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DEVELOPMENTAL STUDIES OF TWO SPECIES OF NOWAKOWSKIELLA (NOWAK.) SCHROETER: N. RAMOSA BUTLER AND N. PROFUSA KARLING

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DEVELOPMENTAL STUDIES OF TWO SPECIES OF NOWAKOWSKIELLA (NOWAK.) SCHROETER: N. RAMOSA BUTLER AND N. PROFUSA KARLING

By

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INTRODUCTION

The first species of the genus of polycentric chytrids, now called Nowakowskiella, was described by Nowakowski (1876) as Cladochytrium elegans. In 1893, Schroeter established the operculate genus Nowakowskiella as distinct from the inoperculate Cladochytrium. Constantineanu (1901) questioned the validity of this genus, but described a second species, N. endogena, which is now considered to be a variation of the type species. A valid second species, N. ramosa, characterized by a pseudoparenchyma preceding the formation of resting spores was found by Butler (1907) on rotting Triticum vulgare stems in India. Domjan (1930) recalled Constantineanu's old specific name when he found N. ramosa in Hungary on the rotting leaves of Typha. N. elegans and N. ramosa were the only valid species which had been described in time to be included in the preparation of Sparrow's monograph on the Aquatic Phycomycetes in 1943.

Since 1940, six new species of Nowakowskiella have been described: N. profusa Karling (1941), N. hemisphaerospora Shanor (1942), N. delica Whiffen (1943), N. granulata, N. elongata, and N. macrospora Karling (1944 and 1945).

The position of this genus in the phylogenetic scheme is still undecided. In calling his species Cladochytrium, Nowakowski placed it in a group which has been considered to contain possible transition forms between the monocentric chytrids and the filamentous Phycomycetes with true mycelium. This entire group, the cladochytriaceous fungi, has remained among the chytrids on the basis of their zoospore structure, not possessing true mycelium,

and other features which will be discussed later with reference to the present report of findings with Nowakowskiella.

Karling (1932) proposed the descriptive term "rhizomycelium" for the thallus of all the polycentric members of the Chytridiales, which were then included in the Cladochytriaceae. Whiffen (1943) questioned whether the swellings in the thallus of Nowakowskiella were homologous with those in Cladochytrium, since the former were non-septate. She suggested that it might be better to separate these two genera on the basis of the presence of septa in these swellings, rather than on the presence of an operculum.

In his monograph on the aquatic phycomycetes, Sparrow (1943) divided all the chytrids into two parallel series: the Operculatae and the Inoperculatae, thus further removing Nowakowskiella from the family Cladochytriaceae.

It is true of all the lower phycomycetes that the final decision as to the tenability of a phylogenetic scheme must await further studies of the cytology, development, and reproduction of all forms, especially those which appear to represent transitions. At the present time, only five species of chytrids have been fully investigated: Polyphagus Euglenae (Wager, 1899, 1913; Dangeard, 1900-1901), Cladochytrium replicatum (Karling, 1937), Endochytrium operculatum (Hillegas, 1940), Catenomyces persicinus (Hanson, 1945a), and Rhizophydium coronum (Hanson, 1945b).

The purpose of this study is to fill in one more gap in our knowledge of chytrid structure so that future workers may proceed in greater clarity, rather than to criticize the existing theories on the basis of investigating two species of a single genus.

MATERIALS AND METHODS

The two species of Nowakowskiella examined in this study were N. ramosa Butler and N. profusa Karling. The original collection of N. ramosa was found by Professor E. A. Bessey on grass leaves in distilled water to which had been added a half teaspoonful of debris from a rain water cistern. During the course of the study additional specimens were found on hemp achenes that had been submerged in a garden pool. The first and subsequent collections of N. profusa were obtained on hemp achenes as bait in the same pool and from the relatively still water along the edge of a river.

Stock cultures were found to persist longer and to develop more luxuriantly if the water used contained some organic matter. Sterilized river water fitted this purpose very well. For substratum, both in maintaining the cultures and studying the forms cytologically, cellophane sterilized in 80% ethyl alcohol and rinsed with sterile distilled water was used. Sterilized hemp achenes were added to the river water and cellophane medium to keep the growth vigorous in stock cultures

Cultures were kept in petri dishes in which they could easily be observed with the low power of the microscope. For the study of living specimens at greater magnifications (high dry and oil), use was made of hanging drops suspended from the cover slips resting on rubber washers. The cover slips were sealed to the washers with vaseline to prevent drying and the washers were fastened to the slides with either vaseline or a water-proof cement.

Permanent preparations were stained at first with Harris's

or Delafield's haematoxylin or with Mayer's haemalum. These stains had been preceded by Belling's modification of Navashin's fluid. Most of the cytological data included in this paper, however, are taken from material which had been killed and fixed in Sass' modified Bouin's fluid or Belling's Navashin' fluid and stained by the smear modification of the haematoxylin method developed by Tuan. Also used and found helpful in some instances for fixing certain images were Flemming's weak fluid, undiluted and one-half strength, Carnoy's fluid, and the Zirkle-Ehrliki fluid. Among the other stains used was Flemming's triple stain.

Since the material was so fragile, rather than wash the specimens from the cellophane chips on which they were being mounted by passage through a dehydration series, that which was stained other than by Flemming's method was mounted directly from the last water rinse of the staining technique into glycerine jelly. In this way, all the slides studied were whole mounts, alleviating the difficulty of matching cytological structures which is encountered in the use of serial sections. Extreme care was necessary to dehydrate material stained by the Flemming's method. The dehydration solutions in large enough volumes not to evaporate completely were run over the specimens on a large glass plate. Dilute balsam was used for mounting.

A minimum of handling was imperative in staining the specimens. Whenever it was possible to do so without leaving a tenacious residue to spoil the preparation, materials were kept in Soyka or Syracuse dishes and treated and washed by adding the liquids to the dish, rotating slowly to be sure of even exposure, and pouring off the excess. In pouring, care was taken that the specimens did

not pass the shoulder of the dish. Rinsing was accomplished by moving the specimens to a dish of distilled water and adding and decanting several changes of distilled water to and from the dish. Even after a good rinsing in this manner, the material was dipped into another dish of fresh distilled water whenever it was transferred from one solution dish to another.

A drop of a 1% aqueous solution of Poirrier's blue was allowed to diffuse into the mount from the edge of the cover slip in the study of living specimens. This procedure was an aid in the observation of internal and external structures, since it seemed to outline the parts of the otherwise hyaline masses.

Zoospores were stained by the Feulgen reaction, Gram's stain, and the method described by Cotner (1930) in which the spores, in a .005% aqueous solution of crystal violet, were dehydrated over sulfuric acid.

All slides which are the bases of descriptions of cytological details were examined with a 90X anastigmat oil immersion objective coupled with 15X anastigmat oculars.

PHYSIOLOGY*

Solid Substrata and Liquid Media

When the fungi (both species) were first isolated into unifungal cultures, the medium employed was sterile distilled water into which had been placed boiled grass leaves and hemp achenes. The author repeatedly washed infected grass leaves in sterile distilled water and transplanted these leaves into sterile distilled water containing sterile filter paper in order to attempt to rid the cultures of contaminating bacteria and microscopic animals that abounded in the debris from which the isolations were obtained. The fungi grew well in this medium for about a week, then the growth began to wane. The fungi failed to become established after being transferred on filter paper to another culture dish containing sterile distilled water and filter paper. Feeling that the difficulty might be in the lack of nutrients or minerals, the author transplanted other pieces of infected filter paper into sterile water from a nearby river in which were pieces of sterile filter paper and autoclaved cellophane. The growth showed some degree of regained vigor on the filter paper and no growth occurred on the cellophane. However, the opacity of the filter paper, as well as the decolorized grass leaves, made it difficult to observe the initial stages of growth. Cellophane chips which were sterilized in 80% ethyl alcohol and rinsed with sterile water were used as a clear substratum. The alcohol-sterilized cellophane showed more luxuriant growth than the autoclaved and still remained clear enough

* Except where noted otherwise this portion on physiology is based on the behaviour of *N. ramosa*.

for the observation of minute details of structure and cytology. It was a fortunate accident that the author used cellophane that had been shirt and cigar wrappers for these initial experiments with growth; since in later trials, growth was not obtained on any cellophane which had come off packages of cigarettes of several brands. The stock cultures of the fungi were kept thereafter in sterile river water containing cellophane chips and two or three hemp achenes per petri dish. Throughout the continuation of the cultures, the best growth for N. ramosa was on the hemp achenes, while N. profusa grew better on the cellophane chips. Zoosporangia of N. profusa usually developed on the hemp achenes from two days to a week later than they did on the cellophane in the same culture.

The author removed the pericarps from several hemp achenes and placed them into dishes separate from those into which the contained oily seeds were placed, in order to detect if the pericarp was sufficient substratum for the maintenance of N. ramosa. Some of these dishes contained sterile distilled water, others sterile river water. There was no growth on the true seeds in either the distilled or river waters; whereas, growth was sparse on the pericarps in distilled water and abundant on the pericarps in the river water.

Since grass leaves are among the standard media for the culture of chytrids, the growth of N. ramosa on cellophane, quackgrass leaf, and corn leaf was studied comparatively in different solutions. The inocula for the cultures were all vigorously growing cultures on cellophane chips in sterile river water. Table I records the results.

Table I. Growth of Nowakowskiella ramosa on various substrata in different solutions

Substrata	River water	Physiological saline	Distilled water
Cellophane	Good	None	None
Corn leaf	Good	None	Slight
Quack grass leaf	Good	None	Good
Filter paper	Good	None	Slight
Hemp achene	Luxuriant	--	Good

Grains of barley, corn, oats, rye, and wheat were germinated in sterile river water; and the fungus on cellophane was added to each culture in an attempt to find if N. ramosa is parasitic on cereals. In only one of the oats cultures was there any suggestion that the fungus was growing on the living plant. In this one there were several zoosporangia produced from filaments which appeared to be growing from the primary root. However, when the entire seedling finally died, there was no evidence of filaments or sporangia on or in the dead plant. The fungus was not found to be growing on the grain, stem, leaf, or root in any other cultures; although there was good growth on pieces of sterile cellophane which had been placed into the culture dishes as checks on the viability of the inocula.

It was decided to test the ability of N. ramosa to grow on the killed grains of the same species in different solutions and to determine the portions of these grains attacked, since this fungus did not attack living grains and seedlings of cereals.

Some grains had failed to germinate in preparation for the parasitism experiment above. The fungus was seeded into dishes containing these grains. Growth was observed only on rye and wheat. Whole grains of all five of those previously used and rice were autoclaved in the dry state and then were tested for

their ability to support the growth of the fungus in sterile river water as an extension of this study. It was interesting to note that there was no growth on autoclaved rye and wheat grains. The growth was very good on barley and oats, good on corn, and fair on rice. The degrees of growth used here are relative, based on the subjective observation of the vegetative extension of the filaments and the development of zoosporangia.

Extracts of barley, corn, oats, rice, rye, and wheat grains and hemp achenes were made, to follow up the experiment testing the relative usability of the pericarps and endosperm by this fungus. The materials were sterilized in the autoclave at 10 pounds pressure for 20 minutes and were added, at the rate of 1 ml. extract to 20 ml. of the liquid medium in the petri plate. The final percent endosperm was 2.5%, since the original extract was made by grinding 1 gram of endosperm into 1 ml. distilled water. The liquid and liquefiable-solid media used were Knop's solution, distilled water, sterile river water, and 1% agar. These media were seeded with cellophane chips bearing well-established vegetative beginnings of growth.

The growth obtained in this experiment was fairly constant for all types of extract, with exception of the corn and the oats extracts. There were few rhizoids formed in any of the liquid cultures, but the erect sterile filaments became greatly extended from the cellophane chips that carried the inoculum. The formation of zoosporangia was very sparse except in sterile river water, in which there were many sporangia borne on short lateral branches. In cases in which the sporangia were numerous, the filaments appeared to be appressed to the bottoms of the culture dishes and not waving erect as was found in the stock cultures. This appressed

habit was so pronounced in cultures of N. profusa that when the substratum to which the rhizoids were attached was removed from a plate, the "erect" filaments and sporangia remained fast to the bottom of the plate, as though they were cemented there. There was hardly any growth at all in the cultures containing corn extract. There was little formation of zoosporangia except in the river water in the oats cultures. The sporangia were produced on short compact filaments in which the isthmuses between the swellings were much shorter than is the normal length. The growth seemed fairly abundant in Knop's solution and in distilled water to which oats extract had been added, but the filaments were very much shortened and looked knobby because the swellings were formed so close together. Vegetative growth was more extensive than ever observed in a completely liquid medium in all the cultures on the plain agar to which the endosperm extracts had been added. There was a difference in sporangium production, however. There were numerous sporangia formed close to the cellophane inoculum in the barley, rice, and hemp cultures. The greatest extension of sterile filaments among these three agars was 9mm. in the hemp agar. No sporangia at all formed in the oats, rye, and wheat agars; but in the rye and wheat cultures the filaments extended so far and were so thick that they gave the impression of being contaminants at first observation. The growth proved to be made up of slender filaments of Nowakowskiella with swellings formed only at the bases close to the cellophane inoculum. The corn cultures resembled the last three in most respects, except that there were present a few sporangia close to the cellophane chips.

The next step in this study of substrata was to take the pericarps of the above cereals and of hemp and attempt to grow the fungus on them in the same solutions and on agar. The data obtained in this phase of the study are given in Table II.

Table II. Growth of Nowakowskiella ramosa three weeks after inoculation on six different pericarps in three liquids and on the surface of 1.0% plain agar

		Vegetative Growth			Sporangium Production			
Peri-carp	Agar	Knop's Sol.	Distilled Water	River Water	Agar	Knop's Sol.	Distilled Water	River Water
Barley	Fair	Good	None	Good	Slight	Good	None	Good
Corn	Fair	None	None	Fair	Fair	None	None	Fair
Oats	Slight	None	Good	Fair	Slight	None	None	Fair
Rice	Slight	Abund.	None	Good	Slight	Abund.	None	Good
Rye	Abund.	None	Slight	Fair	Abund.	None	Slight	Fair
Wheat	Abund.	Good	None	Slight	Abund.	Good	None	Slight
Hemp	Abund.	Good	Good	Good	Abund.	Good	Good	Good

The above data demonstrate that the pericarp of the hemp achene is definitely superior to that of the cereal grains for the growth of N. ramosa. This observation has practical value for the mycologist who wishes to escape from excessive bacterial contamination in a culture of this fungus. It is also of interest to know that in the hemp pericarp cultures the filaments of the fungus were found to extend as far as 2 cm. from the pericarp into the agar. A portion of the agar in which were some of the filament tips was transferred to a dish containing cellophane and hemp achenes in sterile river water. By this means a pure culture of the fungus was obtained.

The growth had become initiated by the end of the first

week after inoculation in all the cultures used in the study above, except those of the pericarps in Knop's solution. Although growth of N. ramosa will occur in distilled water and in Knop's solution, on the average the most uniform growth on each of the solid media was found in river water. This indicated to the author that although much of the food matter is obtained from the carbohydrate parts of the solid, something more than the inorganic salts is obtained from the surrounding liquid.

The rhizoids have been found to penetrate the cellophane chips and to lie free in channels of liquid digested in this solid, as an indication of the utilization of cellulose-like breakdown products and the ability of N. ramosa and N. profusa to digest materials of a higher polysaccharide nature. This channeling of the cellophane has been observed in the absence of microscopic animals or bacteria, and the channels follow the same outline as the rhizoids. Apparently, there is an exoenzyme produced by the rhizoids which digests the "polysaccharide" to a soluble substance or substances to which the rhizoid wall is permeable. The composition of the breakdown products is beyond the scope of this report.

The action of N. ramosa on cellulose was tested with sterile cotton and a technical grade of cellulose acetate in agar, distilled water, Knop's solution, and river water. It was found that zoosporangia were borne as far as 2.5 cm. away from the initial inoculum in the agar containing cotton, whereas cellulose acetate was not attacked at all. Plates with nothing but the wrapping cellophane inoculum showed good growth at the end of three weeks when the experiment was ended. Some vegetative growth and few sporangia developed on the cotton in Knop's solution, but none on

the cellulose acetate. Also in Knop's solution, and in oats extract, the thalli on the cellophane were made up of short knobby filaments and bore zoosporangiain clusters on short erect filaments. The growth was sparse on cellophane in distilled water, while cotton and the technical cellulose acetate were not attacked at all. There was no growth on the cellulose acetate in river water, but the filaments and zoosporangia were abundant on cotton and cellophane. The presence of digested portions of the cotton fibers and the cellophane chips resembled the effect in the channeling by the rhizoids already described above. Whether or not the rhizoids actually entered the cotton fibers was difficult to ascertain. In most cases, the association appeared to be one of the rhizoids lying along side of the fiber or the shadowy outline left by the digested fiber.

Temperature

All of the above experiments with N. ramosa and N. profusa were done at room temperature varying from 20° to 25°C. When the first specimens of N. ramosa were obtained from their cistern source, it was not possible to keep a culture alive for more than ten days at a temperature above 20°C. At this time the cultures were kept in a water-cooled incubator that maintained a temperature between 16° and 18°C. The incubator was made by keeping a constant flow of tap water running through the jacket of an old slide dryer. This arrangement gave better results than a refrigerator set between these temperatures, probably because the humidity was kept higher so that drying-out was less rapid. Repeated attempts during the first year that this species was in culture were made to get cultures to develop

normally at room temperature. Satisfactory growth began to be obtained in cultures kept on the sill of an open window in the middle of September of that year. As continuous sub-culturing of these was carried on, there finally was found to have developed a strain that on some occasions grew well for three to five days in a room where the temperature varied between 25° and 28°C.

The author added fresh river water to an old culture that had been completely dried up for six months, near the end of the observations on N. ramosa. This culture was left standing at room temperature on a table top. After three days, new filaments appeared extending into the liquid surrounding one of the hemp achenes, and cellophane chips were added to this culture and were attacked by spores from the sporangia formed after a week. The strain arising from this culture continued to grow normally in the stock medium described above through several sub-cultures for over a year, at room temperature. In fact, the growth at room temperature seemed to be more abundant than that in the cooled incubator.

It would be well to mention here that this is the only evidence of a rejuvenation of old cultures of N. ramosa observed by the author during the four years he maintained this species in culture. Never by accident or design has he observed the germination of resting spores. The resumption of growth in this case may have been due to the germination of resting spores under the pericarp of the hemp achene, but proof of this assumption was not found. Karling (1944a) has observed the resting spores of a Brazilian strain of N. ramosa to germinate, either directly as zoosporangia or indirectly as prosporangia.

N. profusa grew readily from the first isolation at room

temperature in summer or winter. Growth in the cooled incubator was definitely inferior to that on the table top. The author's strain of this species even withstood the high temperatures developed in the trunk of a black automobile standing in the sun all day for two weeks, and grew readily in sub-cultures again after this exposure.

The author sometimes used the quick change of temperature method for forcing the release of the zoospores from the sporangium. Some of the cultures were allowed to freeze solid and to remain frozen for as long as three days. When these cultures were thawed and examined, the vast majority of the spores were formed with normal structure. Less than one percent of them showed the uneven cleavage of the sporangial cytoplasm to form multiflagellate spores as reported for Blastocladia by Cotner (1930). These abnormal spores will be discussed further under "Development".

Oxygen

Indications were found that N. ramosa is dependent on the presence of a ready supply of oxygen for normal growth. The author has never been able to get this fungus to continue growth in the confines of a test tube. All the cultures mentioned in this paper were in petri or soyka dishes. Even here, the growth was always better in the more shallow, wider petri dish. The fungus was found to be growing most luxuriantly at the surface of the medium, in almost all the cultures where growth and reproduction appeared normal. Sporangia were hardly ever produced much below the water level.

N. profusa differed from N. ramosa in response to oxygen also. Although the former species was never grown in a test tube, it grew well in 500 ml. flasks containing 200 ml. of river water. In addition, the sporangia of N. profusa are produced in abundance appressed to the bottom of a petri dish, and hardly ever were found floating in the immediate surface of the culture liquid.

Light

Although bright and direct sunlight seem to inhibit the growth of these two species, the growth is as good in diffused light as in complete darkness.

Miscellaneous

An attempt was made to cut down the growth of the bacterial contaminants by the bacteriostatic action of crystal violet. The author found that the strain of N. ramosa in question tolerated and grew satisfactorily in a 1:100,000 dilution of this dye, but not in 1:10,000.

Halos were frequently formed around the fungal growth in some of the agar cultures of the same species, which indicated that N. ramosa may be slightly bacteriostatic itself. The bacteria became established in some instances around the site of inoculation before the fungus did; and as the fungus developed a halo appeared in the bacterial growth. Bacterial contaminants rarely overran areas where sporangia were being produced, in agar where the fungus was established. This phenomenon was not exhibited in liquid cultures. Bacterial zoogleal masses around the solid substrata would completely

envelope the thalli. The fungus responded to this condition by exit papillae which extended beyond the limits of the slime.

Microchemical Tests

Thallus walls of both species react to the chitosan test as described by Johannsen (1941). The walls of sporangia and apophyses are stained red-violet by iodine-potassium iodide solution in conjunction with 1.0% sulfuric acid, if the thalli have been previously boiled in saturated potassium hydroxide. Picric acid gives, as Nabel (1939) described, a yellow coloration that cannot be washed out. When 75% sulfuric acid is added to material treated in this way, the entire thallus dissolves. The sporangium and apophysis walls of both these species also reacted slightly to chlor-iodide of zinc, indicating the presence of some cellulose. Although an inner layer appeared to be present in the sporangial wall of N. ramosa (this was not apparent in N. profusa), this inner portion did not stain. Both the chitin and the celloluse seemed to be present in the same layer in both species. There was no evidence of an evanescent inner wall of chitin such as Nabel described for his species, Rhizidiomyces bivellatus. These species of Nowakowskiella do agree with Rh. bivellatus in that the walls of the filaments and rhizoids do respond to tests for chitin and not for cellulose. Hillegas (1940) found the walls of the zoosporangia of Endochytrium operculatum to give a weak cellulose reaction, as is true with the two species being considered here, but does not mention their behavior with tests for chitin.

Sparrow (1943) writes, "The operculum is probably formed of wall material....", in reference to the operculate chytrids in general. It is not clear to the present author whether he means that the operculum is of the same composition as the wall or is made of a transformed wall-substance. In the case of both N. ramosa and N. profusa, treatment with ruthenium red stains none of the fungus except the operculum. The reddish violet color acquired by this structure indicates that it is primarily of a pectic nature.

The "oil" globules and refractive matter reacted similarily, whether in the zoospores, filaments, spindle-organs, or rhizoids. With Sudan IV they were stained golden yellow, became light brown after standing in osmic acid, and were dissolved by acetone. These findings do not add any more information as to the composition of the "oil" in the chytrids than Karling and Hillegas found with Cladochytrium replicatum and Endochytrium operculatum, respectively; that is, they seem to be of a fatty nature but may be more complex. However, treatment with saturated picric acid or with dilute eosin did not show any indications of proteinaceous matter being associated with the fatty globules. This failure to stain may have been due to the immiscibility of the water-dissolved stains and the fatty matter. With picric acid the nuclear caps and cytoplasmic strands stained a bright greenish yellow.

DEVELOPMENT

Structure of the Zoospore

Figs. 1, 2, 3, 14, and 15

The zoospores of N. ramosa and N. profusa are typically posteriorly uniflagellate, uninucleate, and spherical.

The single refractive globule in the spore of N. ramosa is colorless and excentric. The position may be anterior or lateral to the centrally located nucleus, but never posterior. In this respect the zoospore resembles that of Solutoparies Pythii (Whiffen, 1942). Posterior to the nucleus is a large shadowy body. Because of its lunate shape, this structure resembles a nuclear cap; except that in the stained spores the nuclear cap is anterior or lateral to the nucleus. Occasionally one to three refractive globules are found in the spore, instead of the usual one. A similar deviation from a typical single globule has been described for N. elongata by Karling (1944a) and for Sporophlyctis rostrata by San Chiun Sen (1944). A single refractive globule is also typical of N. profusa. The position differs from that given above for N. ramosa in being lateral to and usually posterior to the central nucleus. Smaller refractive granules may be present in the area posterior to the nucleus in some spores of N. profusa, as described by Karling (1945) for N. macrospora. A lunate granular body such as Ajello (1942) described in Polychytrium may also be seen in the living cell. The position of this body has been observed as posterior, lateral, or anterior to the nucleus. Whether this structure is homologous to the similar structure in N. ramosa, the

vesicular side-body in Blastocladiella laevisperma and B. asperosperma (Couch and Whiffen, 1942), or is a nuclear cap without a fixed position was not determined. Butler (1907) described the oil globule of the N. ramosa zoospore as a very mobile one, and Hillegas (1940) reported the globule in Endochytrium operculatum as fluid and changing shape when the amoeboid spore squeezes through a tight space. Neither of these characteristics was observed by the author in the globules of N. profusa zoospores. When the spore is escaping from the zoosporangium or is resorting to amoeboid motion, as it may do repeatedly, the globule remains in its posterior position and keeps its spherical shape. Under such circumstances the spore extends clear, hyaline pseudopodia anteriorly or laterally while the granular and refractive matter of the spore remains faintly delimited as an internal spherical mass. Variability as to the refractive globule in the genus Nowakowskiella is common. Among the other species, N. elegans, N. hemisphaerospora, and N. delica (Matthews, 1928; Shanor, 1942a; Whiffen, 1943) have been described as having a single spherical globule; N. elongata (Karling, 1944a) usually has a single globule but may have two. The refractive material in N. macrospora (Karling, 1943) is composed of a disc-shaped globule and minute granules; and N. granulata (Karling, 1944a) is characterized by the presence of numerous golden brown granules.

Posterior to the nucleus a small vacuole in which may be seen a minute granule is also visible in the living spore. From this granule the single flagellum extends out of the vacuole, through a thin layer of cytoplasm and the plasma membrane, to the surrounding medium. In some of the spores of N. ramosa which contain more than

one refractive globule, a second granule, located in the same or a different vacuole as the first, is present. A second flagellum may or may not extend from this granule. A few of these biflagellates have flagella of unequal length. Some of the biflagellate forms are the same size as the typical uniflagellate, although they are usually somewhat larger (10.6-21.3μ as compared to 4.8-9.7μ). It is possible that these "giant" spores with the two flagella are formed by an upset in the cleavage of the sporangial cytoplasm, but there is no evidence of two nuclei in them. This biflagellate-uninucleate condition of Nowakowskiella sp. spores has also been reported by Ellison (1945). This lack of the extra nucleus may be explained in that miniature, non-flagellated spores, consisting of little more than a nucleus with a thin film of cytoplasm,are sometimes found. It is admitted, however, that the attachment of the flagellum of one spore to the nucleus of another seems improbable. Since fusion of two spores has never been observed, it is believed that these biflagellate spores are not evidence of sexuality in this species. Tri- and quadriflagellate giant spores have also been observed with only one nucleus, and the author prefers to feel that the multiflagellate condition may be due to improper cleavage,as reported by Cotner (1930) for Blastocladia and by other workers for other chytrids. Whatever the reason for the alignment of more than one flagellum with a single nucleus, it appears to be evidence that at least the initials of these appendages are laid down between the times of division of the sporangial plasma into the spore initials by furrowing and the final separation of the mature spores preceding their escape. The formation of the flagellum while the spore is still in the sporangium is indicated

by their presence being evident immediately on those spores not escaping through the orifice in the first mass to escape. Likewise, is this indicated in that spores attempting to separate themselves from the mass at the orifice may be observed held by a fully formed flagellum caught in the exit papilla by another spore. In N. profusa, the author observed this entanglement of flagella in the papillae so frequently that he wonders if this might not be the mechanism explaining the massing of spores on discharge. Some of these caught flagella appear to have loops or vesicles at the ends. The loop is no longer evident when the spore has freed itself. Hillegas (1940) described similar loops on the flagella of immature spores of Endochytrium operculatum; Berdan (1941b) found loops in various positions on the flagella of Catenochytridium carolinianum zoospores; and Ajello (1942) reported them on the flagella of occasional zoospores of Polychytrium. That these looped flagella are not results of developmental irregularities but are homologous to the knobbed modification of the whip-lash flagella found by Ellison in some of the Mycetozoa is doubtful. Karling (1945b) and Hanson (1945a) have recently described loop formation in the absorption of the flagella by spores becoming sessile. The observations on these two species of Nowakowskiella could not support or detract from Hanson's (1945b) report that the flagellum of Rhizophydium coronum is wound about the spore and unwinds as the spore escapes from the mass at the tip of the exit papilla. The author does feel that the belief that the flagellum is formed within the sporangium is true. This theory has been advanced by Karling (1937), Hillegas (1940), and Couch (1945).

Usually there is a single vacuole, other than that contain-

ing the blepharoplast, in the spores of both N. ramosa and N. profusa. In N. profusa this vacuole most often is about the same size as the refractive globule or slightly smaller. Its position is lateral or anterior, frequently opposite the globule in relation to the nucleus. When a spore is kept in distilled water or is exposed to a solution of Poirrier's blue at a slightly toxic concentration, this vacuole begins to swell until the spore is much distended, as little more than a tonoplast about a huge (in relation to usual spore size) vacuole. Seldom is it the small vacuole containing the blepharoplast which undergoes this great increase in volume. In this distended condition of the spore, the nucleus and nuclear cap are compressed into the posterior end of the spore near the point of insertion of the flagellum. When the vacuole is quite large the spore ceases to swim or to move by amoeboid action. The flagellum of a spore in this condition waves weakly but with insufficient power for locomotion. Whether this is due to the great size of the spore or is due to the effect of pressure on the internal motor structures is not conjectured here.

The most conspicuous structure in stained preparations of the zoospores of both N. ramosa and N. profusa is a darkly-staining crescent which resembles a nuclear cap, surrounding a third or more of the centrally-located nucleus. This body is usually tilted so that it is oriented with the thicker part of the crescent to the side and not directly anterior to the center in the spores of these two species. A nuclear cap in a similar position has been figured for Endochytrium operculatum by Hillegas (1940). The finding of nuclear caps in the zoospores of the chytrids prompted Ajello (1942)

to suggest that these extra-nuclear structures are not as important phylogenetically as once thought. The shape of this structure may vary from a more or less thin crescent to a cup or sphere in Nowakowskiella. The spherical form appears to be a hollow ball which is filled with the nucleus. Lateral to the nucleus is a large clear space, the origin of which is not certain. This space may be left by the dissolution of the refractive globule found in the living spore. If this space does represent the position of the globule, it indicates a fixed position for that structure relative to the nucleus and nuclear cap. Hillegas considers a similar clear space to be the nucleus in E. operculatum. The deeply-staining portion of the crescent may be less heavy, showing a second clear sphere containing a nucleolus-like structure, all surrounded by the cap, in N. profusa, especially.

In most of the stained spores, the flagellum is found to be attached to a granule located in a small vacuole posterior to the center of the spore with a rhizoplast passing through the cytoplasm from this granule to the nucleus. Such distinct rhizoplasts and blepharoplasts have been described in chytrid zoospores before (Berdan, 1941; Karling, 1937; Hillegas, 1940). Ellison (1945) uses the presence of these structures for designating that the swimming organelle of the phycomycete zoospore is a true flagellum. In most cases, the fixed spore lies so that the flagellum enters the cytoplasm adjacent to one side of the darkly-staining crescent; and in such cases, the thin rhizoplast can be seen passing diagonally through the cytoplasm, so that the point of attachment to the nucleus is at one of the pointed horns of the cap. Karling (1942) described

the nucleus tapering to the point of attachment of the flagellum in the zoospore of Septochytrium macrosporum, in a similar manner to the sub-triangular nuclei in the zoospores of Blastocladiella simplex and Blastocladia (Matthews, 1937; Cotner, 1930). Either a similar tapering structure or two granular cytoplasmic strands (one or both functioning as a rhizoplast) extend from an apex at the blepharoplast to the posterior side of the tilted nuclear cap in some spores. Only one of the strands joins the central structures at the tip of a horn of the cap. If this really is a conical extension of the nucleus, a second opening in the cap must be presupposed; if it be strands, the structure is like that found in Monoblepharella Taylorii (Springer, 1945) and occasionally in Blastocladia (Cotner, 1930).

The cytoplasm constituting the remainder of the internal structure of the spore is made up of a minutely granular reticulum.

Atypical forms were present in most of the stained preparations of zoospores. Rarely were spores with two nuclei found, more frequently with two flagella, two blepharoplasts, and/or two rhizoplasts. Ellison (1945) also found the biflagellate spores to be unimucleate. Another common atypical spore form was the "midget" spore which measures 3μ if spherical or 2x4μ if oblong and which contains nuclear structures similar to the typical spore but very little cytoplasm, no refractive globule or food body, and shorter or no flagella. The non-flagellate forms are those referred to above as miniature spores which might have lost their flagella in uneven cleavage.

The well-defined cap covering the nucleus disappears

when the spore becomes sessile (between 4 and 12 hours after release from the sporangium for N. ramosa, 6 and 24 hours for N. profusa), in contrast to the occurrence in Cladochytrium replicatum, Endochytrium operculatum, and Polychytrium stromaphilum in which the cap may persist during the early stages of germination (Karling, 1937; Hillegas, 1940; and Ajello, 1942). In place of the cap, there is a deposit of large granules around the outside of the nuclear membrane. Larger granules also become apparent in the cytoplasm, radiating in elongated masses from the nuclear to the plasma membrane, lining the inside periphery of the cell, and surrounding small vacuoles. Examination of spores which appear to be in transitional stages in the disappearance of the cap indicates that this structure is the source of the majority of these large granules. The clear space which is adjacent to the nucleus and nuclear cap in the swimming spore may or may not be present or may be partially filled in with granules. Here again the behaviour of this clear space in the stained spore parallels that of the globule in the living. The nucleolus now takes a position in the center of the nucleus, which is centrally-located in the sessile spore. The rest of the nuclear substance is marked by a fine reticulum. The nucleus rarely divides at this time, although some sessile spores may be found which contain two nuclei. There were two nucleoli in one nucleus in one spore of N. ramosa.

Germination of the Spore

Figs. 4, 5, and 16

Between 6 and 10 hours for N. ramosa and 6 and 24 hours for N. profusa after having become sessile, the spore puts forth a single slender germ-tube which usually sends off one branch before it has grown longer than the diameter of the spore. As the papillary swelling which is the forerunner of the germ-tube becomes evident, a mass of densely-staining granules aggregates at this point. As the tube develops the aggregation remains in the tip and becomes less pronounced during the extension of the tube. After the first offshoot, the tube continues to branch more or less dichotomously until the mature vegetative absorptive portion of the thallus is established. Although the germ-tube may branch two or three times before there are any swellings formed, the most common development results in a primary swelling, which may be homologous to the primary spindle organ in Cladochytrium replicatum (Karling, 1937), before the second dichotomy occurs. This enlargement is usually first noticeable twenty-four hours after the spore germinates in N. ramosa and up to seventy-two hours in N. profusa. During this time the nucleus remains in the spore-case, where it may or may not divide. As the swelling enlarges, cytoplasm from the spore flows to it, and the spore-case begins to shrivel. Berdan (1941a) described the spore of Cladochytrium hyalinum as empty before the formation of the primary spindle organ. When the swelling is about 2-4μ across its broadest point, the nucleus leaves the spore-case and passes through the narrow tube to the swelling. A deviation from the normal is that in this, and subsequent swellings formed,

the nucleus may arrive in the filament before the swelling is initiated, as Hillegas has described for Endochytrium operculatum. In the event that the nucleus has divided in the spore, only one of the daughter nuclei passes to the swelling at this time. These species differ from Cladochytrium replicatum, Endochytrium operculatum, and N. hemisphaerospora (Karling, 1937; Hillegas, 1940;and Shanor, 1942a) in the occurrence of nuclear division in the spore-case. The nucleus becomes elongated and narrow in order to pass through the narrow filament from the spore-case to the enlargement, in the same manner as Karling reported for Cladochytrium replicatum. After the primary swelling has formed there is no further growth originating from the spore even though the spore-case may persist and enlarge to a diameter of 5 to 12μ . Throughout the life of many thalli of N. profusa the spore-case persists and enlarges as an anucleate, rarely nucleate, spherical swelling joined to the functional primary swelling by a short, fine or broad isthmus. The isthmus may be so short and broad that the spore-case appears as a lobe of the functional swelling. The persistence of the spore-case may be ontogenetic evidence of rhizideaceous ancestry. Although a nucleus left in the spore-case may divide, the daughter nuclei, except as noted above in N. profusa, pass to the primary swelling or to others which have begun to form in the first branch of the germ-tube. On rare occasions as many as three nuclei have been seen in the primary swelling. The spore and the branching of the germ-tube may be so close together that, with the high-dry objective of the microscope, there appear to be as many as three germ-tubes developing from the same spore. However, closer examination with oil-immersion shows this to be a false

impression. The fact that only one germ-tube is formed seems to be important in light of the use of Catenaria's forming two being one of seven reasons given by Couch (1945) for moving that genus from the Chytridiales to the Blastocladiales. Nuclear division during these stages has demonstrated some typical mitotic figures.

An obortive method of attempted germination was discovered among some spores of N. ramosa that had become caught in the vessels of a grass leaf. These cells had assumed an amoeboid habit, then the nuclei had divided one or more times, and at least one cell had apparently begun to bud off bits of cytoplasm containing nuclei.

Establishment of the Thallus

Vegetative Portion

Figs. 6 and 17

The spore-case may empty and the shriveled wall become obscured, or as described above may persist after the primary swelling and its rhizoidal offshoots have become established. No cell walls are formed as the nuclei in the swellings divide and separate so that the resulting thallus is without any septations, differing in this respect from Cladochytrium, Catenomyces, and Catenaria. There may be one to four nuclei in each of these swellings.

The thallus continues to grow, branching dichotomously as it does so. Some of these branches remain appressed to the substratum and form additional nucleated enlargements from which, or from the isthmuses, fine or coarse rhizoids are produced. For one or two days in N. ramosa and up to a week in N. profusa most of the growth of the thallus consists of the establishment of spherical, fusiform, and irregular swellings, appressed to and imbedded in the substratum. In the mature thallus numerous much-branched rhizoids arise from the swellings and from the isthmuses between. The swellings may occupy part or all of the cavity of the host cell; and the rhizoids dissolve their way into and ramify in the cell walls when the fungi are grown in grass leaves. These swellings when mature may possess walls about the thickness of the walls of the mature zoosporangia; usually, however, little thicker than the walls of the larger filaments. The rhizoids are short and may be expanded in spots, especially at the scene of much-branching, into bladder-like formations with thin walls and large vacuoles. The resulting

mature rhizoids are typically relatively short, blunt, and knobby. Another polycentric operculate chytrid exhibiting thick, short rhizoids in cellophane is Catenomyces persicinus (Hanson, 1945a). Some of the bladders are so great in diameter in places of pronounced branching that they can be distinguished from the centers of development only by the lack of nuclei, and some by the thinner wall. This network of rhizoids and swellings resembles the thallus of Megachytrium Westonii (Sparrow, 1933) more closely than it does any of the other polycentric chytrids. The notable difference is that Nowakowskiella contains no cross-walls delimiting the swellings from the rest of the thallus.

The most pronounced inclusions in the living protoplasm in the vegetative system described are the refractive globules. These globules resemble closely the globules of the zoospores, except for their great variety of sizes (0.2 to 2.0 in diameter). The presence of similar refractive globules in the intramatrical portion of the thalli has been reported before for N. profusa (Karling, 1944a), as well as for Cladochytrium replicatum (Karling, 1937, Catenomyces persicinus (Hanson, 1945a), Cladochytrium hyalinum, Catenochytridium carolinianum (Berdan, 1941 a, b). Few scattered minute globules are present in the rhizoids which have digested their way into the substratum; but wherever a swelling occurs, there is an accumulation of globules. The mass of globules may completely fill the swelling in mature thalli. The hyaline cytoplasm appears to fill the thin absorptive rhizoids. There are large vacuoles, surrounded and divided diagonally by thin strands of cytoplasm in the swellings. The vacuolate nature of the

cytoplasm of the vegetative portions of Clad. replicatum, Endochytrium, and Catenaria has been described by Karling (1937), Hillegas (1940), and Couch (1945), respectively. The globules are grouped as to appear intra-vacuolar, held in the cytoplasmic strands. Strictures in the isthmuses between the swellings may be filled with cytoplasm, often of a denser appearance than that in the swellings, forming a type of pseudoseptum. These pseudosepta occur more frequently in the older thalli. Similar bands of material were found in the rhizoids of Septochytrium variabile by Berdan (1942).

Fixing and staining dissolves out the refractive matter and accentuates the structure of the cytoplasm and the nuclei. If osmic acid is used as a fixative and is not bleached out, the place occupied by the refractive matter contains a brownish amorphous mass. Otherwise the spaces occupied by the globules are clear, except for loose clumps of densely-staining granules. Similar granules are found scattered throughout the cytoplasmic strands. The number of granules present in N. ramosa and N. profusa seems to be greater than that for Clad. replicatum (Karling, 1937). The nuclei are surrounded by masses of these granules in many instances. In their affinity for the nucleus these granules resemble those which go to make up the nuclear cap, as will be discussed under zoosporogenesis.

Flexuous Filaments

Figs. 8 and 18

Long, flexuous, usually extramatrical filaments which branch by repeated dichotomies arise from the sides or ends of the vegetative swellings or from the isthmuses between. These filaments may be isodiametric throughout their entire length, especially N. profusa (Karling, 1941), or may vary considerably in diameter. Elongate fusiform swellings, which are even in outline, may usually be found regularly in the proximal one-third of each filament in the strains of both species studied. Rarely, were secondarily developed, typical absorptive-vegetative centers arising from these swellings found. Distal to this area, the swellings are irregular in shape and length: being fusiform, spherical, even triangular at points of branching, to mere undulations in the otherwise parallel filament walls. The narrower portions range in diameter from 1.5 to 2.5μ in the more distal segments, while the swellings in these segments are only 2.0 to 4.0μ. The length of the narrower portions is 4.0 to 40μ, as opposed to 11.5 to 30μ for the swellings. It is difficult to draw a clear-cut differentiation as to what constitutes a swelling and what an isthmus in this area. If one overlooks the small differences in diameter, it is possible to consider segments as long as 300μ as lacking true swellings. The swellings and isthmuses in the proximal portion are more easily differentiated, since their sizes are 2.5 to 5.0μ x 6.0 to 11μ and 1.5 to 2.5μ x 4.0 to 30μ, respectively. It is not uncommon to find one of these flexuous filaments almost twice as wide in the distal portion as it is in the proximal. Lateral branches may form at almost right angles and remain only 1.0 to 1.5μ in diameter. Numerous extramatrical

flexuous filaments may arise from five to eighteen main "trunks" originating at a single rhizoidal system in N. ramosa.

These filaments may resemble true tubular coenocytes internally. The cytoplasm in the younger thalli contains many deeply-staining granules and small refractive globules, and is netted with small vacuoles. The vacuoles in older thalli are longer, surrounded and crossed by thin strands of minutely-granular cytoplasm. As in the vegetative system, pseudo-septa of thick cytoplasm may be found in points of constriction.

The presence of nuclei in these filaments is variable. They are usually absent in the narrow lateral branches, and are usually present in the swellings. Up to six nuclei have been found in a single enlargement. Mature thalli may bear irregularly swollen filaments lacking swellings, in which rounded nuclei (2.0 in diameter) are scattered throughout their lengths. As many as 14 nuclei have been observed evenly distributed in a filament of N. ramosa 485 long from its origin to the cross-wall separating the zoosporangium from the rest of the tube. The regular distribution and the rounded shapes and lateral nucleoli of the nuclei make it appear that these structures are fixed in their places, rather than passing through the filaments. Karling (1937) described the moving nucleus in Clad. replicatum as elongate and densely-staining. Hillegas (1940) reported that the nuclei of Endochytrium operculatum elongate only when passing through a constriction in a rhizoid. Since the author has observed nuclei scattered in the majority of the filaments which were alive at the time of killing and fixing, it is his opinion that these structures are

typically nucleate in both N. ramosa and N. profusa.

Formation of the Zoosporangium

Figs. 9, 10, 19, and 22

Each of the flexuous filaments described in the preceding section may bear one or more zoosporangia. These reproductive bodies can be terminal, intercalary, or on short lateral branches. Most of the zoosporangia develop extramatrically, rarely intramatrically. However, even the intramatrical sporangia are outgrowths of short specialized filaments. The author has never observed a typical vegetative swelling to produce zoospores. In this way these species of Nowakowskiella differ from Clad. replicatum (Karling, 1937), and Megachytrium Westonii (Sparrow, 1933). Physocladia (Sparrow, 1932) seems to exhibit similar specialization, even though the vegetative swellings are extramatrical. The differentiation of the thalli into vegetative and reproductive portions is pronounced in N. ramosa and N. profusa. There is a superficial resemblance to Rhizopus with shortened "stolons" in the general habit of a single thallus, except for the presence of the intercalary swellings and zoosporangia.

The manner of bearing zoosporangia on the young, actively-growing thalli differs slightly for N. ramosa and N. profusa. The most readily observed difference is that the sporangia of N. ramosa are more nearly terminal than those of N. profusa. The continuation of the filament distal to the sporangium in N. ramosa is usually a fine thread which shrivels as the sporangium matures. In the few instances where this thread has persisted, it has been found to be anastomosed with another larger flexuous filament. The same structure may be wide and branch one or more times before narrowing to finely blunt ends or being terminated by a zoosporangium in N. profusa.

Most often a filament of N. ramosa will branch near its end, and each of the equal-lengthed branches will be terminated by a sporangium. Both species may produce sporangia on the tips of short lateral branches. The filament below the sporangium of N. ramosa is swollen into an infundibiliform apophysis-like structure. If such a swelling occurs in N. profusa, it is more nearly spherical; and a constriction is found at the cross-septum between the swelling and the sporangium. The present strain of N. profusa, as with Karling's (1941) original strain, is rarely "apophysate"; but it is not uncommon to find a short tenuous filament leading to an intercalary swelling as in N. elongata (Karling, 1944a).

True internal proliferation of the zoosporangium has not been observed by the author in either of the two species. An unusual type of proliferation is common in N. profusa. As the culture becomes older, the swollen or non-swollen filament above and below the original sporangium begins to enlarge. Cross-walls limit these swellings from the remainder of the filament, the walls thicken, and these become secondary sporangia. Chains of two to eight sporangia have been observed on old thalli. If the swellings form close together, their adjacent walls are flattened where they have met in enlarging. Less often there is a short isthmus between two sporangia in a chain. In either case, each individual sporangium lays down its own thickened wall; so that if a sporangium were pulled out of a chain, the walls of those adjacent to it could remain intact. There is extreme variability in the shapes of the sporangia in such chains. These chains of sporangia would resemble the multiseptate sporangia of N. elongata as described by Karling (1944a) except for the progressive formation and the double walls in the present species.

Elongate exit tubes are more common in N. ramosa than in N. profusa. They develop on those sporangia that are intramatrical or surrounded by a zoogleal slime, and do not extend beyond the outer edge of the enveloping material. The zoosporangia of N. ramosa may bear one to three exit tubes or papillae, one or all of which may be branched; but only one is functional.

The swellings which develop into zoosporangia are usually intercalary at their inception in both species. The tip may be included in the rounding out of the sporangial rudiment until the mature sporangium is terminal, if the enlargement begins very near the tip of the filament. The swelling is fusiform-elongate at first, resembling very much those in the lower third of the flexuous filaments. These swellings begin to increase in circumference more rapidly than they do in length early in their development until they have become spherical in shape. Variations from the typical spherical form are pyriform, ovate, obtusely-branched, obtusely-triangular, and hour-glass shaped. When the rudiment is about one-half its mature size a cross-wall forms, dividing the swelling into two unequal parts in N. ramosa and the "apophysate" sporangia of N. profusa, or at a point near which the swelling begins in those lacking "apophyses". The time of delimiting the incipient sporangia differs from that observed by Whiffen (1943) in N. delica. The smaller portion continues to develop into an evenly shaped funnel form in N. ramosa so there is no constriction at the cross-wall. The portions above and below the septum continue to enlarge, the incipient sporangium the more rapidly, in such a manner that there is a constriction between the two unequal-sized spherical swellings in the "apophysate" forms

on the thalli of N. profusa. The septum protrudes as a convex arc into the sporangial cavity when it first begins to form, but is soon inverted as a protrusion back into the filament.

The wall begins to thicken when the incipient sporangium has reached mature size; and the cross-septum attains a thickness equal to that of the rest of the sporangium wall. At one point; lateral, subapical, sub-basal, or rarely apical; the sporangium wall forms a disc-shaped area thicker, but more refractile than the rest, which is beginning to take a light tan cast. Around the circumference of this disc the wall is very thin, seeming to consist of little more than the external layer of the immature thin-walled swelling. If such structures as exit tubes or papillae have formed, this disc is at the tip of a tube or a papilla; otherwise it is flush with the surface of the wall. This disc becomes the pectinaceous operculum which bulges as a slight convexity on the mature sporangium wall.

Zoosporogenesis

Figs. 10, 11, 19, 20, and 23

Appearance of Living Material: The behavior of the protoplasm in the developing sporangia of N. ramosa and N. profusa is essentially the same as that described for other chytrids (Berdan, 1941a and b and 1942; Couch, 1945; Hanson, 1945a and b; Hillegas, 1940; Karling, 1937, 1944a and b, 1945a, b, and c; Whiffen, 1942 and 1943). Because of this similarity, the author will refer to the observations of other workers only where differences in structure and behavior of the organisms seem significant.

The refractive content of the otherwise hyaline cytoplasm begins to increase in the enlargement as the swelling which develops into a zoosporangium grows. At the time of cross-wall formation the refractive matter appears to be more concentrated in the center of the swelling. At this same time several large vacuoles may be observed in the incipient sporangium, and a few large globules are present. Differing from the monocentric chytrids, the filaments supporting the sporangia of these two species are not devoid of cytoplasm when the cross-walls are formed. The large globules in the growing sporangium increase in size and number shortly after the formation of the cross-wall. Later these globules again disperse, and the evenly granular appearance returns to the cytoplasm. If exit tubes are formed, they also are filled with the same type of cytoplasm. The sporangium attains its mature size during this stage of evenly granular appearance. Also during most of the granular stage the vacuoles are not as evident as before. Soon, however,

elongate, narrow vacuoles can be seen forming in the cytoplasm, dividing it into irregularly-shaped masses. This cleavage progresses primarily from the outer edge toward the center, but additional vacuoles may be found arising independently of the primary furrows, cutting the cytoplasm from the inside out, radially or tangentially. The minute oil droplets coalesce into a single, or at the most two, globules in each of the masses so formed. Except for the refractive globules, the cytoplasm acquires a homogeneous appearance and swells until the vacuolar divisions are obscured. The cytoplasm completely fills the sporangial swelling, in this stage, except for a clear space which has formed below the operculum. This space appears to be filled with a viscid substance of low refractive properties, because of its spherical shape, maintained at the expense of the rest of the contents. In the event a long exit tube has been formed, it may be filled with cytoplasm such as in the body of the sporangium, up to the clear space which is just below the operculum at the tip of the tube. Appearance of Stained Material: The development of the sporangium has been followed in the stained material by the study of cells of different sizes, using the size as an indication of relative maturity. This method seemed to be practical because the size of the swelling and the number of nuclei ordinarily paralleled each other.

When the small intercalary swelling first appears it is filled with a gramular cytoplasm and exhibits one nucleus, about 2 in diameter. The deeply-staining granules are small, with larger granules massing in streaks through the cytoplasm. Often these

streaks are associated at one end with the nucleus, similar to the observation of Karling (1937) in the newly nucleated spindle-organ of Clad. replicatum. The author's observations on these two species do not add or detract from the theory that the strand represents the line of passage of the nucleus. As the swelling enlarges, these strands disappear and the granules become evenly distributed throughout the sporangium rudiment. The tapering portion of the enlargement which is to become the subsporangial swelling is filled with cytoplasm and may contain one or more nuclei like those found in the sporangium rudiment, in N. ramosa and in N. profusa if such a swelling is formed. Several large nuclei are found in the rudiment before it has enlarged very much. The first nucleus in the swelling has been observed to be dividing in a few preparations; but there was no proof discovered that all the nuclei in the rudiment came from continued divisions of this single nucleus or that some of them might not have migrated into the swelling from the proximal or distal filaments. This lack of proof is further confused since there are several nuclei in the swelling before the septum cuts it off from the rest of the filament.

The deeply-staining granules aggregate loosely in the center, leaving the remainder of the cytoplasm less densely granular as the swelling continues to enlarge into a spherical form and more nuclei are found in it. When the rounded form of the swelling is established, the adjacent portion of the proximal filament has swollen to be infundibuliform in N. ramosa, and spherical or lacking in N. profusa. The distal continuation of the filament begins to shrivel back to the rounded swelling, in those sporangia which appear terminal at maturity although intercalary in origin.

The distal filament may be swollen or not, if it persists. The flexuous filaments on which the sporangia are borne are usually almost devoid of nuclei and highly vacuolate by the time the sporangium is mature. This appears to be more true for N. ramosa than for N. profusa. Possibly the presence of more intercalary swellings in the former is a factor affecting the presence of nuclei in the non-swollen filaments. The vacuolate nature of the cytoplasm is less prominent nearer the sporangium; and in both species nuclei may be present in the swollen or non-swollen filament immediately below the sporangium. This is also true of the distal continuation of the filament if the sporangium is truly intercalary, or if this portion has anastomosed with another filament as described above. However, in the formation of chains of secondary sporangia in N. profusa, the filaments become emptied of nuclei and cytoplasm.

The granular mass in the center of the sporangium disintegrates as a densely granular reticulum spreads throughout the swelling. The nuclei become aligned on strands of this reticulum in such a way that observation of subsequent nuclear divisions is made difficult. Some strands of reticulum extend down to where the tapering of the swelling begins; and at this place, a thick plate of granules extends across the filament. As this plate becomes more apparent, the outer wall of the sporangium begins to thicken, and the papillar swelling begins to grow through any material present that will inhibit the free escape of the zoospores. As the plate which will form the cross-septum becomes more compact and densely granular, it bulges up from the filament into the

sporangium, as though the pressure exerted in the filament were greater than that in the sporangium itself. The granules forming the reticulum again become dispersed, and the cytoplasm is again uniformly granular. The granules present at this time are minute and deeply-staining. The area immediately below the exit papilla is devoid of stainable cytoplasm at this stage.

The peripheral wall of the sporangium becomes thick and deeply-staining, and the arched operculum is evident as a lightly-stained dome which is thick in the center and tapers off toward the edges. At the point where the operculum is attached to the rest of the wall, the wall is not thickened but has remained a thin ring. By the time the operculum looks fully-developed, the cross-septum between the sporangium and the filament has the same structure as the rest of the sporangium wall.

The cytoplasm begins to be furrowed at the periphery by the elongated vacuoles mentioned above, after the walls are completely formed. This furrowing by vacuoles progresses centripetally and laterally until each nucleus has a mass of cytoplasm cut out around it. The rest of the cytoplasm in each of these masses becomes clearer as the deeply-staining granular material collects around each of the nuclei. The granular mass around the nucleus consolidates and takes the form of a loosely-packed, over-sized nuclear cap. After the cytoplasm swells so as to obscure the divisions and fills the sporangium case, the nuclear caps become more compact and more evenly and deeply-staining. The nucleolus must function independently of the nuclear cap, because it is evident as a small, dark body in the center of the nucleus before and after the granules begin to clump about the nucleus.

The above observations as to the formation of the nuclear cap tend to support Karling's (1937) belief that this structure arises from chromatic bodies or granules in the cytoplasm and is external to the nucleus itself. As with Karling's and Hillegas' (1940) material, the present species demonstrate easily-stained nuclear caps after non-chondriosomal fixatives. It is of interest to note the similarity of behavior between the granules which form the nuclear cap and the refractive globules in their aggregation and dispersion in the incipient sporangium. The presence of similar deeply-staining granules in the vegetative portion of the thallus which in the living state contains a great deal of the "oil globule" material indicates that the dispersed cap material and the "oil" may be closely associated throughout the life of the thallus. It may be that the granular material is carried dispersed in a lipoidal medium; and extrudes the lipoid as the "oil globule" of the zoospore in the process of aggregating as the compact nuclear cap. The proximity of the cap and the globule in the swimming spore appears to support this belief. Since both the globule and the cap lose their distinctive identity before and during germination of the spore, the suggestions by Karling and Hillegas that the cap is food-material which is used up in the process seem to apply to N. ramosa and N. profusa.

Although the present author has not attained the perfection of staining the dividing nuclei demonstrated by the investigators at Columbia University (Karling, Hillegas, and Hanson), he was able to observe definite mitotic figures in both species of Nowakowskiella studied. The nuclear behavior differs in these

species from that in Clad. replicatum and Endochytrium operculatum in that the nucleus does not increase appreciably in size before division and divides repeatedly during the enlargement of the incipient sporangium before and after the formation of the cross-wall. Division of all the nuclei in a single sporangium is not absolutely synchronous although it occurs simultaneously, since nuclei in various stages of division may be observed in a single swelling.

Zoospore Escape

Figs. 11 and 21

No further visible change takes place in the sporangium for an indefinite time after maturation of the zoosporangium and zoosporogenesis. The entire contents begin to revolve in an undivided mass within the zoosporangium wall when conditions are naturally or artificially conducive to zoospore discharge. The operculum is forced clear off or is thrown back as on a hinge while the cytoplasm is in motion, and the subopercular material is expelled. After the operculum dehisces, the cleavage lines in the sporangium again become distinct and the irregularly-shaped individual spores are evident.

The spores escape as individuals in each species. The first to pass through the orifice flow through rapidly, apparently forced from behind by those adjacent to them. In both N. ramosa and N. profusa a mass of zoospores may form at the outside of the exit papilla. However, the individuality of these spores is evident both before and after their escape. The present author would like to suggest that the spores escape into a mass, rather than en masse. In these two species of Nowakowskiella a single spore must be elongated and distorted to pass through the exit orifice; since the opening is usually but one-half the diameter of the typical spherical zoospore, and the surrounding sporangium wall is rigid compared to the naked protoplast of the spore. A vesicle or any other confining material was not found enclosing the mass of spores at the tip of the papilla in either species. Whenever the outline of such a structure was suspected, continued observation showed the line to be the entangled flagellum of one of the spores in the mass.

The existence of the mass is short-lived, only long enough for the spores to round up, free their flagella, and swim away.

The number of zoospores formed per sporangium varies in both species. The zoosporangia producing the fewest spores have been observed in cultures of N. ramosa when very small sporangia on short filaments were formed early in the life of the thallus and contained only four or eight spores. The average number of spores for a normal zoosporangium in this species has been determined as about thirty-six. No such small sporangia were observed on the thalli of N. profusa, and the average number of spores in a typical sporangium is between sixty and eighty.

All the secondary sporangia in a chain do not mature and discharge their spores at the same time, even though they may be adjacent on the same filament.

Fusion of Cells and the Resting Bodies of N. ramosa

Fig. 13

Resting bodies were formed, developing from a pseudo-parenchyma as described by Butler, in many of the older cultures. These cells have yellowish walls of considerable thickness, which are smooth in most cases. However, it is not uncommon to find resting bodies with the outer wall corrugated and with the inner wall exhibiting striations.

Cultures were ordinarily transferred every two or three weeks. The flexuous filaments begin to produce short lateral outgrowths instead of numerous zoosporangia, in cultures older than a few weeks or in strains under laboratory cultivation for two or three months. These outgrowths may be merely irregularly-shaped enlargements which are formed by the filament swelling on one side only, or they may be short side branches consisting of thin isthmuses terminated by club-shaped tips. Two of these swellings come into contact and become superimposed upon each other. The author had difficulty in determining whether these cells actually fuse or if their cell walls merely adhere to each other. Whichever occurs, they are found in this appressed condition often enough to indicate that they are fastened together in some manner. Both cells retain their rounded shape, being flattened only where they are in contact. The cytoplasm in these cells is minutely granular, and each of them contains a single nucleus. The resultant structures are pretty

definitely single cells with two rounded hemispheres, produced by the fusion of the two swellings in some cases. Whatever the case may be, nuclear fusion has not been observed. Karling (1944a) has described the formation of pseudoparenchyma from single swellings and single lateral branches, as well as from the fused tips of branches in strains of N. ramosa collected in Brazil. This "pooling" of the contents of two cells from different filaments may be homologous with the behavior of the two cell-potentials from which arise the resting spores of N. hemisphaerospora (Shanor, 1942a). It is possible that a procedure such as has been reported for N. hemisphaerospora occurs during the formation of the pseudoparenchyma in the Brazilian strains in which the pseudoparenchyma is produced without the "fusion" of cells, but from a single cell. The first change that occurs in the "fused" cells is the formation of large vacuoles with the tonoplasts extending across the cells so as to separate them into four to eight clear areas. A cell wall is laid down where each line of cytoplasm is present, in such a way that each of the "fused" cells is divided into as many daughter cells as there are vacuoles. Not all of these daughter cells contain nuclei. Those that do are either continuous with the mother filament or give rise to apophyses as described below.

The nucleate cells in the pseudoparenchyma produce short off-shoots which have clavate tips. These tips are filled with a granular cytoplasm as they enlarge. After they have attained a more or less spherical shape, the nucleus from the pseudoparenchyma cell migrates into the apical swelling, and a plate of cytoplasm forms across the base of the rounded resting-cell-initial. The nucleus

takes a central position in the cell, surrounded by a loosely-packed mass of granules. Strands of granules radiate from the center to the periphery of the cell. The presence of similar chromatic granules has been reported for Polyphagus Euglenae, Clad. replicatum, and Endochytrium operculatum (Wager, 1913; Karling, 1937; Hillegas, 1940). The nucleus in this resting cell was observed not to divide but to remain single and centrally placed. The unimucleate condition until mature size is attained has also been observed in Clad. replicatum and Endochytrium operculatum (Karling, 1937 and Hillegas, 1940).

The living cell shows a dispersion of oil droplets evenly throughout the cell and then a re-coalescing of the droplets into about eight large globules. Stained material shows the deeply-staining granules to follow the same procedure. The cytoplasm remains reticulate. Although the peripheral wall is thickened slightly by the time the cross-septum is completed, after the cell has become more stabilized as to its internal structure, a striated and thicker inner wall is laid down inside the original. These resting-bodies have not been observed to germinate in this present strain, but Karling (1944a) has observed them to produce zoospores directly or to function as prosporangia.

TABLE OF MEASUREMENTS FOR STRAINS STUDIED
(expressed in microns)

Structure	N. ramosa	N. profusa
Reproductive Structures		
Zoospore		
Diameter of spherical spore	4.85-9.7	3.9-4.7
Dimensions of amoeboid spore	5.82-7.7x 9.7-15.5	3.5-7.7x 3.5-4.5
Length of flagellum	24-35	20-30
Diameter of nuclear cap	2.0-2.3	1.5-2.0
Diameter of oil globule	2.5-3.0	1.0-2.0
Zoosporangium		
Diameter of spherical sporangium	27.16-31.37	17.0-40.0
Dimensions of sporangium other than spherical	35.9-56.3x 37.0-77.6	11-20x 40-50
Length of papilla		
Usual form	2.0-15.0	4.0-6.0
Long variety	50.0-80.0	up to 30
Diameter of papilla	4.5-10.0	4.0-8.0
Diameter of operculum	2.91-8.7	3.0-4.0
Thickness of operculum	0.9-1.9	0.6-1.0
Diameter of sub-opercular space	1.8-5.0	1.5-6.0
Thickness of wall	0.6-1.3	0.6-1.0
Diameter of nucleus	1.5-2.0	1.5-2.0
Subsporangial Swelling		
Diameter	3.9-13.6	0 or 2.0-6.5
Length	6.8-15.5	7.0-16.0
Thickness of wall	0.9	0.5-0.7
Diameter of subtending filament	2.0-5.0	1.5-4.0
Resting Cell		
Diameter	7.5-15.0	-
Thickness of wall	0.5-0.6	-
Diameter of nucleus	2.0-5.0	-
Flexuous Filaments		
Swelling		
Diameter of cell		
Near vegetative center	7.0-10.0	2.5-5.0
In distal portion	2.8-7.0	2.0-2.5
Length of cell		
Near vegetative center	7.0-12.0	6.0-11.0
In distal portion	11.0-35.0	11.5-30.0
Thickness of wall	0.2 or less	0.2 or less
Diameter of nucleus	1.5-2.0	1.5-2.0

TABLE OF MEASUREMENTS FOR STRAINS STUDIED
(expressed in microns)

Structure	N. ramosa	N. profusa
Isthmus		
Length		
Near vegetative center	2.0-25.0	4.0-30.0
In distal portion	5.0-60.0	4.0-40.0
Diameter		
Near vegetative center	1.7-4.0	1.5-2.5
In distal portion	1.5-5.0	2.0-4.0
Thickness of wall	0.9	0.5-0.7
Diameter of nucleus	1.5-2.5	1.5-2.0
Vegetative Center		
Swelling		
Diameter of cell	4.0-20.0	3.0-12.0
Length of cell	4.0-30.0	4.0-24.0
Diameter of globules	0.2-3.0	0.2-2.0
Thickness of wall	0.2 or less	0.2 or less
Diameter of nucleus	1.5-2.0	2.0
Isthmus		
Length	0.0-7.0	0.0-6.0
Diameter	0.5-1.5	0.5-2.0
Thickness of wall	0.2 or less	0.2 or less
Rhizoids		
Length		
Branched	9.0-60.0	9.0-48.0
Unbranched	2.0-30.0	2.0-20.0
Diameter		
Branched	0.5-1.5	0.5-2.0
Unbranched	0.5-0.8	0.5-1.0
Extent of Vegetative Portion	35x50-35x200	40x40-16x160
Extent of Reproductive Portion	up to 2 cm.	up to 2.5 mm.

DISCUSSION

The long extramatrical filaments which arise from the vegetative portion of the thallus, often in great enough numbers to obscure it, are referred to here, collectively, as the "reproductive portion" because each of these filaments may potentially bear intercalary or terminal zoosporangia or, in N. ramosa, resting cells. The "vegetative" and "reproductive" portions of the thallus are distinct from one another in external and internal structure in both the N. ramosa and N. profusa strains studied. The vegetative portion as a separate unit superficially resembles the "rhizomycelium" of the other polycentric chytrids with the nuclei confined to the replicated swellings and the presence of anucleate isthmuses and rhizoids. However, these two species differ from the others in that no reproductive organs are produced within the swellings of the vegetative portion. A possible exception to this is that Karling's original description of N. profusa reports the formation of resting bodies formed directly from the intramatrical swellings. The present strain of N. profusa is sterile in this respect. It might be that the resting bodies described by Karling, as well as those by Whiffen (1943) for N. delica, were not transformed true vegetative swellings, since reproductive bodies may be formed intramatrically on short filaments which do not extend out of the cells of decaying grass leaves.

The sterile swellings confined to the vegetative portion of N. ramosa and N. profusa appear to be similar to the intramatrical prosporangial (the term is used here in the sense of Whiffen, 1944) swellings such as are found in Nephrochytrium, especially those forms which are lobed. Karling (1938) suggested that this monocentric

genus might be a stage in the evolution of the polycentric mode of development. If this conjecture were acceptable the flexuous filaments on which the reproductive organs are borne would be elongated and branched homologs to the isthmuses between the intramatrical prosporangia and the extramatrical zoosporangia. Such a hypothesis is substantiated to some degree by the occasional formation of a single sporangium close to the substratum on a short filament which extends only from the intramatrical swelling in the adjacent cell of the decaying leaf. The swellings in the extramatrical filaments might be the result of progressive sterilization accompanying the process of elongation.

The term "rhizomycelium" does not seem to characterize adequately the reproductive portions of these two strains. The filaments are specialized outgrowths of the vegetative portion and are not intimately associated with rhizoids in structure or function. Unlike the typical rhizoids of the chytrids, they do not become completely devoid of cytoplasm, nuclei, and food materials during the production of a single zoosporangium; but retain enough to produce secondary, tertiary, and chains of zoosporangia in basipetal succession (especially in N. profusa which is lacking numerous swellings in these filaments). Unlike in the "rhizomycelium" of Cladochytrium replicatum, the nuclei are not confined in the swellings of these sporangium-bearing filaments.

The vegetative portion persists throughout the life of the thallus as an absorbtive and storage unit, so that a complete change from a trophocentric to a genocentric thallus does not occur. In this manner the thallus of Nowakowskiella resembles the true mycelium of the higher fungi, and continues to digest the

substratum and produce asexual spores until the available food supply has been diminished or the concentration of metabolic waste in the medium inhibits the growth.

Similar to the higher fungi, N. ramosa produces fewer asexual spores, and initiates resting-cell formation when the culture has aged. So far as has been possible to determine, the production of resting-cells in this species is not preceded by nuclear fusion, Karling (1945c) refers to the fusion of filaments before the formation of the pseudoparenchyma as "vegetative anastomosing". Shanor (1942a) presented some evidence that a fusion nucleus may be present in the resting-cell of N. hemisphaerospora. The same might be true of N. ramosa, for the cytoplasmogamy in N. hemisphaerospora bears some resemblance to the formation of the first cell of the pseudoparenchyma in N. ramosa. The formation of "parthenogenetic" resting spores has been reported for the genus Siphonaria, a genus which supposedly demonstrates sexuality among the chytrids (Karling, 1945c).

In general, the two species of Nowakowskiella studied do not differ greatly in the behavior of the nuclei and cytoplasm during the development of the thallus and the formation of reproductive organs from that described for other chytrids. Such differences as do occur may be primarily due to the specialization of the portions of the thallus and the extensive extramatrical development.

The most pronounced characteristic by which N. ramosa and N. profusa differ from the other polycentric chytrids is the distinct differentiation of the thallus into vegetative and reproductive portions.

As to the relationships of Nowakowskiella, little can be ventured until other members of the operculate series of the Chytridiales are investigated more fully. This genus might suggest an incompletely known phylogenetic series between the Chytridiales and the Zygomycetes in general habit and in the possible fusion of filaments preceding the formation of resting-cells. This hypothesis can be considered merely as an interesting speculation until much more is known about the trophic mycelium as compared with the reproductive mycelium of the latter and until possible transition forms have been investigated.

SUMMARY

1. The growth of Nowakowskiella ramosa and N. profusa is abundant on solid substrata composed primarily of cellulose if the surrounding liquid contains organic decomposition products, such as found in river water, pond water, etc.

2. The natural optimum temperature for the development of N. ramosa is between 16° and 18°C. and that of N. profusa, 24° and 28°C. Both species may be adapted to cultivation at the other temperatures.

3. The cell walls of both species show a predominance of chitin which is mixed with cellulose in the same layer. The operoula are pectinaceous.

4. The behaviour of the protoplasm during development of the thallus and formation of the reproductive organs does not differ greatly from that in other chytrids.

5. The flagellum appears to be formed within the sporangium and is connected with the nucleus of the zoospore by a rhizoplast near one of the points of a tilted nuclear cap.

6. The center of growth in the developing thallus is early transferred from the spore case to a swelling in the germination tube.

7. The mature thallus is divided into two distinct portions which function concurrently, the vegetative portion and the reproductive portion.

8. The complete thallus is not adequately characterized by the term "rhizomycelium" whch has been applied to the thalli of the polycentric chytrids.

Acknowledgements

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(all except fig. 7, 9, and 22 approximately 25 microns / inch)

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Fig. 1. Living zoospores: a-l, typical spores; m-q, spores with more than one globule, flagellum, and/or blepharoplast

Fig. 2. Stained zoospores: a-k, typical spores; l-s, spores with more than one nucleus, flagellum, rhizoplast, and/or blepharoplast; q, r, s, possibilies of structures in the event that two blepharoplasts are formed

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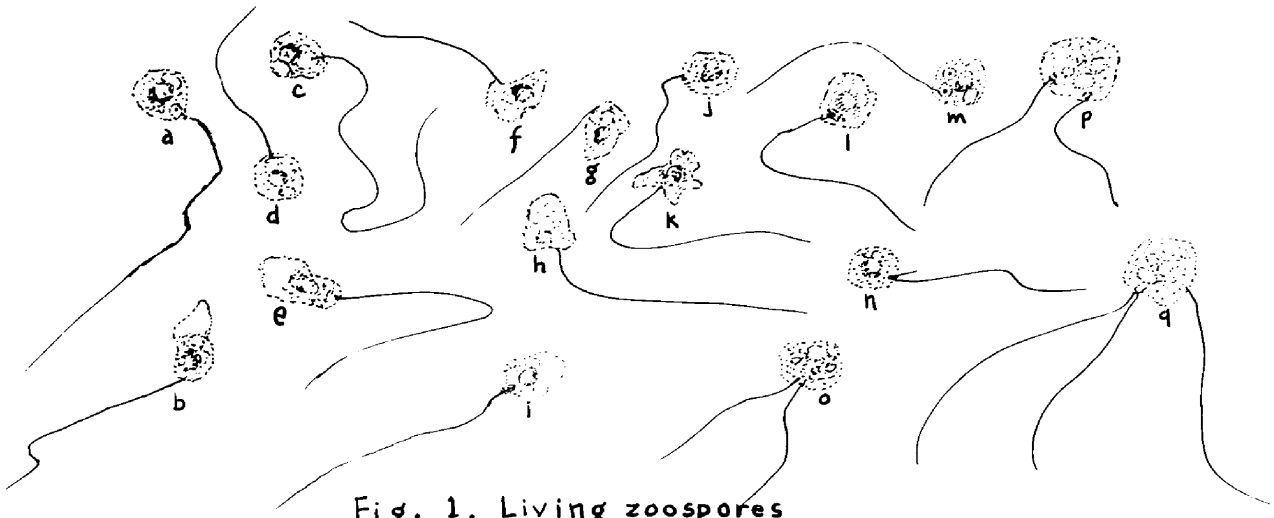

Fig. 1. Living zoospores

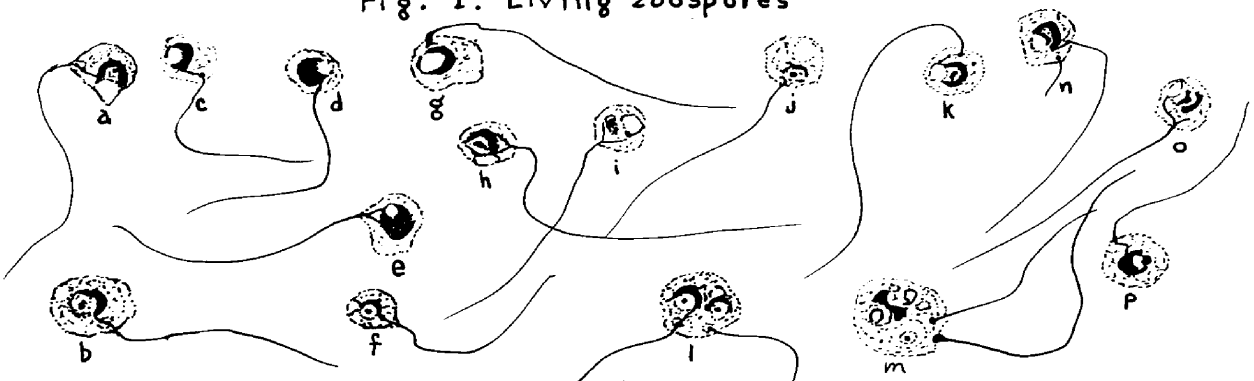

Fig. 2. Stained zoospores

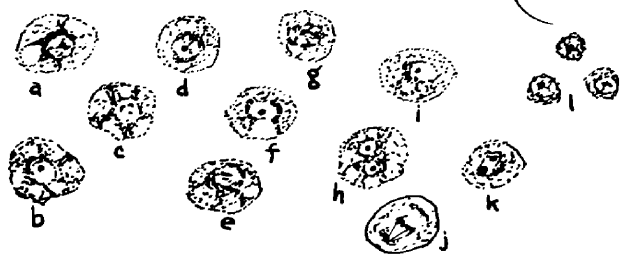

Fig. 3. Sessile spores

Fig. 4. Budding of amoeboid spores

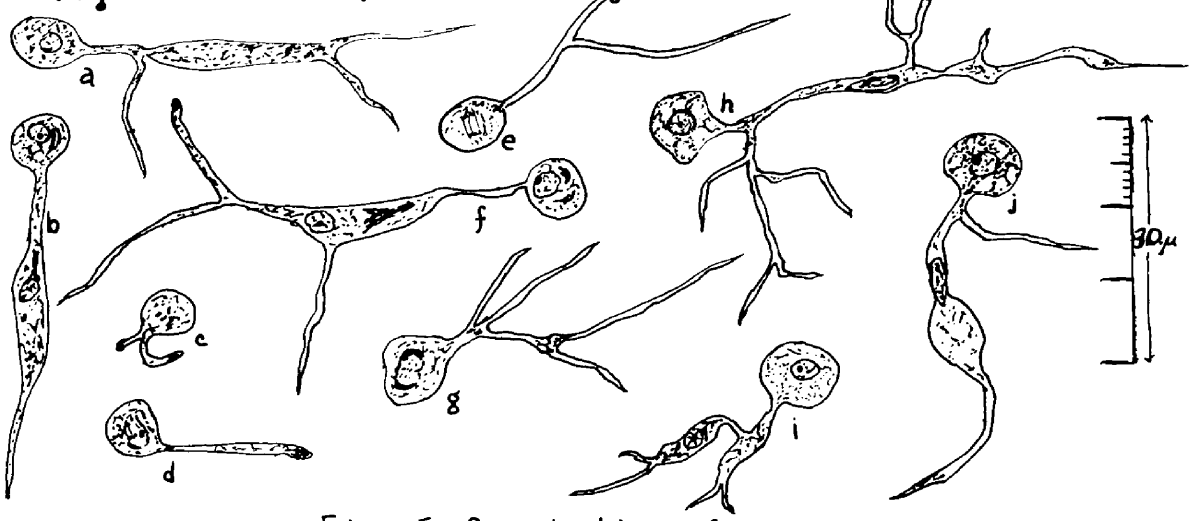

Fig. 5. Germination of spores

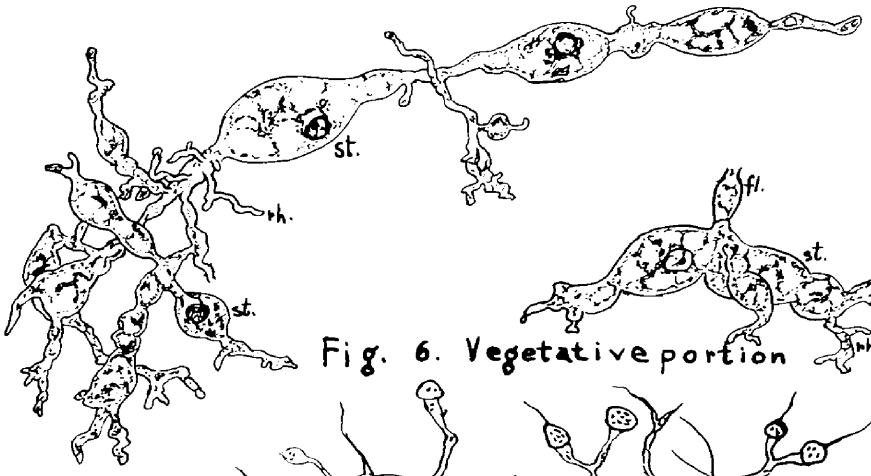

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Fig. 7. Habit sketch of thallus

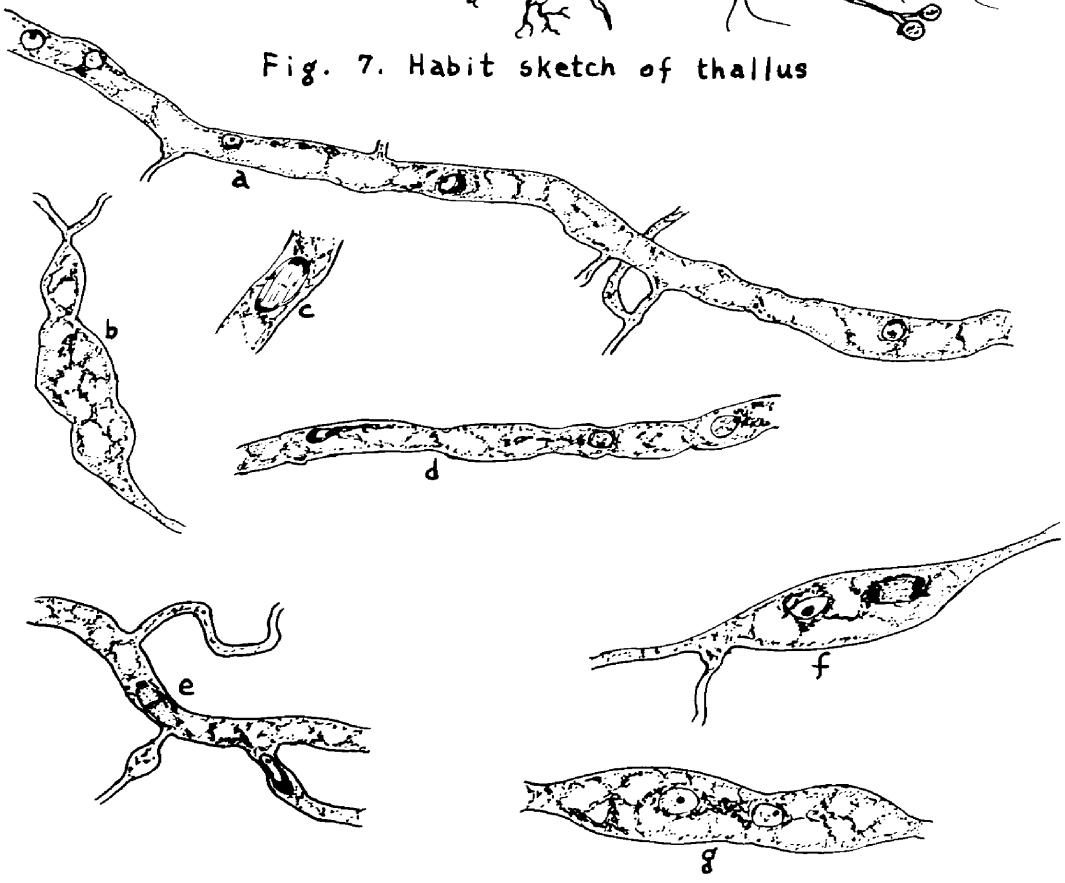

Fig. 8. Flexuous filaments

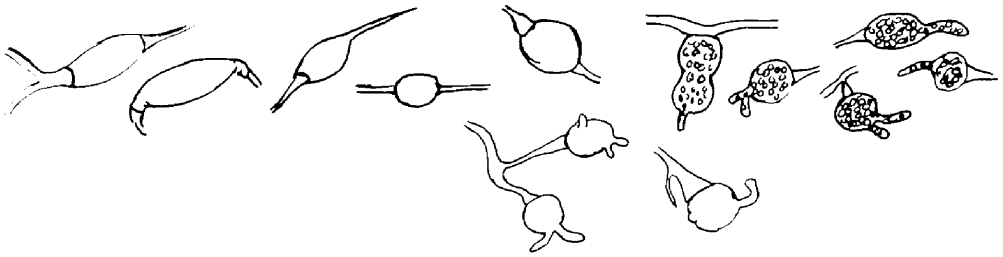

Fig. 9. Zoosporangium shapes

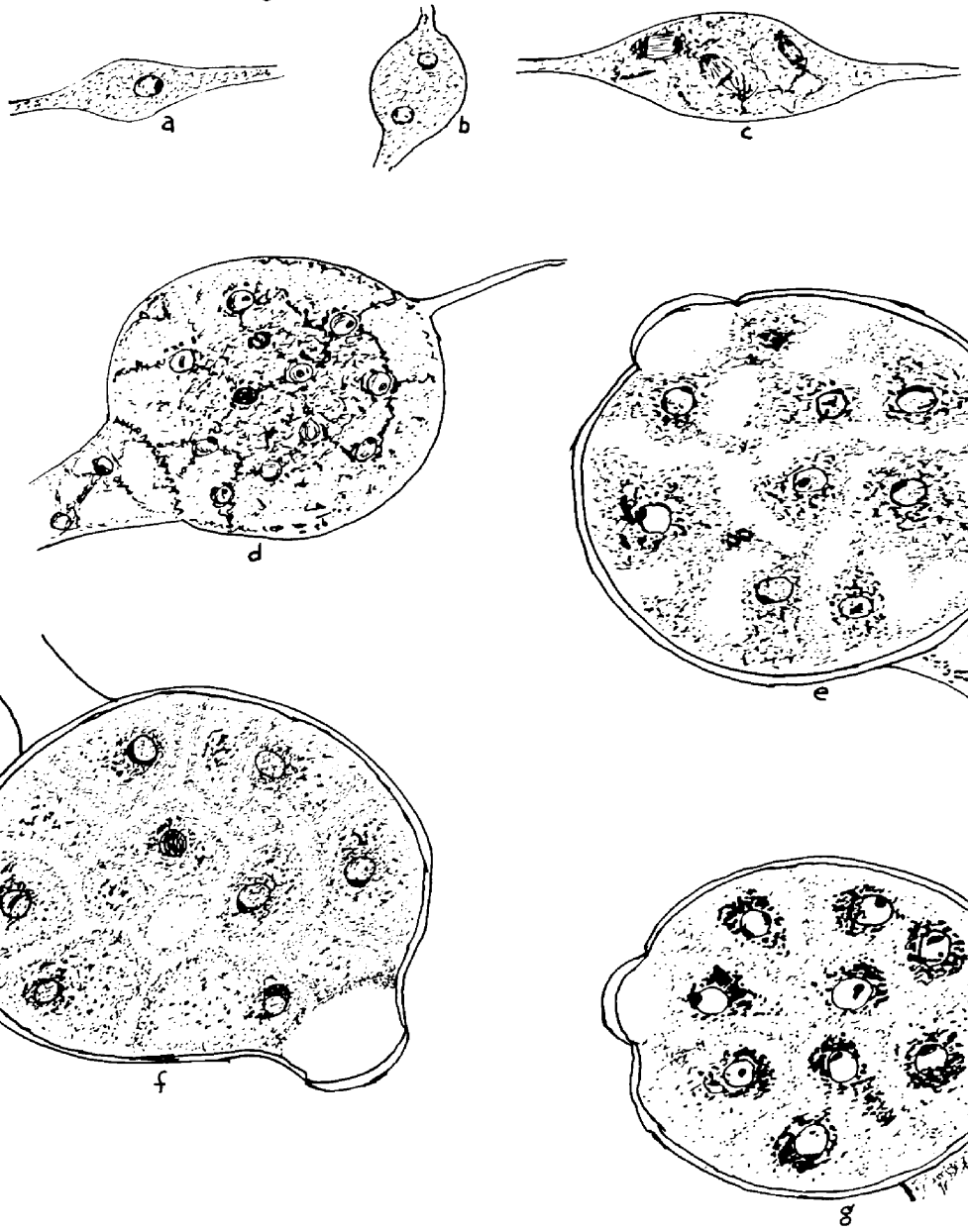

Fig. 10. Development of zoosporangium

Fig. 11. Mature zoosporangium

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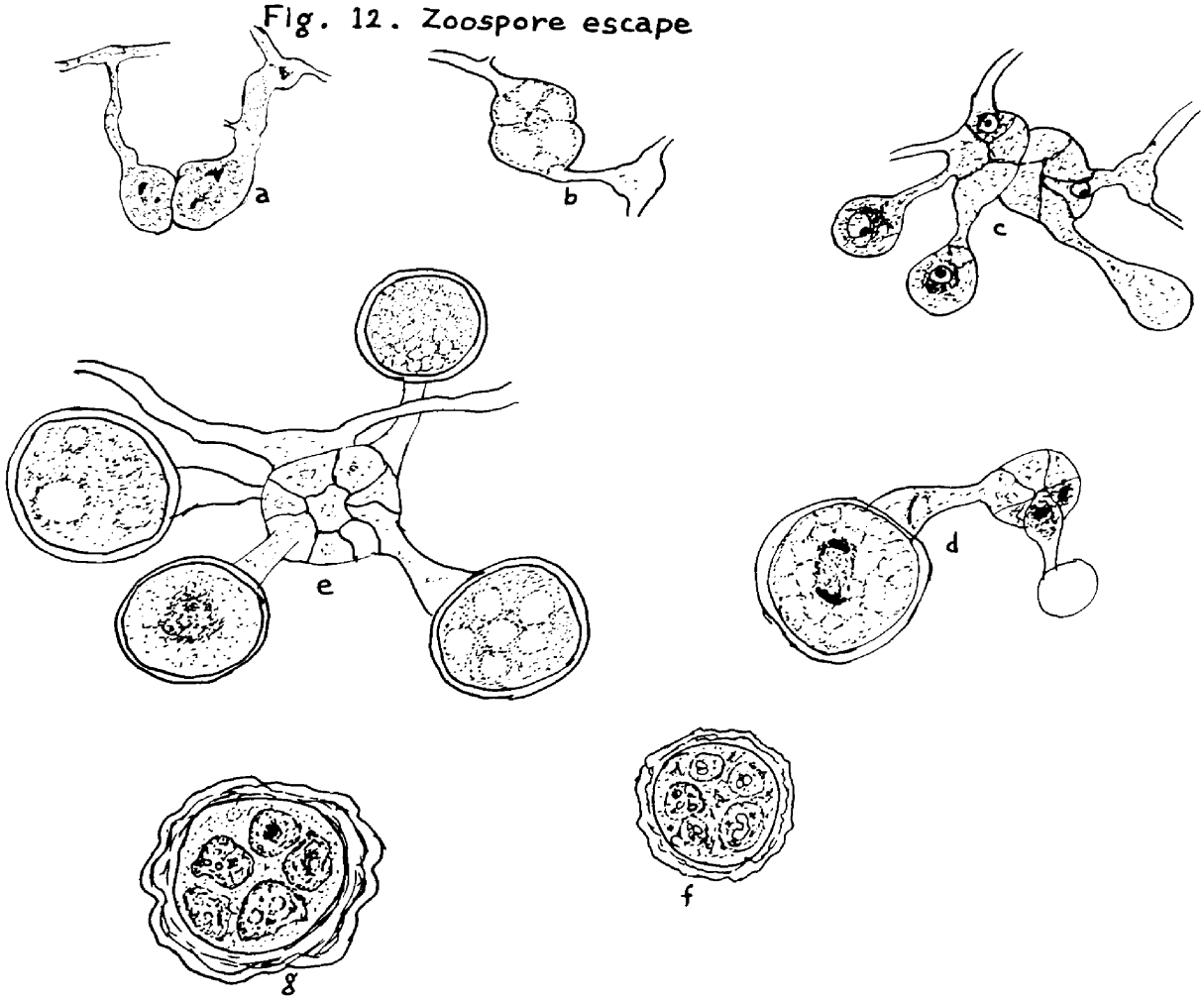

Fig. 13. Formation of resting cell

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Fig. 22. Zoosporangium shapes (about 60 microns/inch): a, chain of sporangia in which a filamentous isthmus occurs; b, "proliferating" chain; d,and f, unevenly-shaped elongate forms
Fig. 23. Zoosporogenesis (stained): a, granules massed in center; b, nuclei aligned on granular strands of cytoplasm; c, cell showing simultaneous but not completely synchronized mitosis; d, late stage in the formation of nuclear caps; e, mature zoospores in the sporangium

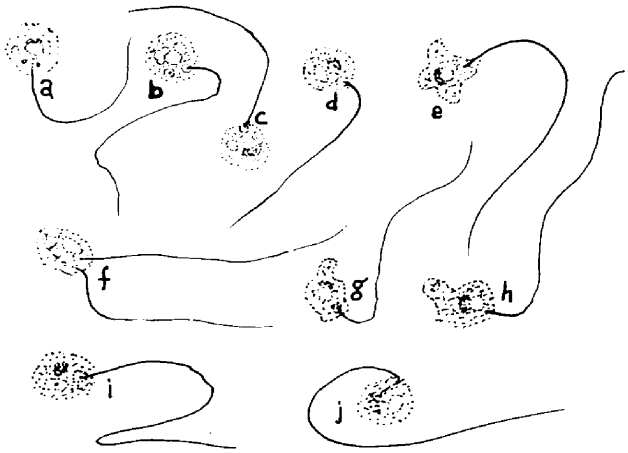

Fig. 14. Living zoospores

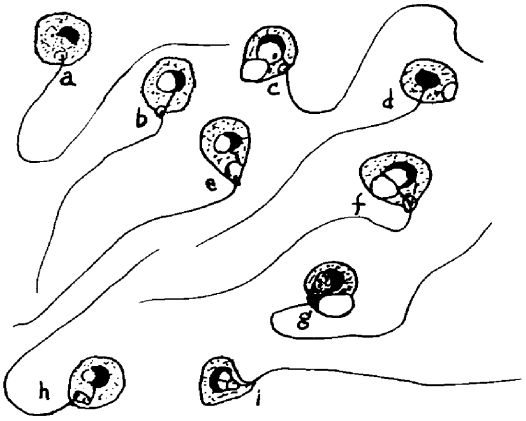

Fig.15. Stained zoospores

Fig. 16. Germinating spores

Fig. 17. Vegetative portion

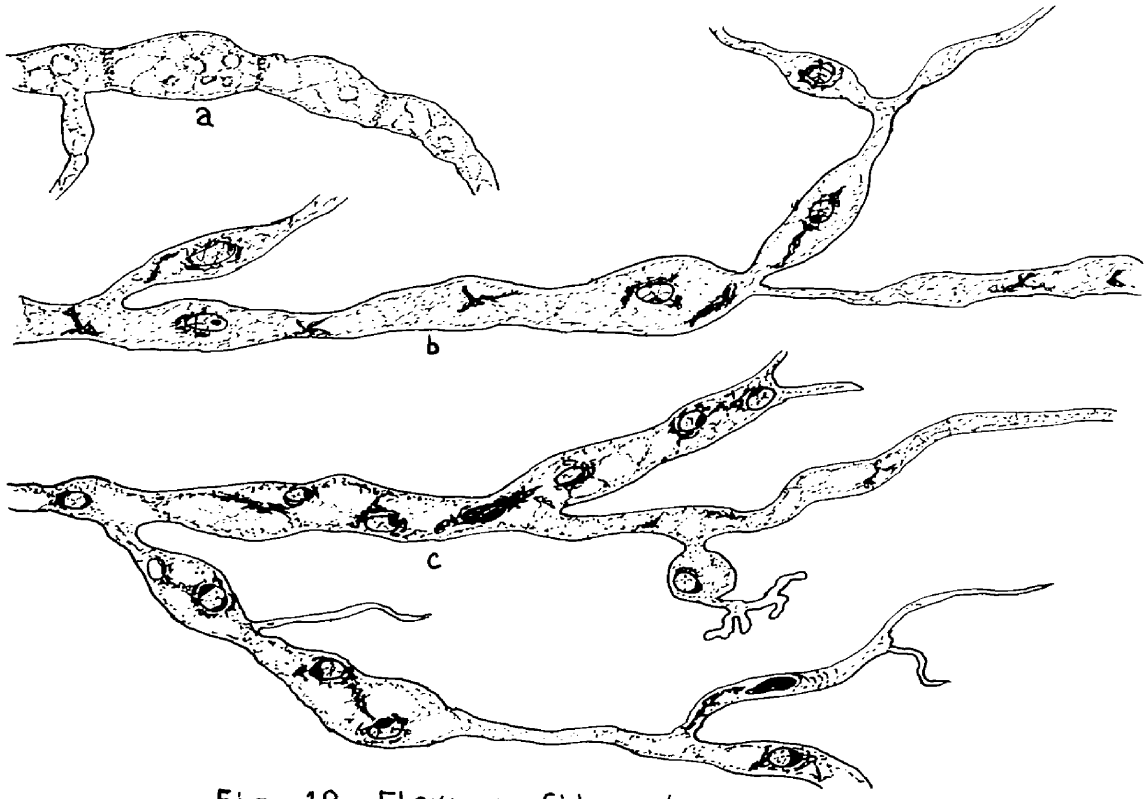

Fig. 18. Flexuous filaments

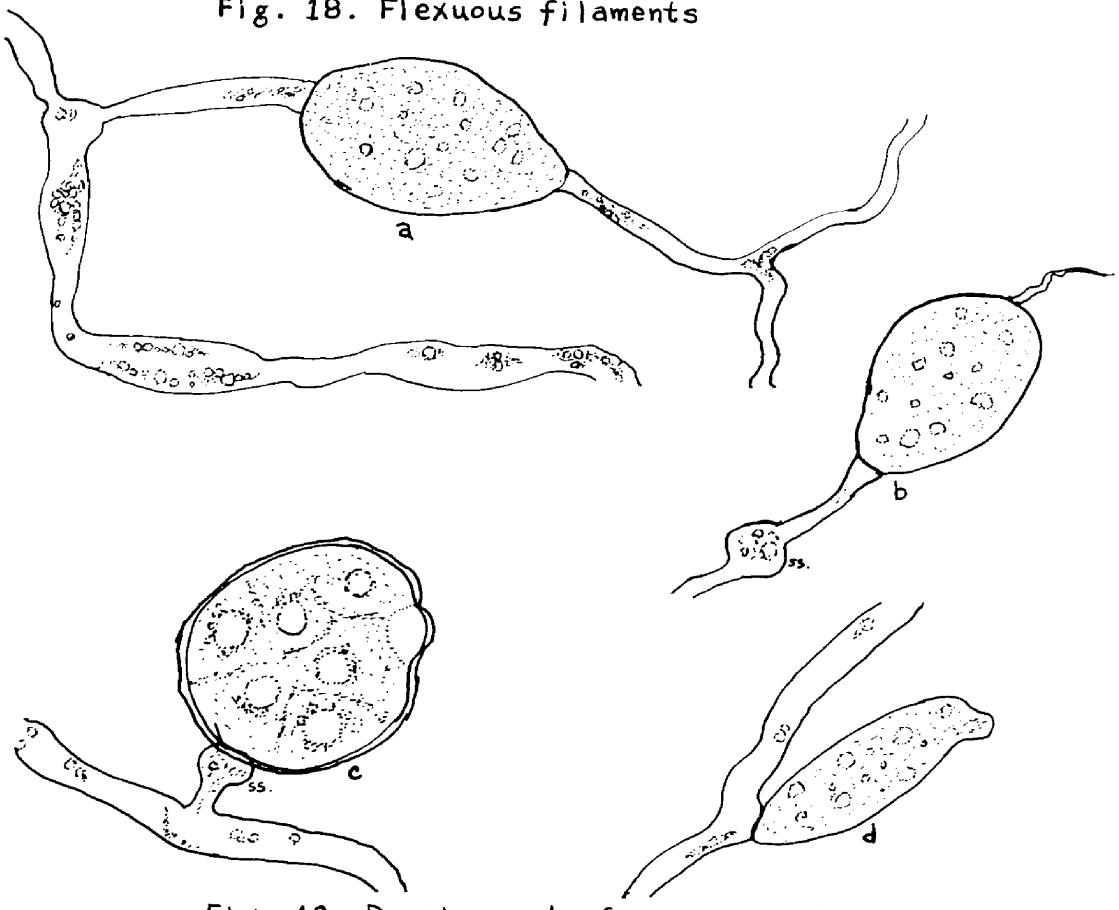

Fig. 19. Development of zoosporangium

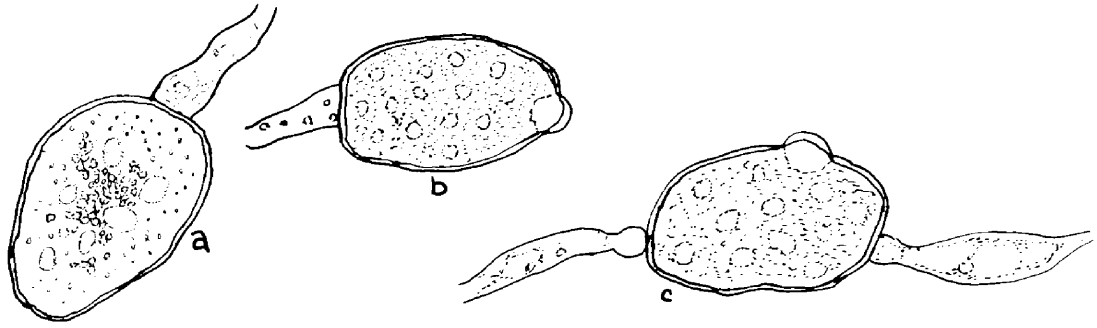

Fig. 20. Maturation of zoosporangium

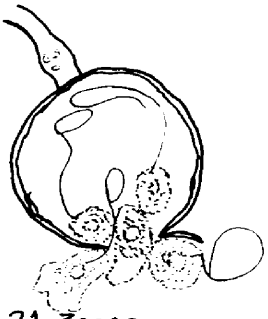

Fig. 21. Zoospore escape

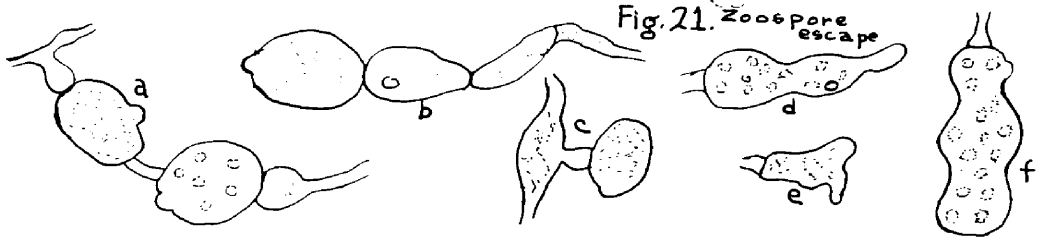

Fig. 22. Zoosporangium shapes

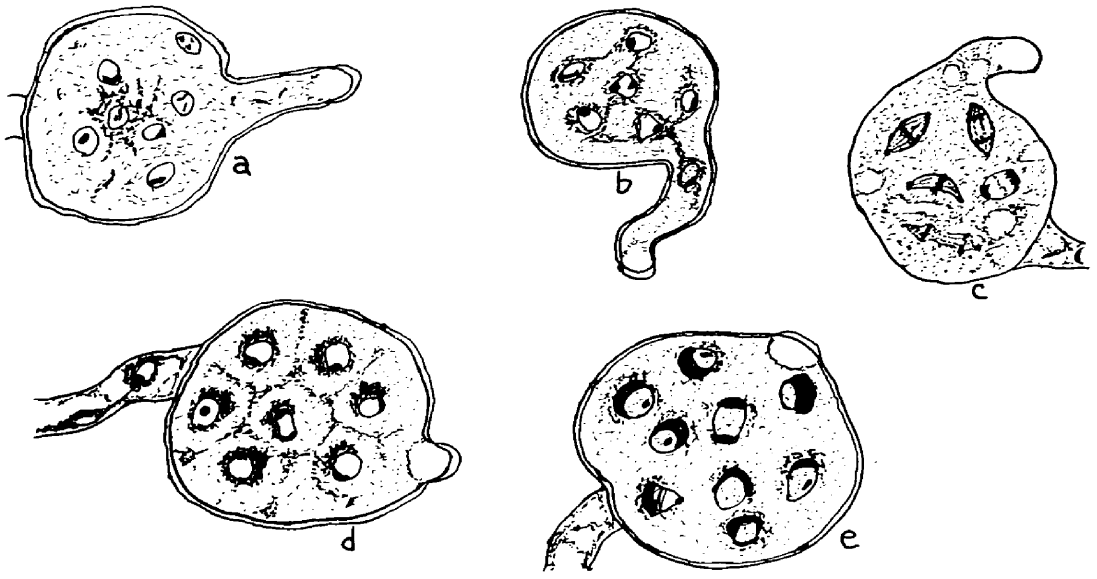

Fig. 23. Zoosporogenesis

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