NOTES ON THE DEVELOPMENT OF THE GAMETOPHYTE AND EMBRYO OF ASPLENIUM ANGUSTIFOLIUM MICHX.

RUSKIN S. FREER,
Lynchburg College.

OCCURRENCE.

Asplenium angustifolium Michx., (*Asplenium pycnocarpon* Spreng.), the Narrow-Leaved Spleenwort, may be found from western Quebec and New Hampshire to Minnesota, and southwards to Georgia, Alabama, Missouri and Kansas (Gray, 24, and Britton and Brown, 6). Eaton (12) states that it probably occurs in the mountains of northern Georgia, and that it is more common in the Ohio valley than in New England. He also states that it has no close relatives anywhere, the nearest being a South African species, *Asplenium anisophyllum.*

There are two records of its occurrence in Louisiana, (Pennell, 21), and it is listed in Mohr's "Plant Life of Alabama," from a mountain region with an elevation of fifteen hundred feet. The Louisiana station is at an elevation of less than two hundred feet. Otherwise its southern distribution seems to be montane.

Asplenium angustifolium is reported as occurring very abundantly in some places in Camden County, Missouri, especially in limestone sinks, where it may cover an acre or more, to the exclusion of other vegetation (Standley, 26). It is generally spoken of as one of the rarer ferns by New England students of the pteridophytes, but when found, is frequently reported as growing closely associated with Goldie's Fern, *A. Goldianum* (5, 11, 16).

Of interest in connection with the occurrence of *A. angustifolium* is the following note by A. H. Young (41) from the first issue of the Botanical Gazette, then called the Botanical Bulletin, published in November, 1875:

"*A. angustifolium* * * * is found in southern Indiana. It is an inhabitant of cooler regions, and here it seeks the coolest and dampest portions of the ravines running back from the Ohio river, and it is always found encircled by a rich carpet of moss to shield its roots from the heat and retain sufficient

moisture. It is by no means abundant, and indeed this remark will apply to all our ferns with the exception of three or four species."

A survey of Ohio botanical literature indicates that the Narrow-Leaved Spleenwort is not a common plant. There are specimens in the herbarium at the Ohio State University from twenty counties, not including Delaware County. Griggs (18) includes it in his list of plants of the Sugar Grove region. Moseley (19) speaks of it as infrequent in Erie County. Schaffner (25) lists it as general, but not common in the state.

The single plant from which spores for this work were obtained, was found in a shaded ravine on the east side of the Scioto River Valley, about four miles west of Delaware, Ohio.

A. angustifolium is usually found in ravines in rich soil, where shade and moisture are abundant. One writer (33) states that it prefers a basic soil, but tests of the soil around the plant used in this work showed results to the contrary.

The stipes grow in tufts, are very erect, and are from one to four feet in length. The leaves are pinnate, with numerous pinnæ, which are linear-lanceolate, acuminate, with entire margins. The fertile fronds are narrower than the sterile. The fruit dots are linear. The fronds are very thin and are easily affected by frost. Woolson, as quoted by Tilton (32), writes of it, "There is nothing in the fern kingdom which looks so cool and refreshing on a hot day as a mass of this clear-cut, delicately made-up fern."

WORK ON RELATED GENERA.

So far as the writer has been able to discover, no work has been done on the gametophyte of *A. angustifolium*, and except for the work of Campbell (9) and Pickett (23), very little has been done on the complete development of the gametophyte of any of the Polypodiaceæ. The former has described the development of *Onoclea Struthiopteris*, and the latter more recently has worked out the development of the prothallium of *Camptosorus rhizophyllus*.

Much has been done, however, in the study of certain phases of the development of the gametophyte of the Polypodiaceæ. Atkinson (2) was the first worker to report polyembryony in the ferns. He found this condition in *Adiantum cuneatum*. Recently Etter (13) has successfully determined

methods for bringing about polyembryony in *Matteucia Struthiopteris*, *Dryopteris mollis*, *Onoclea sensibilis*, and *Pteris longifolia*.

Farlow's (14) discovery of an asexually developed sporophyte on *Pteris cretica* var. *alba lineata* in 1874, was the beginning of numerous studies in apogamy.* Steil (27) has described apogamy in *Pellea atropurpurea*, and (28) in *Notholaena*, *Pteris* and *Aspidium*. Miss Wuist (38, 8) had similar results with *Phegopteris polypodioides* and *Camptosorus rhizophyllus*. Yamanouchi (39, 40) has done a very excellent piece of work on *Nephrodium*, in the study of apogamy, spermatogenesis, oogenesis and fertilization.

Miss Ferguson (15), without knowing of Miss Black's (3) work on imbedded sexual cells in *Dryopteris stipularis* and *Nephrodium molle*, found similar structures in some species of Pteris. Miss Black's paper, which was published first, raises some very interesting questions as to sex inheritance, and the influence of environmental factors, particularly direct sunlight and dryness, on determination of sex. She also raises some question as to the validity of Yamanouchi's assumption that the embryo shown in Plate 10, Figure 29, (40) developed in an apogamous manner from the shaded cell in his Figure 1.

Goebel (17)* has given an account of apospory in *Asplenium bulbiferum*, and Steil (29, 30) has reported this condition in *Pteris sulcata* and *Polypodium irioides*.

Miss Wuist (35, 36) and Mottier (20) have studied the environmental factors controlling sex in the prothallia of *Onoclea Struthiopteris*. A reference is made to their work later.

Miss Black (4) has reported branched cells in the prothallia of *Onoclea sensibilis*, and Miss Wuist (37) records many genera and species of the Polypodiaceæ in which branched prothallia occur. The branched cells reported by Miss Black were developed under water. A deficient oxygen supply was believed to be the cause of this abnormality. Miss Wuist's results were attributed to nutrition.

Several other unrelated papers have been published, bearing on some phase of the development of the gametophyte of the Polypodiaceæ. Some interesting observations on unequal segmentation in the embryo of *Adiantum cuneatum* and *Pteris serrulata* have been made by Atkinson (1). Woodburn (34) found an instance of polyspermy in *Onoclea Struthiopteris*.

*The writer was unable to secure the original papers.

Four neck canal cells with a definite wall between two of the neck canal cell nuclei, were found by Miss Pfeiffer (22) in *Pteris longifolia*. Successful attempts at regeneration from young detached sporophyte leaves of *Phegopteris polypodioides* were made by Mrs. Brown (7). The leaves were placed in sand moistened with nutrient solutions. One produced a prothallus-like structure which in turn produced four new sporophyte leaves. Vegetative reproduction, and development of prothallia and secondary antheridia from both antheridia and archegonia, have been observed by Steil (30, 31) in *Polypodium irioides*. He assigns unusual cultural conditions as the cause.

THE SPORE AND ITS GERMINATION.

Before germination the spores are enclosed in a roughly and irregularly wrinkled perinium (Text-Fig. 1, A). They are spherical and are about 50 micra in diameter. Germination begins with the swelling of the endospore and rupturing of the perinium, followed by the extension of the first rhizoid, which makes rapid growth (Text-Fig. 1, A, B, C). Chloroplasts are numerous in the swollen endospore, though not so densely crowded together as in older cells. The chloroplasts are oval in shape. The perinium adheres to the first cell for a long time, being present usually on gametophytes bearing fertilized egg cells.

Spores were sown on soil in earthenware platters. These platters of soil had been thoroughly sterilized in an autoclave. In addition to the sowings on soil, others were made on Knop's solution and on sphagnum. The platters were covered with battery jars in such a manner as to allow ventilation, and were kept in the greenhouse at a temperature of about 70° and subjected to the usual greenhouse lighting conditions. At least a month was required for germination in some cases, and growth was slow. In one case, small prothallia were visible with the aid of a pocket lens in thirteen days after sowing, and were visible to the unaided eye in twenty-four days. Cultures of *Pteris longifolia* sown at the same time germinated more rapidly and made a great deal more rapid growth all along. Other experimenters report germination and greening of the soil in from three to seven days for the species more commonly studied, but Pickett (23) states that a period of twenty-five days elapsed between the sowing and first appearance of green in the case of *Camptosorus rhizophyllus*. The gametophytes of *Asplenium*

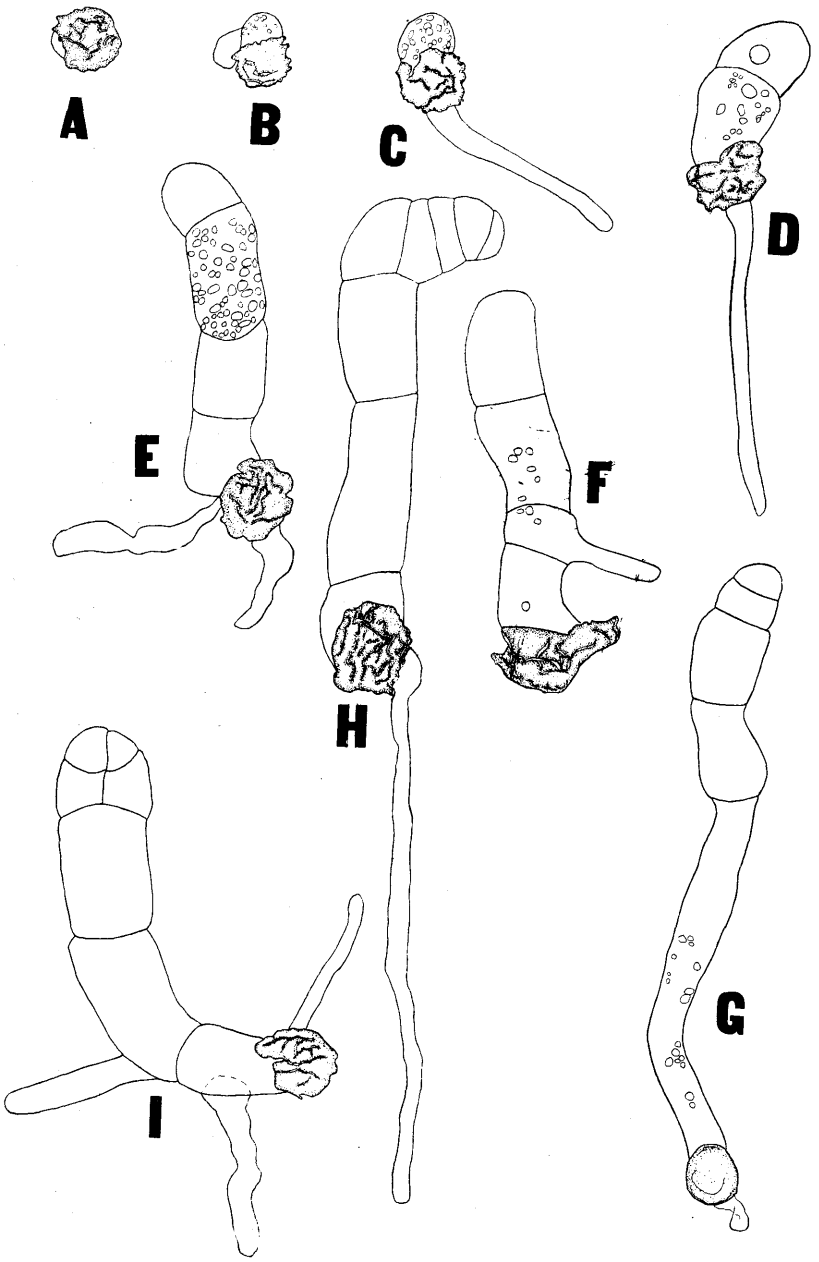

Fig. 1. Germination of spore, and early divisions of prothallium, A-E, and I. F, G, H, some unusual forms of the young prothallium. ×130.

angustifolium were easily affected by a change of environment, as was shown by transference to the laboratory, which was heated by a gas stove. Growth was greatly retarded. The prothallia grew poorly on sphagnum. Most rapid growth took place on sterilized soil, while germination and growth on Knop's solution were somewhat slower. Judging from the slow growth they made, optimum conditions apparently were not obtained with most of the cultures.

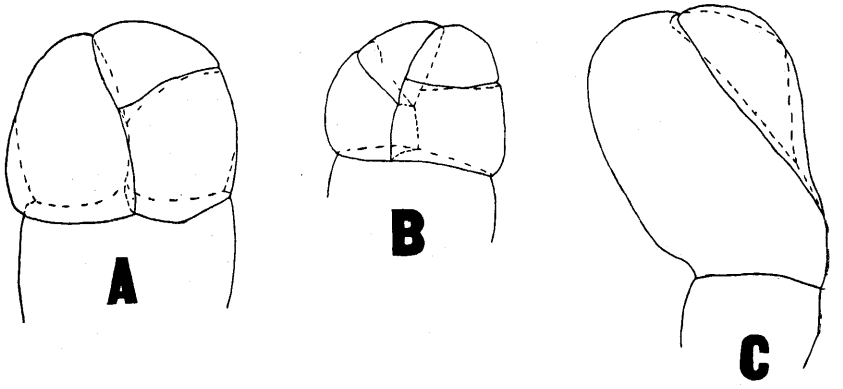

Fig. 2. Methods of division in terminal cell. ×170. *x*, apical cell.

DEVELOPMENT OF PROTHALLIA.

Further growth proceeds by division in a single plane for a time, (Text-Fig. 1, D, E, F, G), resulting in a protonema or filament, containing as many as nine cells (Text-Fig. 3, D), although division in a second plane may occur after the formation of only four cells, as shown in Text-Figure 3, B. An unusual elongation of the first cell is shown in Text-Figure 1, G. The perinium which is usually retained for some time, was not present on this filament. An irregular growth in which one or more cells were developed on the side of the young gametophyte is represented in Text-Figure 3, C. There were only a few such cases, all found growing on Knop's solution. Another peculiar behavior was frequently observed in prothallia grown on Knop's solution. After the development of a filament a few cells long, further growth was at right angles to the older cells, (Text-Figure 1, H). Probably this was caused by changing the position of the glass vessel with respect to the light. The prothallia grew prostrate on the solution. These growths closely resemble Figure 5 of Plate 22, illustrating Miss Black's paper (4) on branched cells.

Division in a second plane in the normal terminal cell takes place after six or eight transverse divisions. This division frequently occurs simultaneously in the last two cells formed

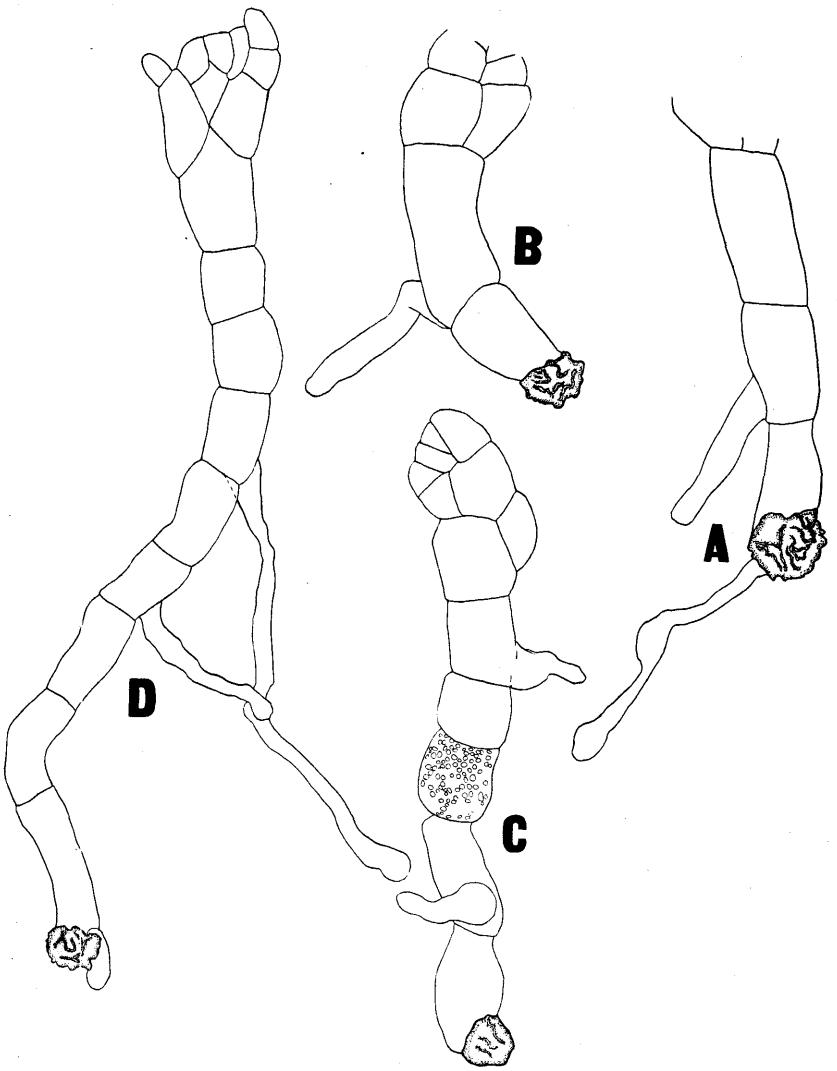

Fig. 3. Young filamentous prothallia. ×145. *x*, apical cell.

(Text-Figs. 1, I; 3, A). This is quite different from the usual behavior of *Onoclea Struthiopteris*, as recorded by Campbell (9), in which the first longitudinal division is oblique, and takes place

only in the terminal cell of the filament. Ordinarily, however, *Asplenium angustifolium* conforms to the method of division illustrated in Text-figures 2, A, B; 3, B. Here the terminal cell divides by formation of a longitudinal wall (Text-Fig. 2, A).

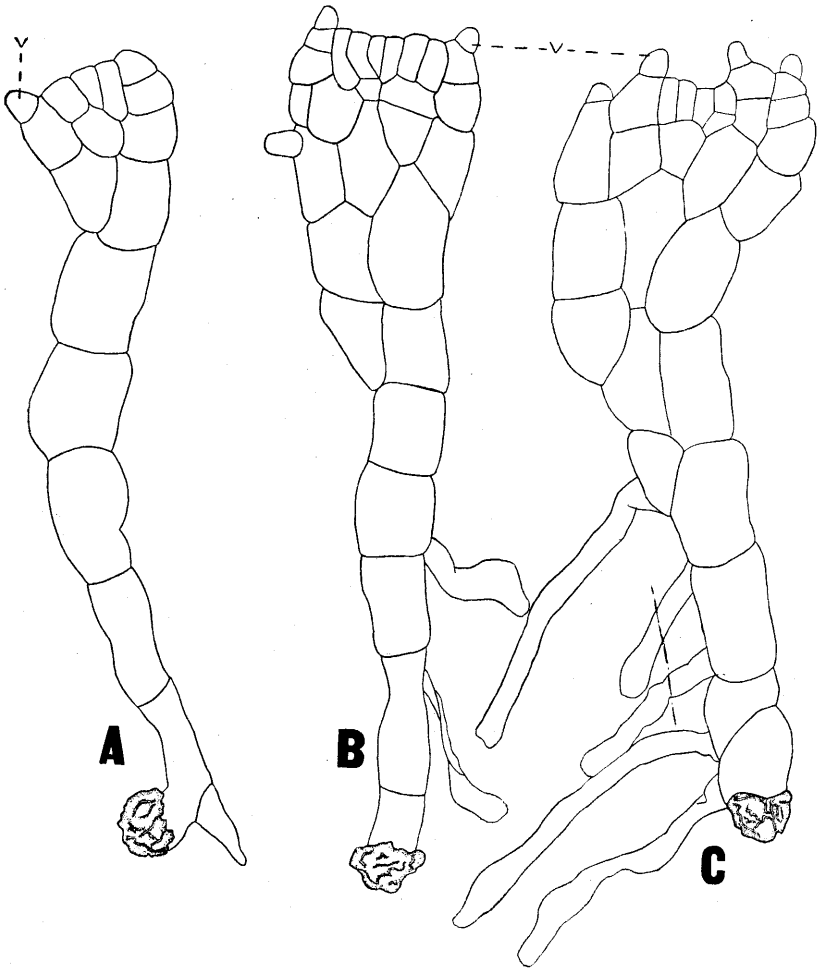

Fig. 4. Young gametophytes. ×145. *v*, young glandular hairs.

A somewhat oblique wall is next formed, resulting in the apical cell (*x*, Text-Fig. 2, A). The next is a transverse wall (Text-Fig. 2, B), then another longitudinal wall divides the large cell immediately below the former terminal cell (Text-Fig. 3, B). Only one case resembling the method Campbell described for *O. Struthiopteris* was observed (Text-Fig. 2, C).

The apical cell (*x*, Text-Figs. 2, A, B; 3, B) is the center of growth during the subsequent development of the gameto-

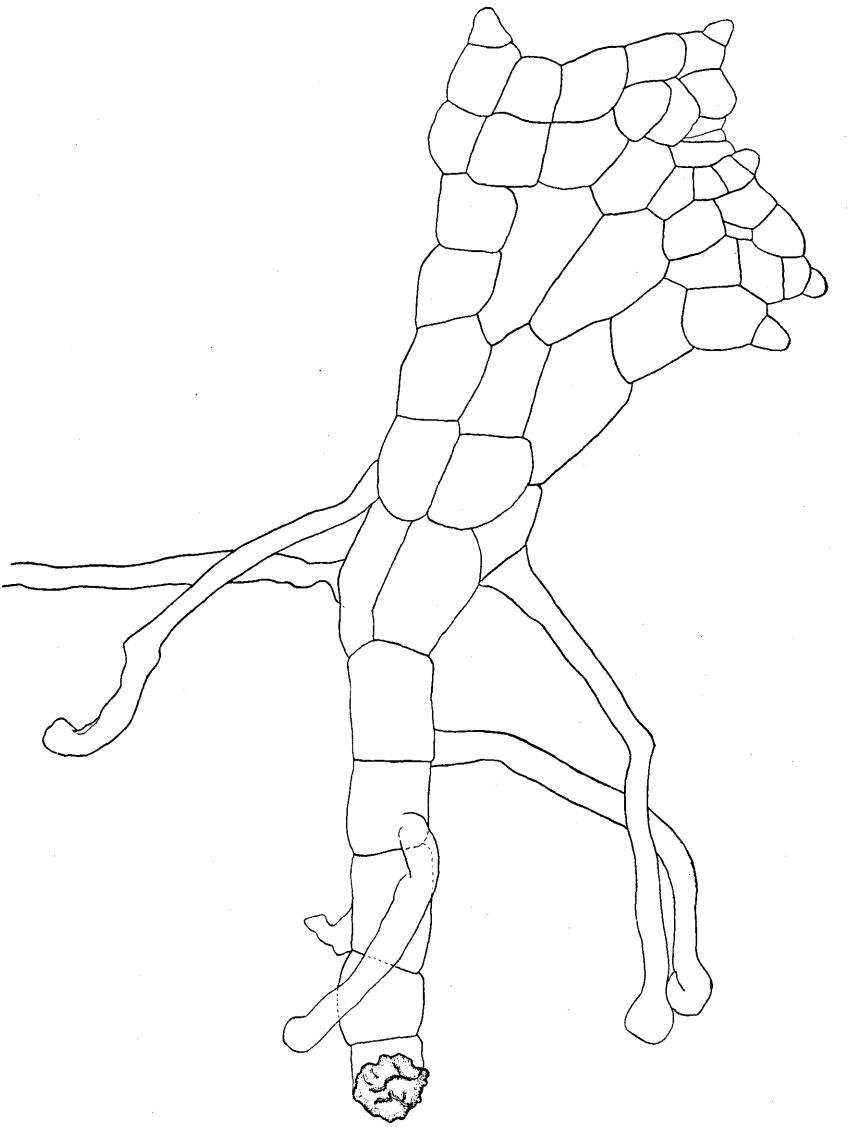

Fig. 5. Young fan-shaped gametophyte. ×150.

phyte. It is sometimes difficult to point out the apical cell in the earlier stages, but it may usually be distinguished by its triangular shape.

Mitotic figures have been observed frequently by the writer in the cells of the archegonial cushion, and not at all in the cells

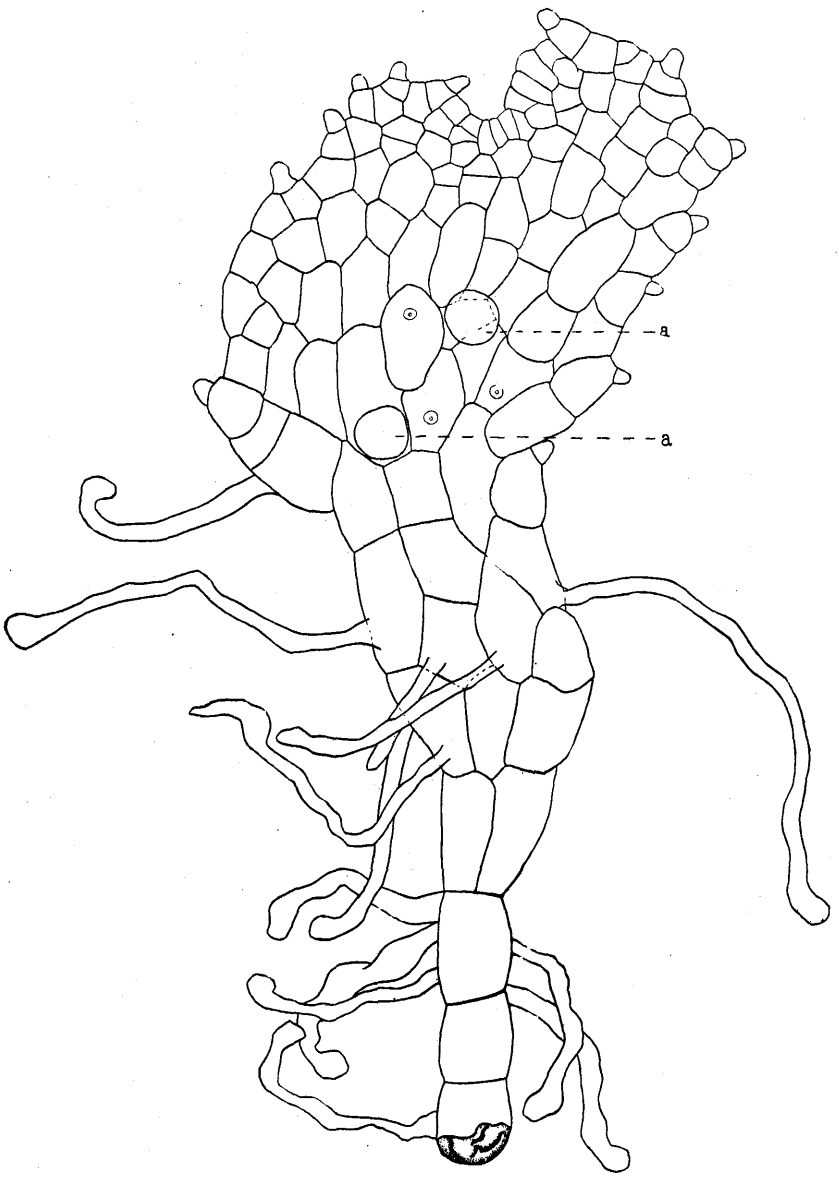

Fig. 6. Young gametophyte bearing two antheridia, *a*. ×100

of the surrounding lobes. They occur only in the anterior part of the cushion, however, (Text-Fig. 9, C). But it is

evident that cell division takes place not only in the apical cells, but in adjacent cells as well.

A rapid widening of the upper part of the prothallium follows the appearance of the apical cell, a fan shape being

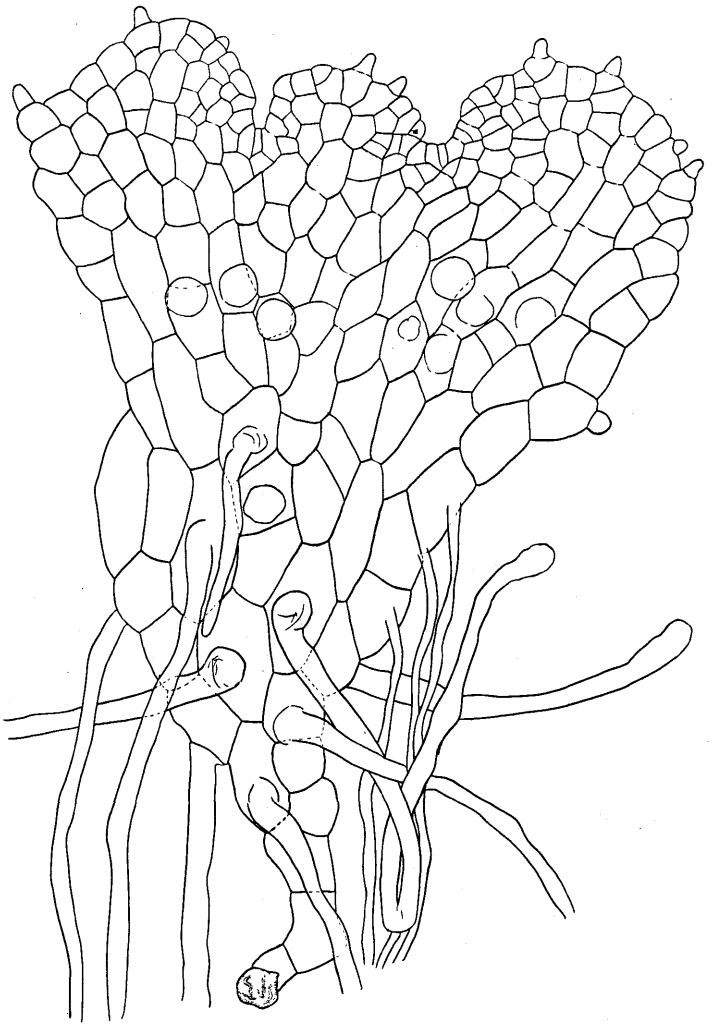

Fig. 7. Young prothallium showing two apical sinuses and several antheridia. ×105.

assumed, (Text-Figs. 3, D; 4, A, B, C; 5). By the rapid cutting off of cells on either side of the apical cell, lobes are developed, resulting in an apical sinus or indentation, which becomes deeper as the prothallium matures (Text-Figs. 6, 7).

This process results eventually in the regular heart-shaped structure which characterizes most fern prothallia.

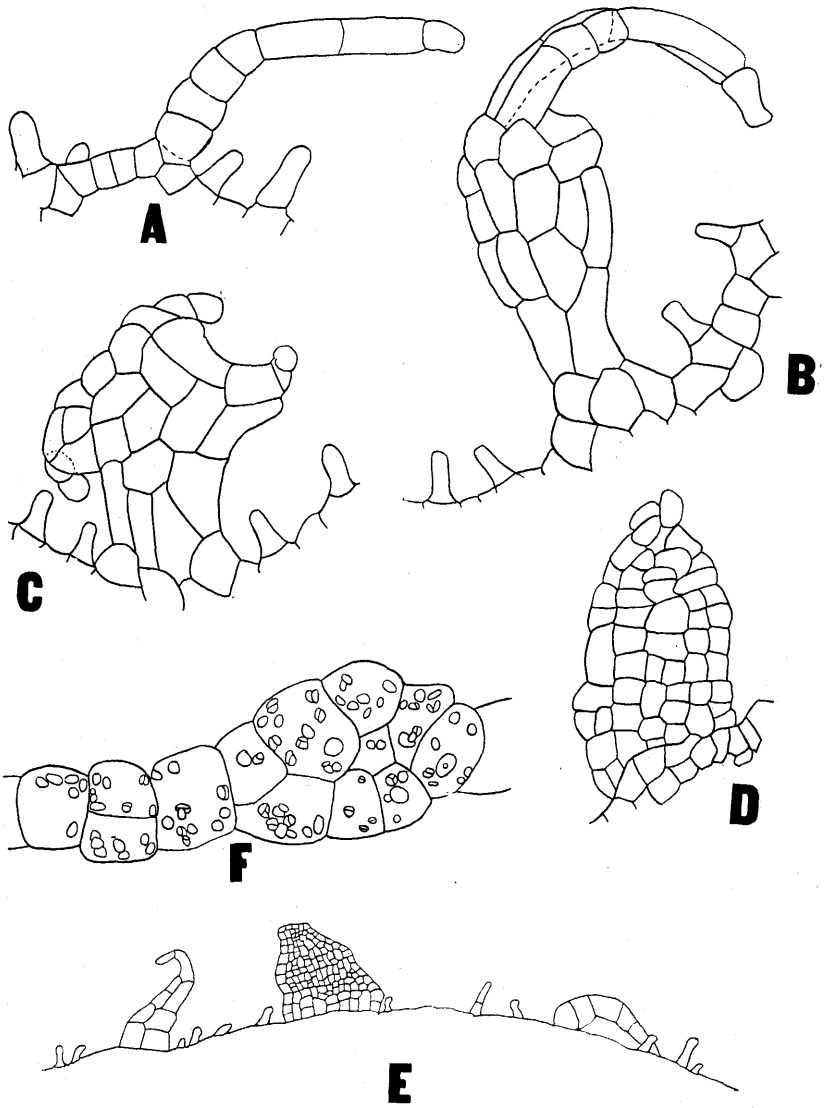

Fig. 8. Proliferations found on prothallia of *A. angustifolium*. A, B, C, D, F, ×125; E, ×50.

Very soon after the apical cell is discernible certain peculiar vegetative cells appear (*v*, Text-Fig. 4, A, B, C), which persist throughout the life of the gametophyte. Steil (28) designates

such structures as glandular hairs, and states that they occur in all species of *Aspidium* in which apogamy has been found, but that they do not occur in *Pellea* or *Pteris*.

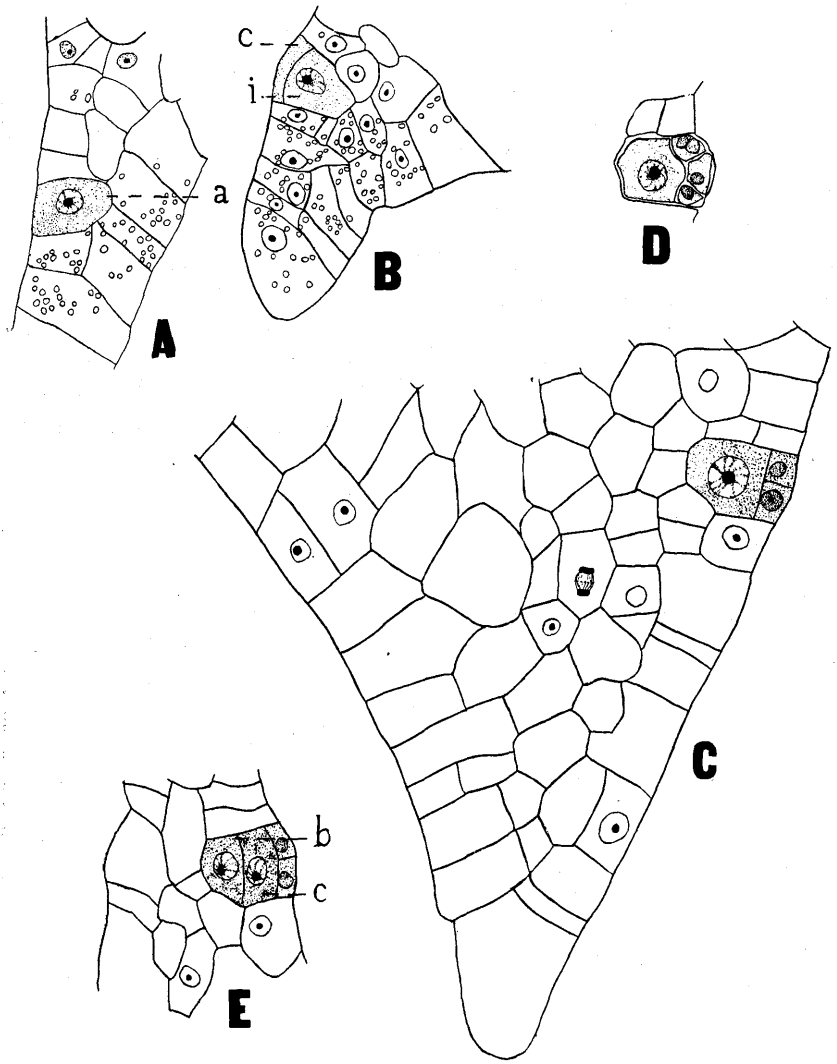

Fig. 9. A. *a*, archegonium initial. B. *c*, cap cell; *i*, inner cell. C. First division of cap cell. D. Second division of cap cell. E. First division of inner cell; *b*, basal cell; *c*, central cell. ×240.

The prothallium remains only one cell in thickness until it is about 2 mm. across, before the midrib or archegonial cushion is developed around the apical sinus. This is accomplished by

the cutting off of posterior segments from the apical cell or cells. These posterior segments then divide in a plane parallel to that of the prothallium, forming a cushion two cells thick. The older cushions are several cells thick, after many more posterior segments have been cut off from the apical cells, and after more divisions in the plane of the prothallium have occurred.

Pickett (23) finds irregularity of shape to be the usual thing in *Camptosorus rhizophyllus*. In *Asplenium angustifolium* the prothallia are remarkably regular in outline. However, a few prothallia showed unusual growths or proliferations along the wings. These outgrowths were devoid of chlorophyll, and took various forms (Text-Fig. 8, A-E). Several cases were found where there were two apical sinuses (Text-Fig. 7). Another interesting irregularity was discovered in one of the prothallia which was sectioned (Text-Fig. 8, F). Preceding and succeeding sections showed this to be a spherical cluster of cells out near the edge of the prothallium. It was entirely surrounded by only a single layer of normal cells. The cells comprising the cluster stained like the rest of the vegetative tissue, hence it was evidently simply a vegetative abnormality or proliferation.

THE DEVELOPMENT OF THE ARCHEGONIUM.

In the selection of prothallia for the study of archegonia, individuals were selected which seemed to be in the desired stage of development, and were examined under the microscope. In this way the particular age of the archegonium sought for may be determined fairly accurately. The wings of each prothallium selected were clipped off almost to the cushion, to reduce to a minimum the number of worthless sections on the slide.

After careful washing in distilled water, the prothallia were placed in chromo-acetic acid containing one minim of a 2% osmic acid solution per 100 c. c., for 12–36 hours. They were then washed in running water for 24 hours, and were run through the usual series of alcohols and alcohol-chloroform mixtures. Some dry erythrosin was added to the 100% alcohol to render the tiny pieces visible in the paraffin. The material was then imbedded in the usual way.

Sections were cut 5 micra thick and stained with the anilin-safranin, gentian-violet, orange G combination. The last dye was used in clove oil. Sections were examined with an oil immersion lens and drawings were made with the aid of a camera lucida.

Archegonia appear on the cushion on the ventral side of the prothallia when they are about 2 mm. across. The initial cell may be easily distinguished by its dense cytoplasm, very large nucleus, and keystone shape (Text-Fig. 9, A). After one becomes familiar with them, they may be found readily on most prothallia of the right age.

The first division of the initial cell results in the formation of the cap cell and the inner cell. The cap cell is thin in comparison with the latter (Text-Fig. 9, B). The nucleus of the

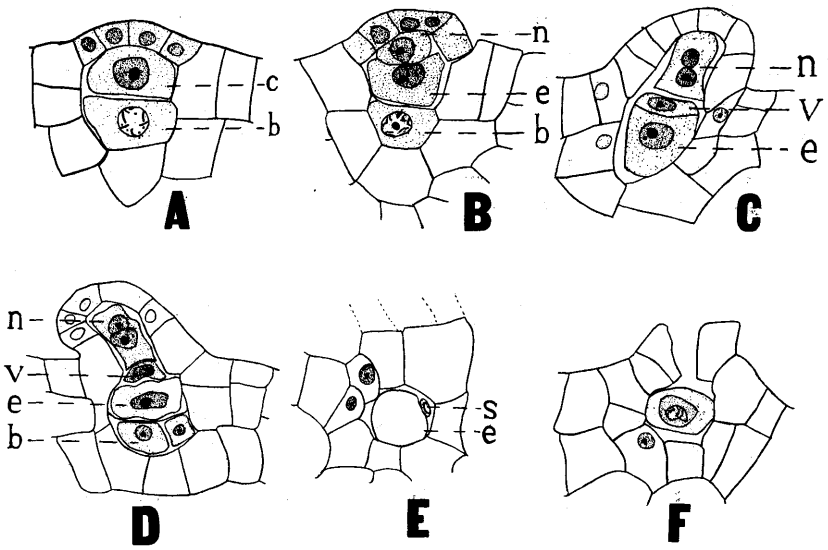

Fig. 10. Stages in the development of the archegonium. A. *c*, central cell; *b*, basal cell. B. *n*, cell which gives rise to neck canal cell; *e*, cell which gives rise to egg and ventral canal cell; *b*, basal cell. C. *n*, neck canal cell; *v*, ventral canal cell; *e*, egg. D. *n*, neck canal cell; *v*, ventral canal cell; *e*, egg; *b*, basal cells. E. *e*, egg; *s*, sperm. F. Fertilized egg, sperm within egg nucleus. ×230.

inner cell is larger than that of the first cap cell or its daughter cells. The first division of the cap cell is soon followed by divisions in the two daughter cells. These divisions which are at right angles to the first, result in the formation of the four cells constituting the chimney foundation (Text-Fig. 9, D). The inner cell divides into a basal and a central cell (Text-Fig. 9, E). The sequence of these divisions varies somewhat, but usually the second division of the cap cell precedes the first division of the inner cell. Text-Figure 10, A, B, representing mitotic stages in the basal cells, shows that the order of division may

vary also in the basal and central cells. In subsequent divisions the basal cell gives rise to the four cells (Text-Fig. 10, D) which form part of the wall of the venter. The central cell divides to form the neck canal cell, and a cell which gives rise to the egg and the ventral canal cell (Text-Fig. 10, B). The neck canal cell nucleus divides, forming two nuclei without a corresponding division of the cell, as shown in Text-Fig. 10, C, D. The arrangement of archegonial contents usually observed is best shown in C of this Figure. The basal cells, which are left out in C because of oblique sectioning, stained darkly in the section represented in D, just as they did in sections of younger archegonia (Text-Fig. 10, A, B). The last named divisions, accompanied by the development of the archegonial neck from the four cells of the chimney foundation, complete the growth of the archegonium. When mature, the necks of the archegonia are bent backwards toward the rhizoids, as is usually the case in the Polypodiaceæ. The degeneration of the neck canal and ventral canal cells gives rise to a gelatinous substance, which swells on absorption of water, and bursts open the lid cells of the archegonium. The gelatinous substance emerges, and forms a frothy mass at the opening of the neck. In this condition fertilization takes place.

SEX IN ASPLENIUM ANGUSTIFOLIUM.

Antheridia occur on the prothallia when the latter are quite young, and sometimes persist until archegonia appear, although usually the plant seems to be dioecious. No attempt was made to develop purely monoecious or dioecious plants by controlling environmental factors, but in one culture grown on an unusually concentrated Knop's solution, it was noted that antheridia were developed much earlier than in cultures grown upon sterilized soil. Archegonia were not found in the solution cultures.

Experiments having to do with the control of sex in ferns have been carried out on *Onoclea Struthiopteris* by Miss Wuist (36), Mottier (20), and others. Mottier believes that it is highly probable that the sex tendency is predetermined in the spore, and that the male tendency is dominant under good cultural conditions, but that the development of sex organs may be influenced by varying environmental conditions. This seems to be the case in *A. angustifolium*. In thickly sown cultures, antheridia are borne abundantly on the irregularly shaped prothallia as well as on those possessing the more typical

heart shape. The crowded condition prevents the maturing of the prothallia and proves but little about sex, other than that the young prothallia are largely male.

DEVELOPMENT OF ANTHERIDIUM.

The development of the antheridium in *A. angustifolium* is entirely normal, beginning with the cutting off of a single hemispherical initial on a surface or marginal cell of the prothallium (Text-Fig. 11, A, G). The first division of the initial

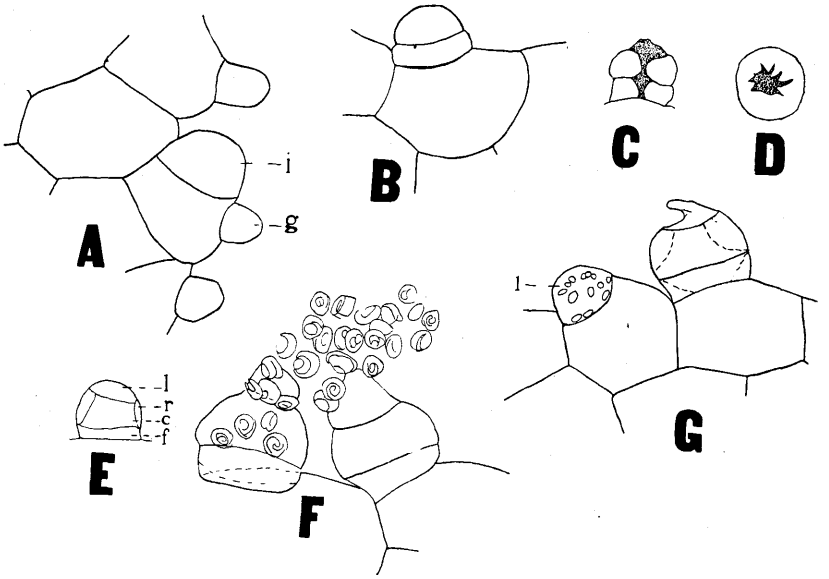

Fig. 11. Stages in development of antheridium. A. *i*, initial cell; *g*, young glandular hairs. B. Two-celled stage. C. Antheridium after escape of sperms, side view. D. Same, top view. E. Mature antheridium; *l*, lid cell; *r*, ring cell; *c*, central cell; *f*, funnel cell. F. Mature antheridia, sperms escaping from one at left. G. *i*, initial cell containing chloroplasts, with old antheridium. A, B, F, G, ×255. C, D, E, ×145.

results in the formation of a lower funnel cell and a dome-shaped upper cell (Text-Fig. 11, B). A periclinal division in the dome-shaped cell gives rise to the central cell and an arching cover cell (Text-Fig. 11, E). A final division of the cover cell results in the cap cell and the ring cell (Text-Fig. 11, G). The formation of the cap and ring cells completes the development of the antheridium. The central cell divides several times, giving rise to the sperm mother cells, from which the sperms or antherozoids are developed. Details of this development are beyond the scope of this paper.

Mature sperms are induced to escape from the antheridium by allowing the culture to become rather dry, and then transferring some of the antheridial prothallia to a drop of water on a slide. The rupture of the cap cell, swelling of funnel and ring cells, from water absorption, and consequent expulsion of the sperms, were observed under the microscope (Text-Fig. 11, F). After remaining tightly coiled at the opening of the antheridium for a few seconds, the sperms suddenly uncoil and immediately swim away by means of numerous cilia. Under laboratory

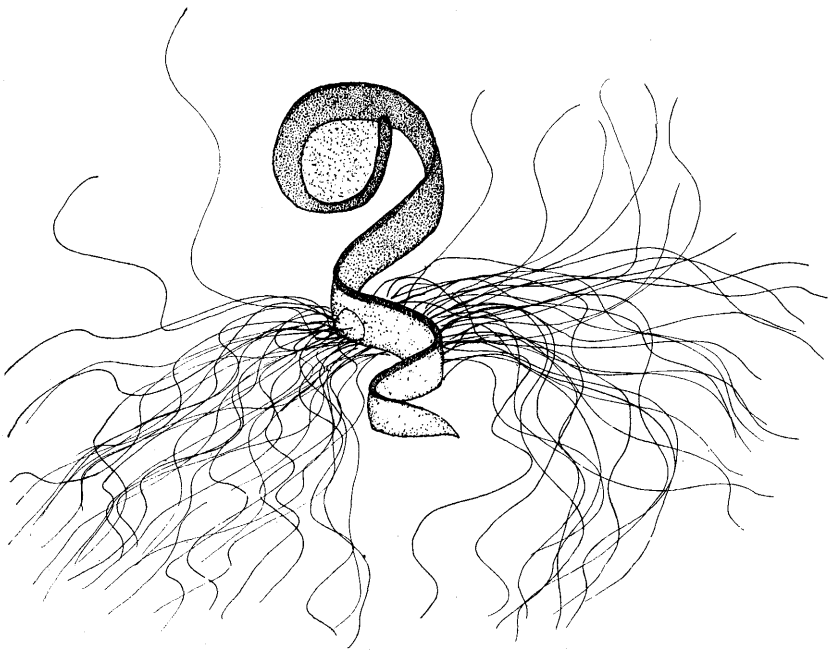

Fig. 12. A mature sperm. ×1465.

conditions locomotion may be kept up for about half an hour. Views of antheridia from which sperms have escaped are shown in Text-Figure 11, C, D, and G. A single mature sperm is represented in Text-Figure 12.

FERTILIZATION.

Several sections prepared for study of fertilization showed great numbers of sperms around the neck of the archegonium. Text-Figure 10, E, shows a sperm within the venter next to the egg, and fertilization is shown in F of the same figure. In this

case, the sperm was distinguishable in the egg nucleus, but no traces of cytoplasm were left on the outside of the egg. The most of the cells of the neck of the archegonium were missing in this section.

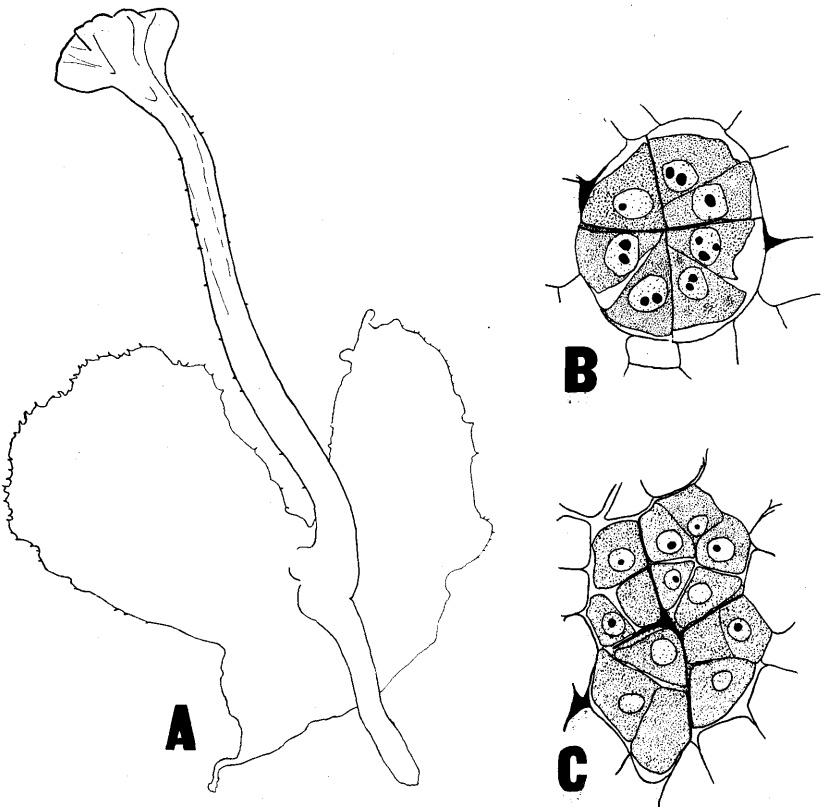

Fig. 13. A. Young sporophyte, ×9.5. B, C. Sections of young embryos, ×255.

In the material prepared for the study of the embryo, no sections contained the first division of the egg after fertilization, but several contained young embryos. Text-Figure 13, B, shows the youngest stage found, while C of the same figure represents one somewhat older. The quadrants, from which are developed the leaf, stem, foot and root of the young sporophyte, showed up very distinctly in the embryo sections. These four parts may be readily identified in Text-Figure 13, A.

SUMMARY.

1. The spores of *Asplenium angustifolium* germinate slowly. About a month is ordinarily required, although in one case small green prothallia were observed in thirteen days with the aid of a pocket lens. The fact of its slow germination, coupled with its scarcity and fragility, may be factors in a losing fight for survival of this species.

2. The steps leading to the formation of the apical cell differ from the method usually described for polypodiaceous ferns. The first division of the terminal cell is longitudinal instead of oblique, and both of the resulting cells divide transversely.

3. The protonemal filament is usually only three or four cells long when the terminal cell divides, but in some cases it is a great deal longer.

4. The perinium usually adheres throughout the life of the gametophyte.

5. The gametophyte is regularly heart-shaped. A few cases were observed where proliferations devoid of chlorophyll were developed around the edge of the prothallium. One proliferation took the form of a localized thickening of the wing.

6. The development of the archegonium conformed to the method usually described with the following exceptions: (a) The first division of the inner cell sometimes precedes and sometimes follows the formation of the chimney foundation from the cap cells. (b) The division of the central cell may precede or may follow the division of the basal cell.

7. In the development of the antheridium and the embryo, the stages usually described for ferns of this group are followed.

The writer wishes to express his great appreciation and gratitude to Professor C. E. O'Neal, of Ohio Wesleyan University, for his helpful suggestions and criticisms in the carrying on of this work.

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