On the Control of Antheridium Formation in the Fern Species *Lygodium japonicum.** (26016)

ULRICH NAF (Introduced by W. Trager)
The Rockefeller Institute, New York City

Döpp(1) demonstrated that an extract from mature prothalli of *Pteridium aquilinum* hastened the onset of antheridium formation in young prothalli of this fern species by a few days and in prothalli of *Athyrium filix-mas* by a few weeks. Döpp envisaged the possibility that the promotion of antheridium formation occurred by way of nonspecific growth inhibition. Subsequent investigations, though, led to the conclusion that the activity of the extract must be attributed to a specific factor which controls initiation of antheridia during the normal process of development(2,3).

An assay was devised which took advantage of the observation that prothalli of *Onoclea sensibilis* failed to form antheridia spontaneously under the prevailing conditions of culture but responded readily when grown on a medium containing extract of mature *Pteridium prothalli*(2). Conditions were further defined under which the extract from 7-week-old prothalli of *P. aquilinum* was usually active to a dilution of 1:30,000. This increased the activity obtained by Döpp about 300 times. Under these same conditions of culture the active substance accumulated to almost as high an activity in the medium(2). The active factor was recently obtained in a highly purified form by Pringle *et al.*(4). It was concluded from these studies that the inducing factor must be active at a concentration of less than 1 part in 10 billion (10^{-4} μg/ml).

Although prothalli of both *O. sensibilis* and *P. aquilinum* responded readily to the active factor at young stages of development, they lost their responsiveness soon after they attained heart shape and a few days prior to attaining the archegonial phase. Once insensitive, the prothalli failed to form antheridia even if supplied with the factor at a concentration 15,000 times higher than that sufficient to induce antheridia in prothalli just a few days younger(3).

Further investigations led to the conclusion that prothalli do not begin to produce the factor at effective concentrations until after they have become insensitive to it. Thus, the antheridia a prothallus forms arise in response not to antheridial factor produced by itself, but to antheridial factor elaborated and secreted into the medium by the more rapidly growing and developing individuals of the gametophyte population(3).

Studies on the activity spectrum of this substance (termed *Pteridium* factor) disclosed that *Pteridium* medium was active toward the tested representatives of 8 out of 9 subgroups of the family Polypodiaceae: Asplenioids, Pteroids(1); Onocleoids, Blechnoids, Dryopteroids(2); Gymnogrammoids (5), Woodsioids(6) and in *Nephrolepis hirsutula* (Davallioids). Among nonpolypodiaceous species only *Dennstaedtia punctilobula* (Dicksoniaceae) was responsive. The substance failed to promote antheridium formation even at the highest available concentration (½ full strength *Pteridium* medium that was active toward prothalli of *O. sensibilis* to a dilution of 1:30,000) in the following fern species: *Polypodium aureum* (Polypodiaceae), *Lygodium japonicum, Anemia phyllitidis, Mohria caffrorum* (Schizaeaceae), *Osmunda claytonia, O. cinnamomea* (Osmundaceae) and *Alsophila australis* (Cyatheaceae).

Subsequent investigations led to demonstration of a further substance (termed *Anemia* factor) which controls formation of antheridia in *A. phyllitidis*, one of the several species found to be unresponsive toward the *Pteridium* factor(6).

The present account is concerned with control of antheridium formation in *L. japonicum* which, like *A. phyllitidis*, belongs to the family Schizaeaceae.

Methods. The procedures used in sterilization and inoculation of the spores have been

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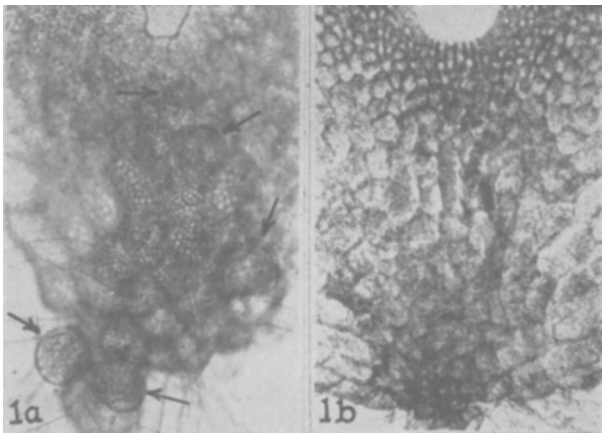

FIG. 1a. Magnification 76.8 ×. FIG. 1b. Magnification 33.6 ×.

described(3). This same report gives a detailed description of the conditions of culture and of the medium which contains the inorganic salts used by Moore(7). Hoagland's earlier-used A-Z solution of microelements was replaced with a Hoagland trace element solution of simpler composition (mg/l of medium: H_3BO_3 2.86, $MnCl_2 \cdot 4H_2O$ 1.81, $ZnSO_4 \cdot 7H_2O$ 0.22, MoO_3 0.07, $CuSO_4 \cdot 5H_2O$ 0.08). Iron was added as ferric tartrate. The prothalli were cultured in 125-ml flasks, each containing 33 ml of medium, or in 50-ml flasks with 12 ml of nutrient. Unless stated otherwise, the media were solidified with 1% agar.

Results. It was found that spores of *L. japonicum,* superinoculated on 25- or 40-day-old gametophyte cultures of this same species. gave rise to prothalli that attained the antheridial phase at a very early stage of development. Prothalli superinoculated on 15-day-old cultures attained the antheridial phase somewhat later although still several days prior to control prothalli.

This observation suggested that as the prothalli of *L. japonicum* reached a certain stage of development, they began to elaborate, and to secrete into the medium, a substance controlling induction of antheridia. This hypothesis received support when medium that had supported the growth of *Lygodium* prothalli was assayed for antheridium-inducing activity. The assays were conducted by inoculating spores of *L. japonicum* on fresh nutrient medium which contained medium of *Lygodium* cultures. The *Lygodium* medium was added at a dilution series with a dilution factor of approximately 3 (1/3, 1/10, 1/30, 1/100, . . .). Fifty-ml flasks which contained 12 ml of medium were used. Assays were read 15 days after inoculation, 6-7 days prior to onset of spontaneous antheridium formation. Medium from 15-day-old cultures or younger cultures was regularly inactive even at ⅓ full strength, the highest concentration of *Lygodium* medium tested. The medium of 25-day-old cultures, however, induced antheridium formation to a dilution of 1/10, the medium of 30-day-old cultures to a dilution of about 1/30, and media of 40- and 50-day-old cultures to a dilution of 1:100 (occasionally 1/300), and 1:300 (occasionally 1/1000), respectively. Fig. 1a shows a 14-day-old prothallus with 5 antheridia (see arrows) which were initiated in response to 1/30 full strength medium of a 40-day-old *Lygodium* culture, while control prothalli have not yet attained the antheridial phase even 19 days after inoculation (Fig. 1b). Whereas the lowest effective concentration of *Lygodium* medium brought about antheridium formation in only 1 out of 4 to 20 prothalli, the percentage of responding prothalli rose to 100 as concentration of the active factor was increased by a factor of from 3 to 10. Antheridial initials could be first discerned about 11 days after the spores were inoculated (the first spores germinated about 5 days following their inoculation), about 10 to 11 days prior to their appearance in control cultures.

Bauke(8) and Heim(9) reported that *L. japonicum,* unlike most other fern species, begins to form archegonia before it attains the antheridial phase. Archegonium-bearing prothalli first appeared in our cultures about 20 to 21 days following inoculation and 1 or 2 further days elapsed until gametophytes with antheridium initials could be seen. The prothalli which had just initiated the first antheridium all bore either one or, mostly, 2 archegonium initials. Thus, these prothalli actually attained the archegonial phase shortly before the first antheridium initial could be seen. Inspection of slightly older cultures, though, showed that the observations of Bauke and Heim were incomplete. All prothalli bore antheridia in about 25-day-old cultures but the smaller ones, *i.e.,* the most slowly growing individuals of the gametophyte population, lacked archegonia. Within a few further days, however, all prothalli bore sex organs of both types. It is inferred from this that the sequence of sex organ formation encountered in the prothalli that first attain the reproductive phase is reversed in more slowly growing individuals of the gametophyte population. This reversal may be understood if we consider that the most rapidly growing prothalli first attain the developmental stage at which elaboration of the antheridium-inducing factor, and its secretion into the medium, sets in. It may thus be postulated that the more slowly growing individuals begin to form antheridia at an earlier stage of development because they respond to this secreted factor before they themselves begin to produce it. This hypothesis was considered established with the demonstration, firstly, that onset of the antheridial phase preceded attainment of the archegonial phase in all prothalli if the medium was supplemented with the antheridium-inducing factor and, secondly, that the opposite sequence prevailed in all prothalli if they were picked randomly from young (13-day-old) cultures and isolated, one per flask, on new medium.

The zone of spontaneous antheridium formation in *L. japonicum* is, according to Bauke and Heim(8,9), restricted to the anterior part of the gametophyte. While this could be confirmed for the majority of gametophytes, a few of the very smallest individuals occasionally bore antheridia in the posterior region.

Moreover, if spores of *L. japonicum* were inoculated on 30-day-old cultures of this same fern species or on medium supplemented with antheridium-inducing factor, antheridia were observed to occur in the basal region, even among the very basal cells, of all gametophytes. As these prothalli continued to grow, the zone of newly formed antheridia became gradually restricted to the anterior portion of the gametophyte, indicating that the basal cells were sensitive to the antheridium-inducing factor during an initial period but lost their responsiveness as they matured. This hypothesis was considered established with the demonstration that antheridia arose on the basal parts of all gametophytes if antheridial factor (0.5 ml of ½ full strength medium from 40-day-old *Lygodium* cultures that was active to a dilution of 1:300) was added to 7-day-old cultures in 50-ml flasks, while occurrence of antheridia was restricted to the anterior region if the same amount of antheridium-inducing factor was added to 14-day-old cultures (again with the exception of the very smallest, *i.e.,* developmentally least advanced, individuals).

The *Lygodium* factor failed to promote antheridium formation in prothalli of *O. claytonia* (as checked 15 days after inoculation, 2 days prior to onset of spontaneous antheridium formation) which were also unresponsive to the *Pteridium* factor and the *Anemia* factor(6).

The antheridium-inducing activity of the *Lygodium* medium was adsorbed on charcoal and destroyed by ashing, as was the case also for *Pteridium* and *Anemia* factors(2,6). Biological activity of the *Lygodium* medium was further found to be stable to autoclaving for 15 min at a pressure of 15 lb at pH 5 and to boiling for 10 min at pH 2 and 12 (it is possible that some inactivation, just at the verge of detectability, takes place upon boiling at both pH 2 and 12). The *Anemia* factor was stable under these conditions(6), while the *Pteridium* factor differed only by its lability at pH 12(2).

As mentioned earlier, *Pteridium* medium

failed to promote antheridium formation in *L. japonicum* throughout the wide range of applied concentrations (⅓ to 1/30,000 full strength *Pteridium* medium that was active toward prothalli of *O. sensibilis, i.e.,* the prothalli used to assay for the *Pteridium* factor, to a dilution of 1:30,000). In the reciprocal experiment 13 out of 16 samples of *Lygodium* medium (active toward prothalli of *L. japonicum* mostly to a dilution of 1:300) were without effect in *O. sensibilis*. Two of 16 samples brought about an antheridial response in *O. sensibilis* if only at the highest concentration (⅓ full strength). These 2 samples were active toward *L. japonicum* to a dilution of 1:300. The remaining sample brought about an antheridial response in *O. sensibilis* to a dilution of 1:10 even though it was active toward *L. japonicum* only to a dilution of 1:100. The last-mentioned sample of *Lygodium* medium was boiled for 10 min at pH 12, under which conditions the *Pteridium* factor was shown to be labile(2). The batch of *Lygodium* medium thus treated failed to bring about an antheridial response in *O. sensibilis* even at the highest concentration. In contrast, its activity toward *L. japonicum,* as tested again by a dilution series, remained unimpaired. This experiment could not be repeated because all subsequently harvested batches of *Lygodium* medium lacked detectable activity toward *Onoclea* prothalli.

Even though a few samples of *Lygodium* medium showed very slight activity toward *O. sensibilis,* the factor controlling antheridium formation in *L. japonicum* would appear to be chemically distinct from the *Pteridium* factor. This is indicated by the unresponsiveness of *Lygodium* prothalli toward *Pteridium* medium even at a concentration 10,000 times higher than that necessary to bring about a minimal response in *Onoclea* prothalli. The selective destruction of the activity of *Lygodium* medium toward *O. sensibilis* upon boiling for 10 min at pH 12 and the indicated variable proportion between the antheridium-inducing activities of *Lygodium* medium toward *O. sensibilis* and *L. japonicum* point in the same direction. The *Lygodium* plants from which spores were obtained grew together with many other fern species, hence the possibility cannot be discounted that the antheridium-inducing activity toward *O. sensibilis* was elaborated by the prothalli of a species other than *L. japonicum* (amount of antheridium-inducing factor produced by a single prothallus of *P. aquilinum* is ample to account for the observed effect(3)).

It was earlier concluded that the *Pteridium* factor is chemically distinct from the substance controlling antheridium formation in *A. phyllitidis*(6). It was of further interest to assay for possible similarity between this *Anemia* factor and the substance that controls antheridium formation in *L. japonicum*.

Lygodium medium failed to bring about an antheridial response in *A. phyllitidis* throughout the wide range of applied concentrations (⅓ to 1/30,000 full strength *Lygodium* medium). The same result was obtained in a dozen repetitions with further batches of *Lygodium* medium which were active toward *L. japonicum* to a dilution of 1:300, occasionally to 1:1,000. In the reciprocal experiment, *Anemia* medium regularly showed activity toward *Lygodium* prothalli. To obtain a minimal antheridial response, however, the *Anemia* medium had to be supplied to the *Lygodium* prothalli at a concentration 10 to 30 times higher than to the *Anemia* prothalli.

Discussion. The results are considered to show that the factor controlling antheridium formation in *L. japonicum* differs from the *Pteridium* factor, *i.e.,* the substance which controls this same process in many species of the fern family Polypodiaceae.

The further question arises whether this *Lygodium* factor is identical with the antheridial factor of *A. phyllitidis*. The need for a higher concentration of *Anemia* medium to bring about a minimal response in *Lygodium* is consistent with this hypothesis if we postulate that this species possesses a greater capacity than *Anemia* to inactivate, or otherwise neutralize, the active substance. In the reciprocal experiment, though, this hypothesis calls for greater activity of *Lygodium* medium toward *Anemia* than toward *Lygodium* itself. Instead, the *Anemia* prothalli were found to be unresponsive to *Lygodium* medium even at the highest applied concentration. The hypothesis that the same substance controls an-

theridium formation in the two species may be retained by invoking complex subsidiary assumptions. It seems more likely, however, that control of this developmental event in *L. japonicum* will be traced to a different factor than in *A. phyllitidis*. If we assume that the 2 substances are similar, then the factor controlling antheridium formation in *Anemia* might itself be effective also in *Lygodium*, provided it is supplied to the latter species at a high enough concentration. This postulate is compatible with the experimental result. In the reciprocal experiment, failure of *Anemia* prothalli to respond to even the highest applied concentration of *Lygodium* medium is also consistent with this hypothesis if we consider that the *Anemia* factor might more easily "fit" the *Lygodium* acceptor molecule than the similar *Lygodium* factor "fits" the corresponding molecule in *Anemia*. It seems quite as likely, however, that the antheridium-inducing activity of *Anemia* medium toward *Lygodium* is referable to a different substance than its activity toward *Anemia* itself. Work now in progress should clarify some of these questions.

It is hoped that these investigations will contribute to the classification of Pteridophytes which remains in a state of considerable fluidity. More important, they should provide information on the mode of evolution within a closely delimited segment of metabolism. Kluyver and Van Niel have directed attention to the similarity, even identity, of many basic biochemical patterns in taxonomically widely separated organisms. The proposed investigation should be of special interest because of its concern with the less frequently considered question: How similar or dissimilar among organisms are the biochemical processes associated with a given developmental event (*viz.* antheridium formation) that we conceive of primarily in morphological terms and which in different groups of ferns proceeds similarly in terms of morphology and is identical in terms of function?

Summary. It is demonstrated that prothalli of *L. japonicum* elaborate, and secrete into the medium, a substance which controls formation of antheridia in this fern species. The antheridium-inducing activity of the medium is stable to boiling for 10 min at both pH 2 and 12. It is adsorbed on charcoal and destroyed by ashing. The results are considered to show that this antheridium-inducing substance differs from the substance which controls the same developmental event in many species of the fern family Polypodiaceae. The results further indicate that the *Lygodium* factor is also chemically distinct from the substance that controls antheridium formation in *A. phyllitidis* which, like *L. japonicum*, belongs to the fern family *Schizaeaceae*. The individuals that first attain the reproductive phase in a gametophyte population of *L. japonicum* give rise to one or 2 archegonia before the first antheridium initial appears. This sequence of sex-organ formation is reversed in the prothalli which subsequently attain the reproductive phase. All prothalli attain the archegonial phase first if they are isolated, one per flask, at an early stage of development. In contrast, all prothalli attain the antheridial phase first if they are all provided with the antheridium-inducing substance at an early stage of development. It is concluded that the more slowly growing individuals in a gametophyte population of *L. japonicum* attain the antheridial phase first because they respond to the antheridium-inducing substance that is elaborated, and secreted into the medium, by the more rapidly growing gametophytes.

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