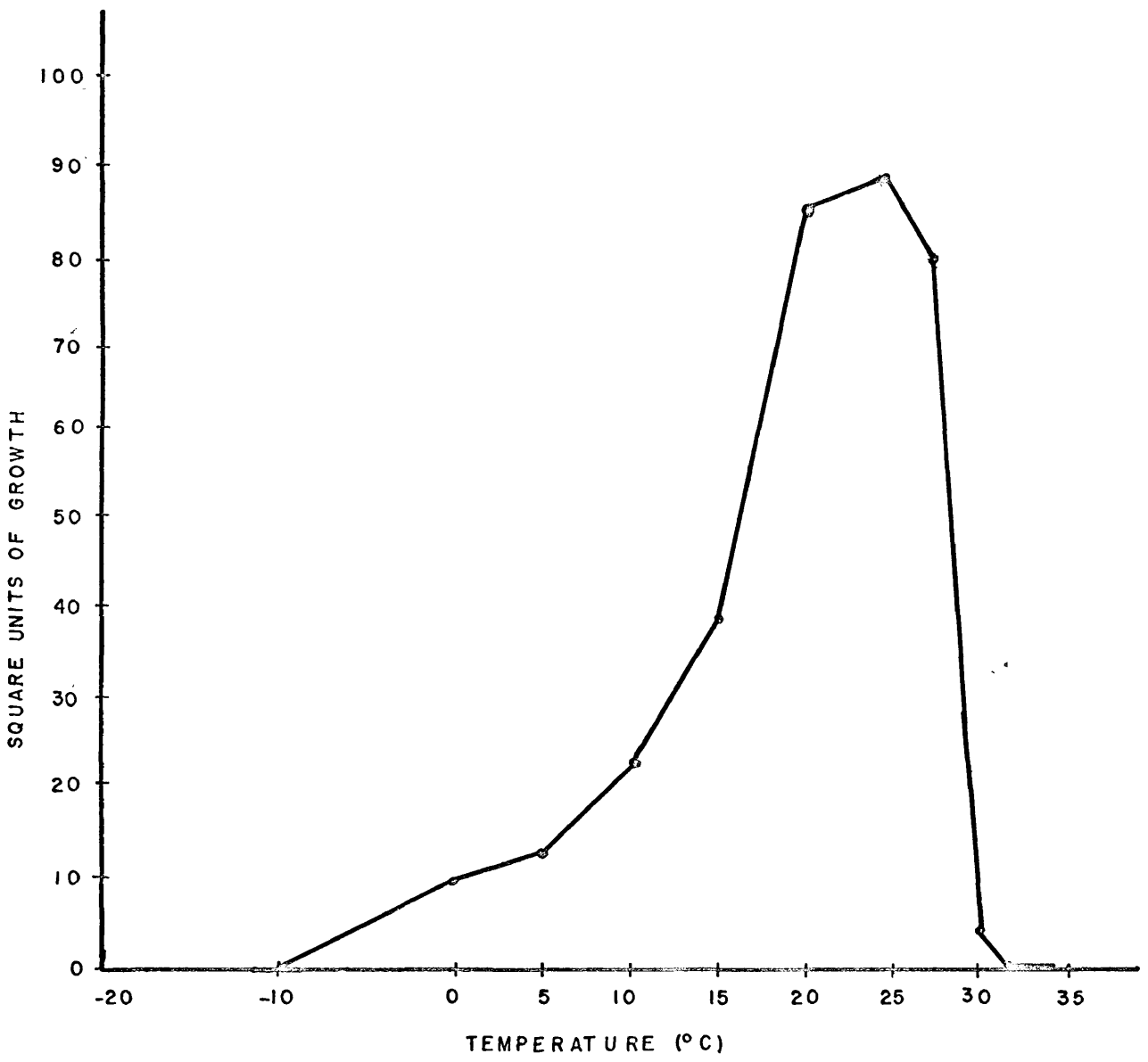

SQUARE UNITS OF GROWTH
100
90
80
70
60
50
40
30
20
10
0
-20
-10
0
5
10
15
20
25
30
35
TEMPERATURE (°C)

Fig. 6. - Growth of isolate L1 as a function of pH.

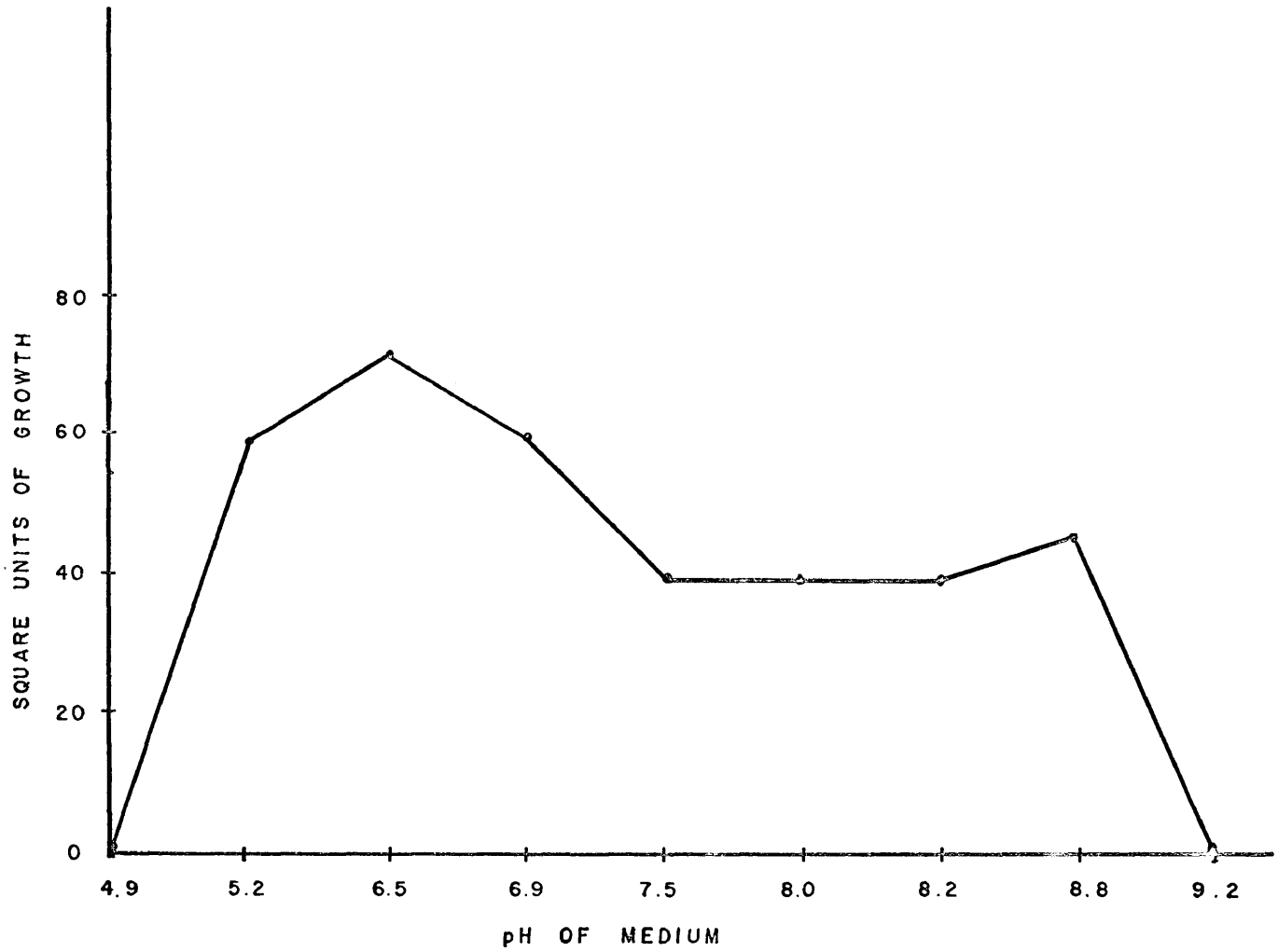

SQUARE UNITS OF GROWTH
80
60
40
20
0
4.9
5.2
6.5
6.9
7.5
8.0
8.2
8.8
9.2
pH OF MEDIUM

Fig. 7. - Zoosporulation in isolate 67.

a) Forming sorus with few spindle cells still visible. Dark-medium phase. 300X.

b) Mature sorus showing sporangia containing zoospores. Each of the dark bodies is a chromatic body of a zoospore. Bright field. 1500X.

c) Swimming zoospore; plus seven hours after release from sporangium. Dark-medium phase. This zoospore is the same cell as in Figs. 7d, e, and f. 2100X.

d) Zoospore has settled down and lost one flagellum (anterior); plus 8 hours after release from sporangium. Other cells which have already transformed are beginning to crowd the zoospore. Dark-medium phase. 1300X.

e) Posterior flagellum has shortened; plus 9 hours after release from sporangium. Cell is still at place where it settled. Dark-medium phase. 1250X.

f) Flagella have disappeared and the cell has assumed the typical spindle cell shape; plus 16 hours after release from sporangium. The light area in the central portion is the nucleus. Dark-medium phase. 1700X.

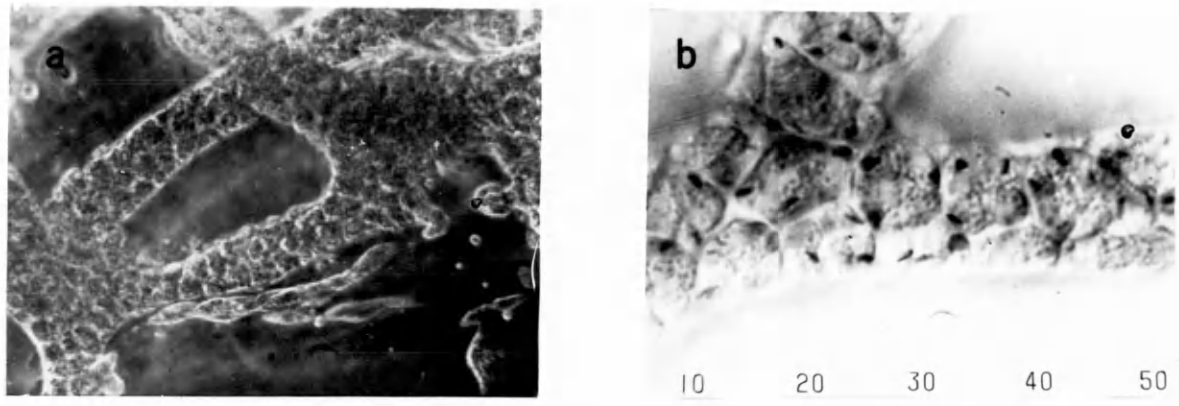

a
b
10
20
30
40
50
c
d
e
f

Fig. 8. - Fine details of Labyrinthula zoospores.

a) A diagramatic representation of a zoospore showing the nucleus (N), stigma (S), tinsels (T), anterior flagellum (A), posterior whiplash flagellum (W), and the lipid inclusions. Scale = 1 μ.

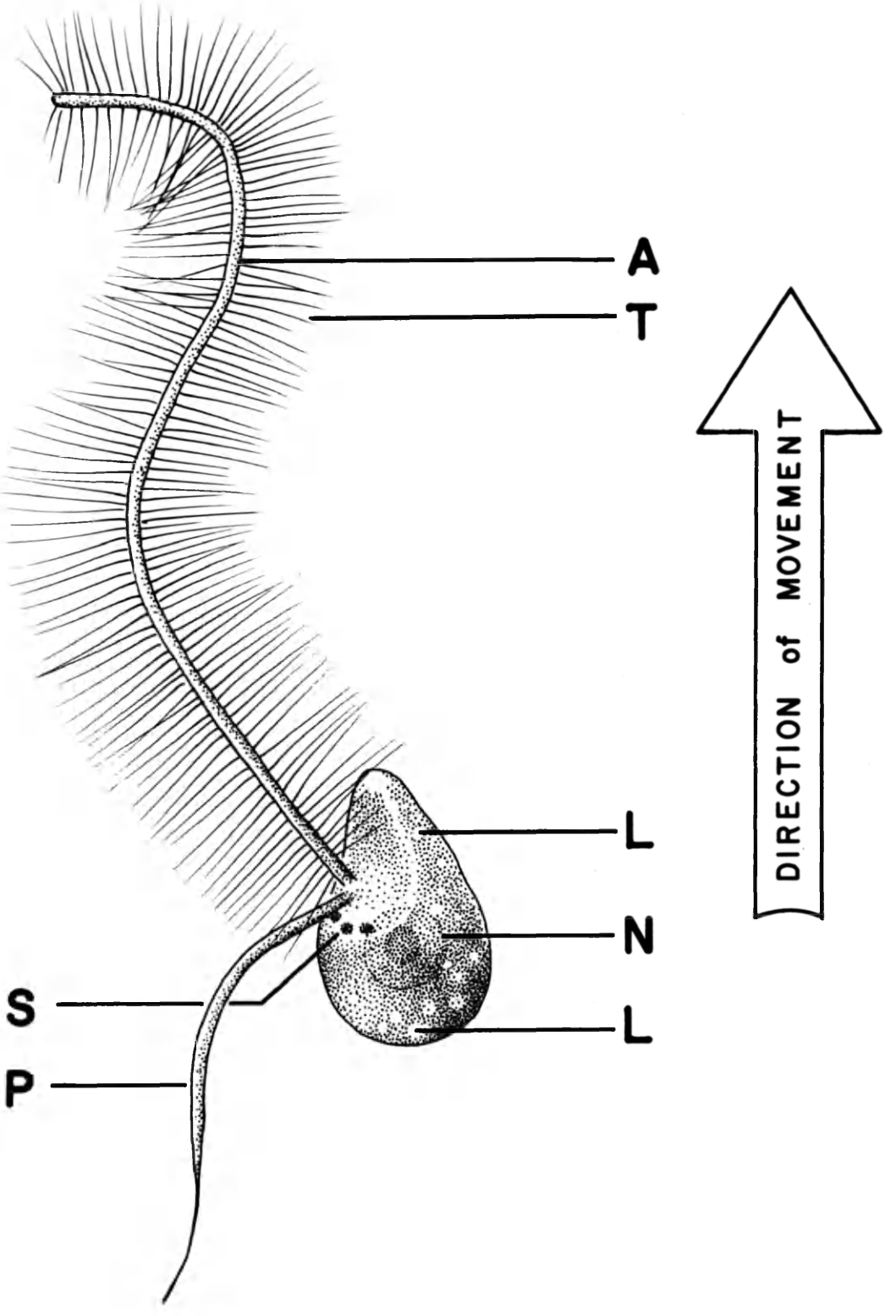

A
T
L
N
L
S
P
DIRECTION of MOVEMENT

Fig. 8. - b) An electron micrograph of a shadowed zoospore showing the flagella. 12,700X.

c) An enlargement of the above showing the detail of the tinseled anterior flagellum. 29,500X.

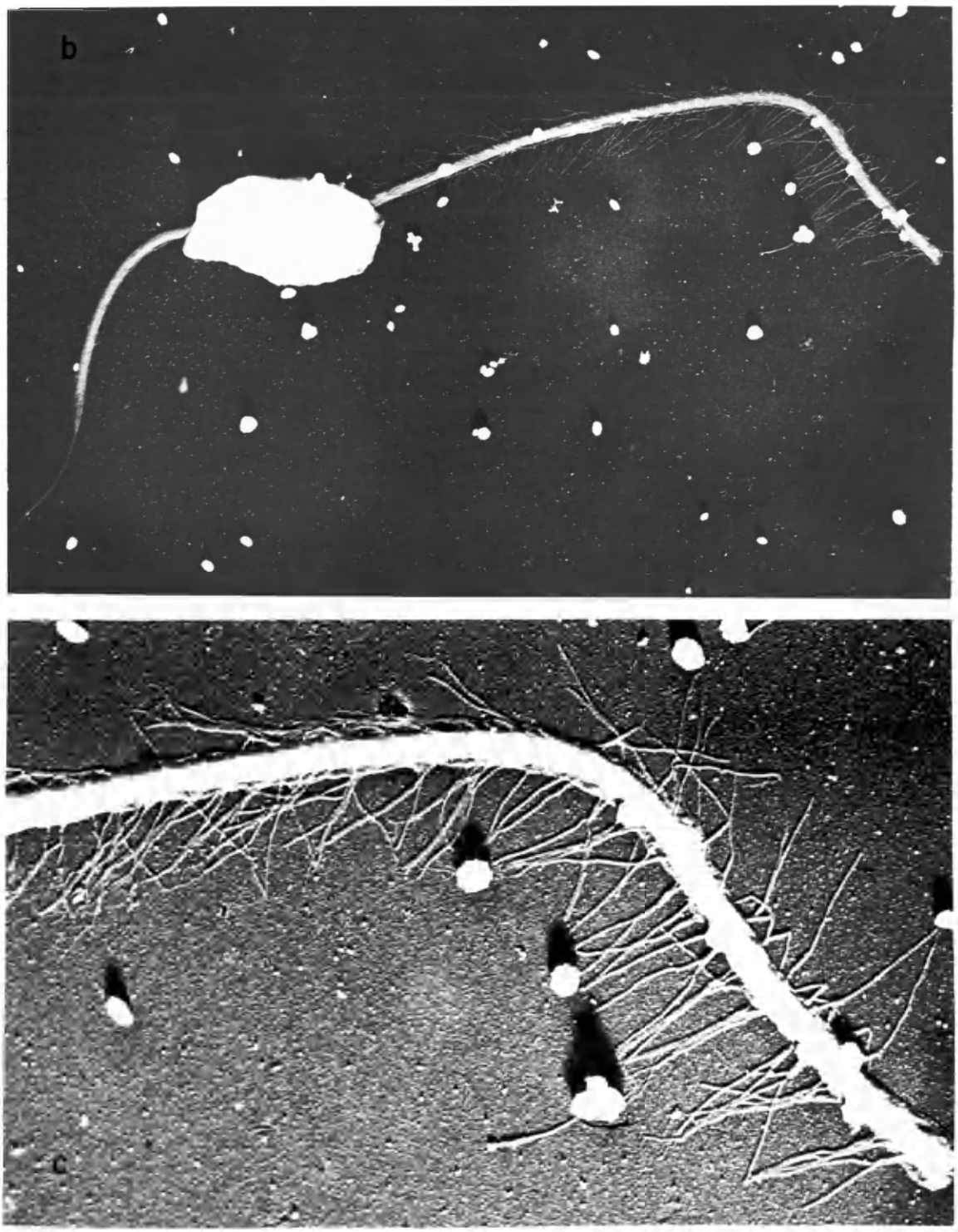

b
c

Fig. 9. - Plasmodium formation in isolate L8 of Group II type isolates.

a) Early stages of plasmodium formation. Dark-medium phase. 400X.

b) Massing of cells and formation of plasmodia. Dark-medium phase. 400X.

c) Multinucleate plasmodium either forming from several cells or dividing to form several multinucleate cells. Dark-medium phase. 1100X.

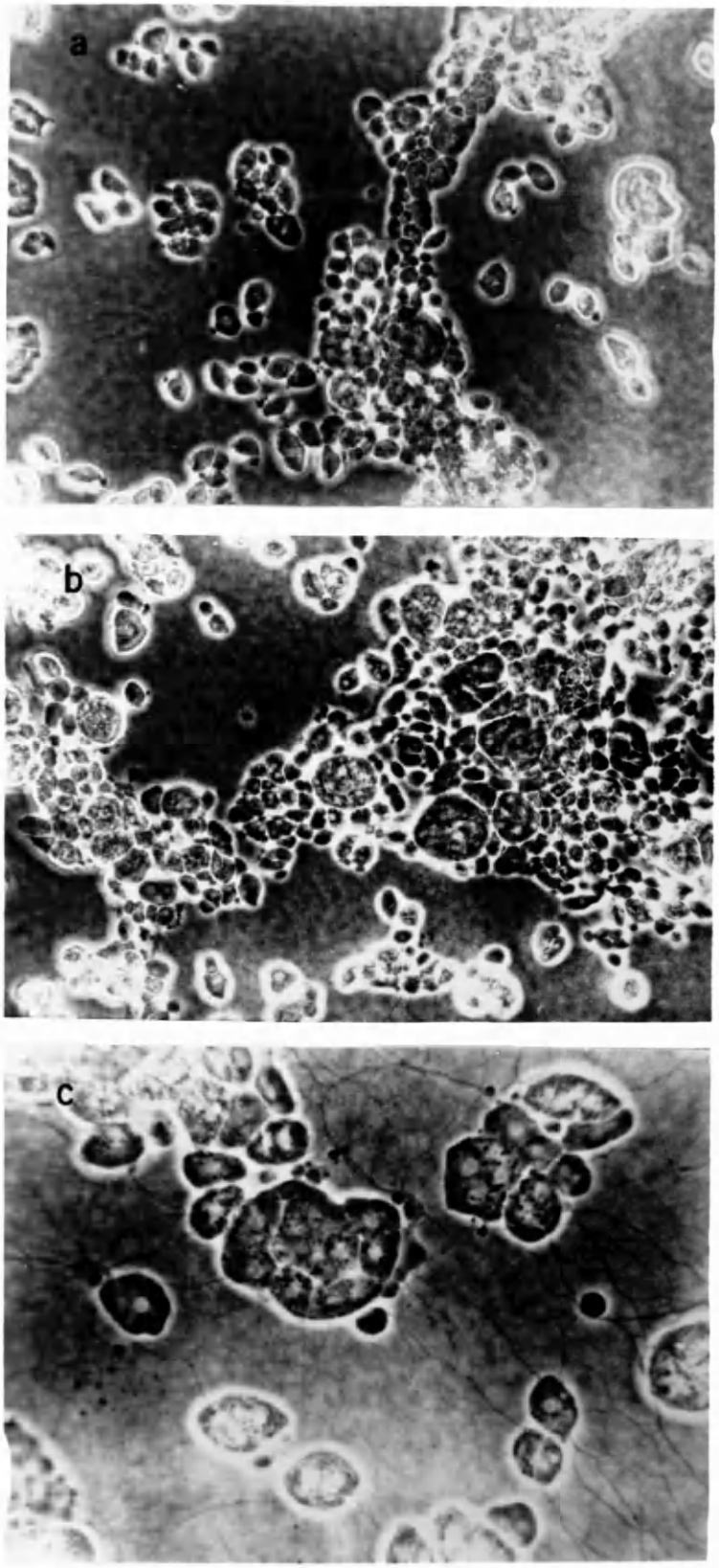

a
b
c

Fig. 10. - General morphology of Labyrinthula cells examined in the present study.

a) Isolate L1 showing interphase nucleus. Chrom-alum stain. 1100X.

b) Isolate L1 showing prophase nucleus. Chrom-alum stain. 1100X.

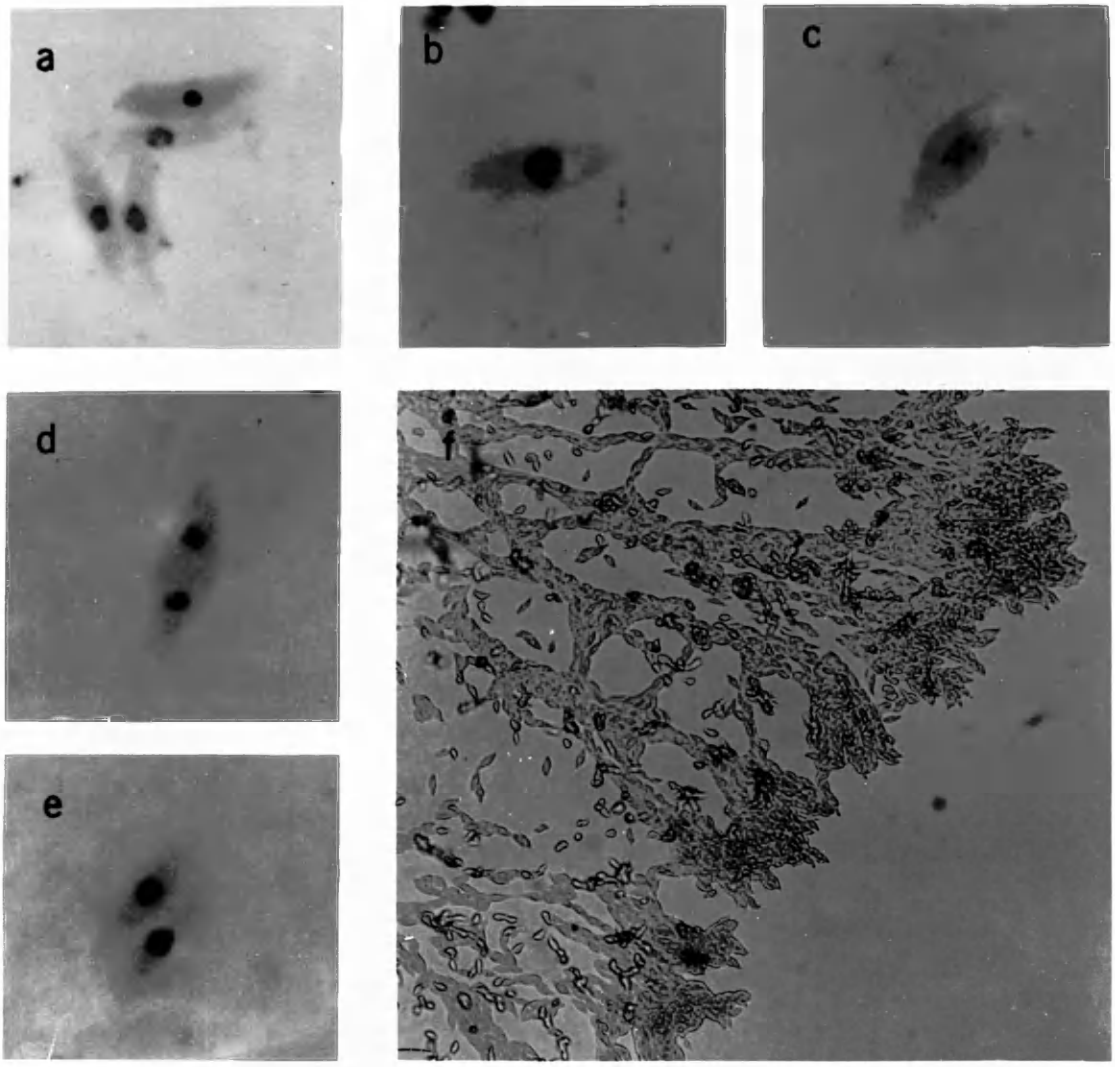
c) Isolate L1 showing anaphase nucleus. Chrom-alum stain. 1100X.

d) Isolate L1 showing late telophase nucleus. Chrom-alum stain. 1100X.

e) Isolate L1 showing interphase nucleus. Chrom-alum stain. 1100X.

f) A photograph showing the characteristic edge of a growing Labyrinthula (L1) colony. 170X.

g) Typical grouping of cells found in isolate L1. 2000X.


a
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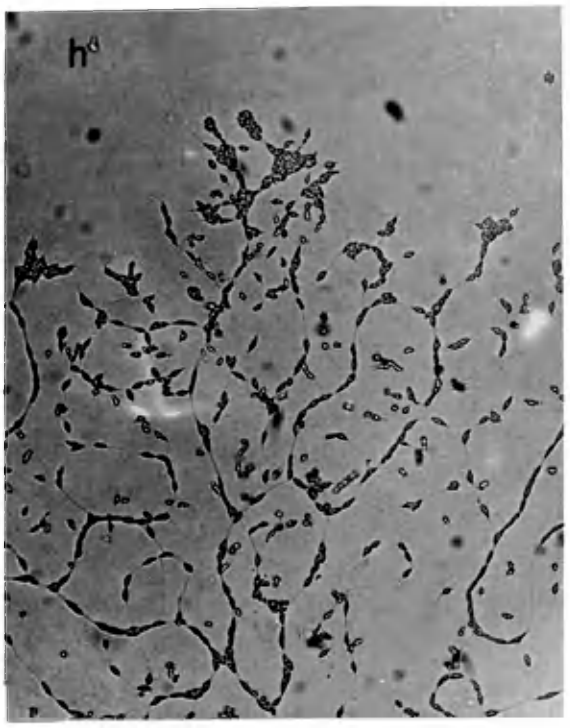
g

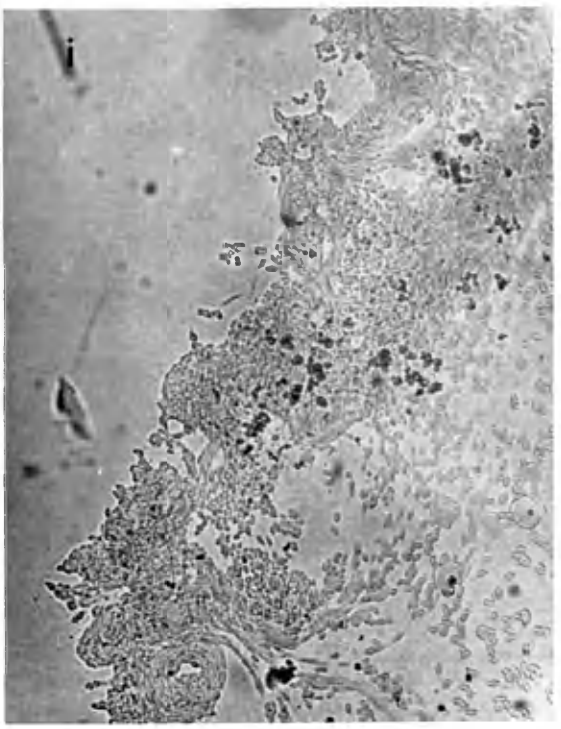
Fig. 10. - h) A photomicrograph showing the characteristic edge of an isolate 44 colony. 150X.

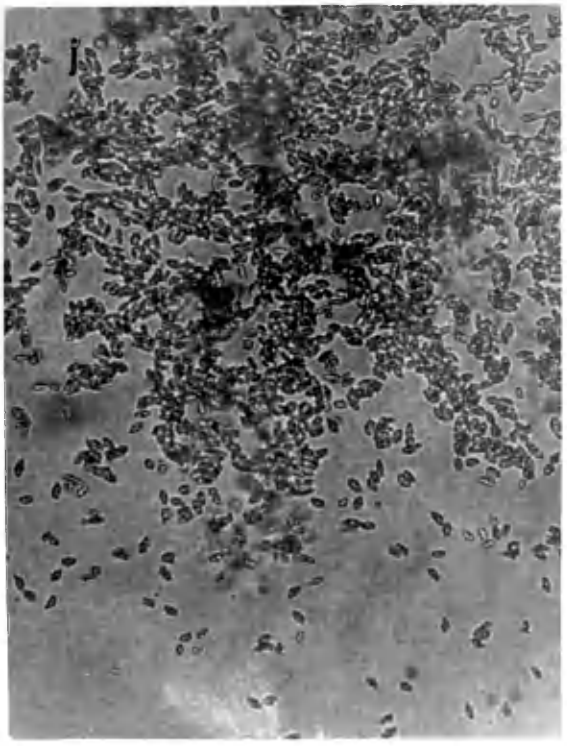
i) A photomicrograph showing the characteristic edge of an isolate L8 colony. 150X.

j) A photomicrograph showing the characteristic edge of an isolate 39 colony. 150X.

k) A photomicrograph showing the characteristic edge of an isolate L11 colony. 150X.


h


i


j

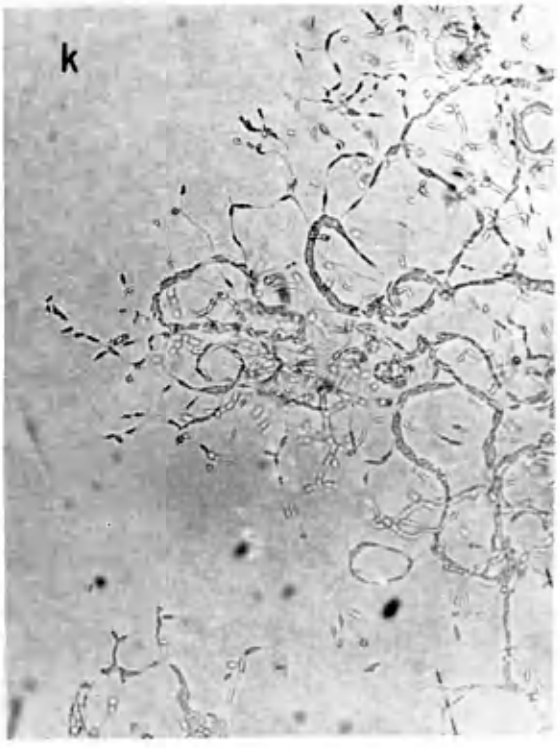
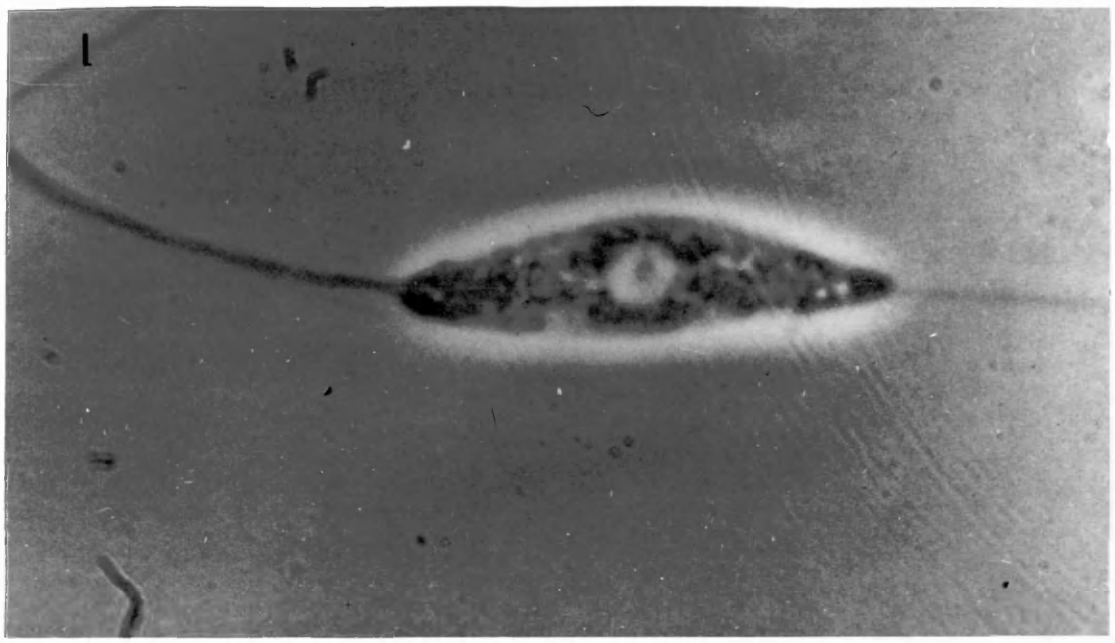
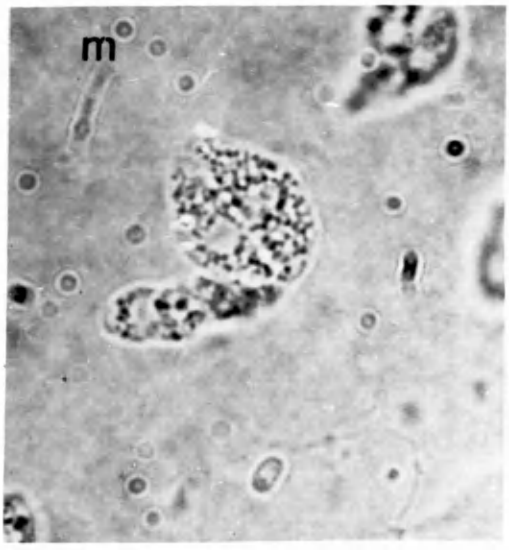

k

Fig. 10. - l) A photomicrograph of an individual cell of isolate 67. Dark-medium phase. 4200X.

m) A photomicrograph showing two recently divided cells of isolate 44. 1000X.

n) A photomicrograph showing the blunted shape of the cells in isolate L8. Dark-medium phase. 1700X.


l


m

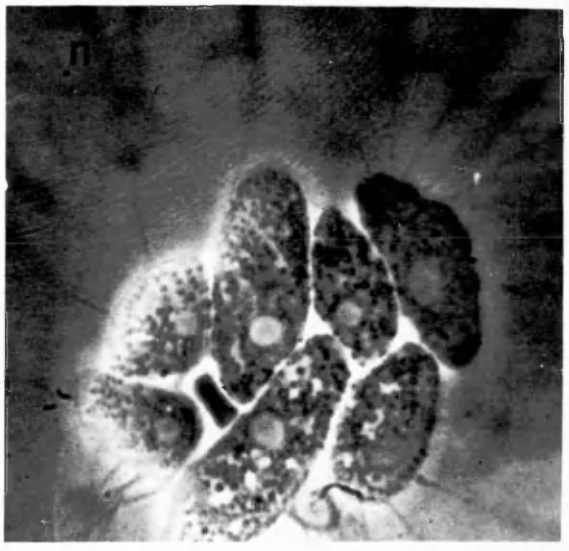
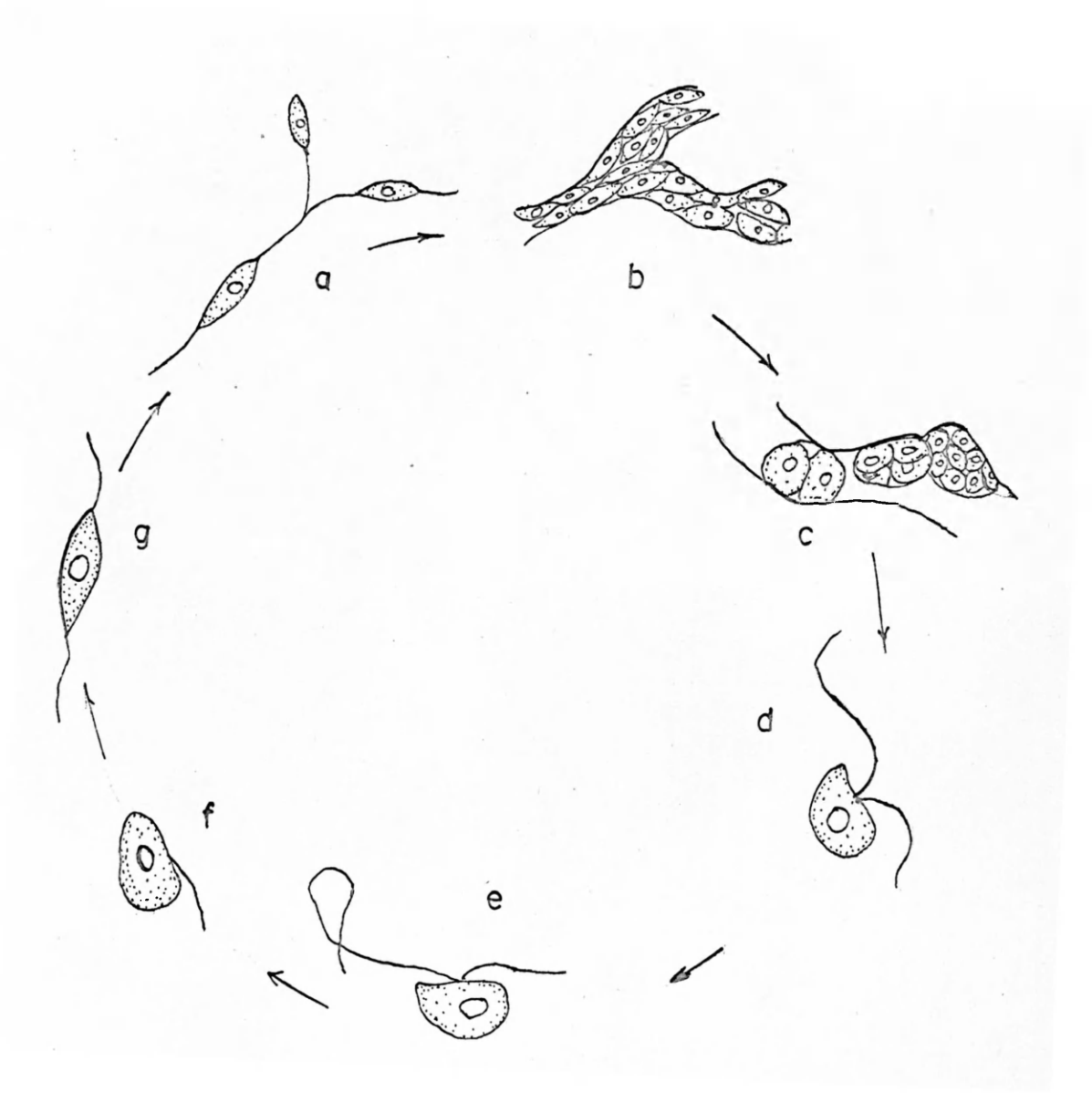

n

Fig. 11. - A diagram summarizing the life cycle for Group I isolates; a) somatic cells, b) aggregation of somatic cells, c) sporangial development by successive bipartition, d) zoospore release, e-f) zoospore settling and loss of flagella, g) new somatic cell.


a
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g

Fig. 12. - A diagram summarizing the life cycle of Group II isolates; a) somatic cells, b) widening of tubes and escapement, c-d) karyokinesis, e-f) cytokinesis, g) formation of new somatic phase, c') aggregation and fusion of cells, d') fusion plasmodium.

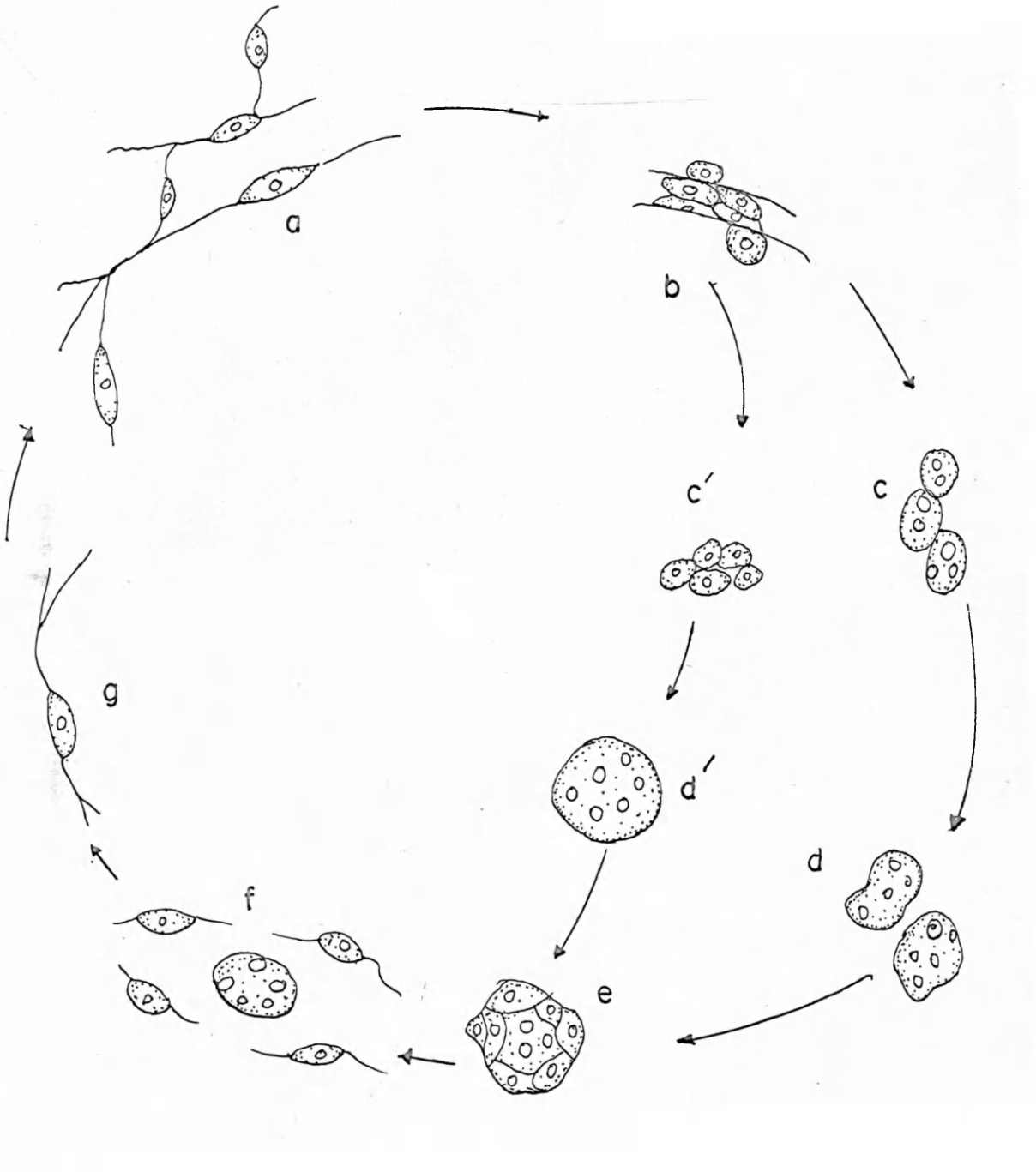

a
b
c´
c
g
d´
d
f
e

Fig. 13. - Slimeways of various Labyrinthula sp. isolates.

a) A branching slimeway of isolate L8 spindle cell. Dark-medium phase. 1500X.

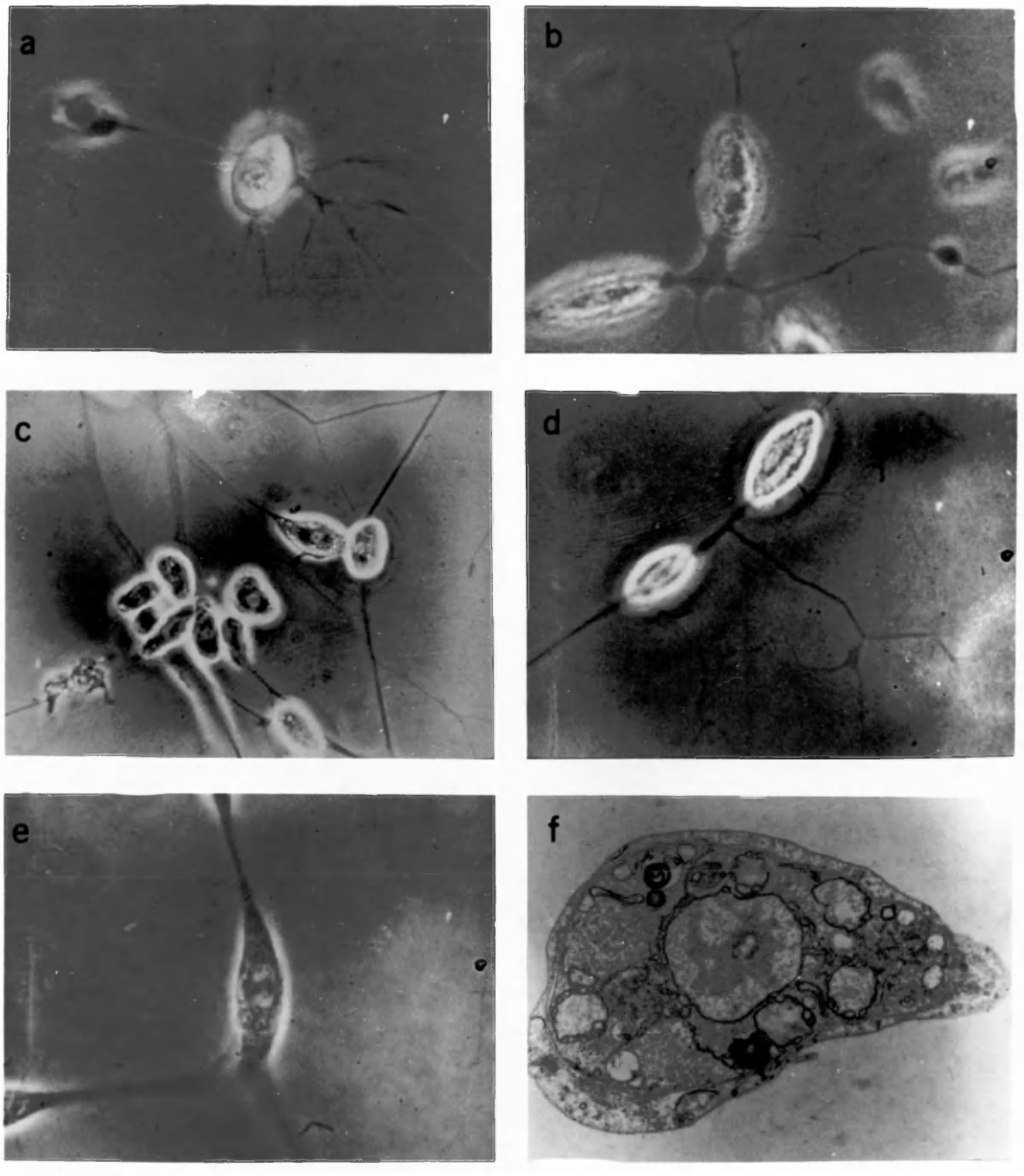
b) Slimeways of isolate L8 showing enlargements or nodes. Dark-medium phase. 1400X.

c) Various slimeway types. Dark-medium phase. 1000X.

d) A right angle branch on slimeway. Dark-medium phase. 1500X.

e) Dividing cell in obviously tubular slimeway. Dark-medium phase. 1500X.

f) Electron micrograph of Labyrinthula in slimeway showing tubular nature of slimeway. Cell is completely enclosed within the tube.


a
b
c
d
e
f

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