On Antheridal Metabolism in the Fern Species Onoclea sensibilis L.,

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Döpp (1) demonstrated that the extract from mature prothalli of the fern species Pteridium aquilinum (L.) Kuhn hastened the onset of antheridium formation in young prothalli of this species by several days and in the prothalli of Dryopteris filix-mas (L.) Schott by several weeks. Döpp's conclusion that the prothalli of P. aquilinum elaborate a specific antheridium-inducing factor was confirmed. Conditions were defined under which the prothallial extract and the medium of 4 to 6-week-old cultures of P. aquilinum were mostly active to a dilution of 1: 30,000 if assayed against the prothalli of the fern species Onoclea sensibilis L. (4, 8, 10). The prothalli of this species proved ideal for assaying antheridium-inducing activity for various reasons (3, 4, 8, 10). Using this assay, the active substance was isolated and partially characterized (11, 12).

The prothalli of O. sensibilis (5) and of P. aquilinum (3, 5) lose their competence to form antheridia in the presence of the antheridial factor soon after they attain heart-shape. In O. sensibilis the loss of sensitivity to the antheridial factor is completed within a period of less than 2 days (5). Once the prothalli are insensitive they fail to form antheridia even if the antheridial factor is supplied at a concentration 15,000 times higher than that sufficient to induce antheridia in younger prothalli. Surgical experiments led Döpp (2) to conclude that this loss of sensitivity must be ascribed to a substance, termed by him the H-Substanz, which is elaborated by the meristem of the maturing prothallus.

The present report is concerned with the question why Onoclea prothalli fail to form antheridia spontaneously under the prevailing conditions of culture. Earlier studies indicated that the maturing Onoclea prothalli did elaborate some antheridial factor, even though they failed to form antheridia at all stages of development. It must be pointed out that the last individuals of the gametophyte population became insensitive to the antheridial factor 2 to 3 days before the first trace of antheridium-inducing activity could be detected in the medium (7). The results thus indicated that the failure of Onoclea prothalli to form antheridia could be attributed to their failure to elaborate the antheridial factor while they were still at the sensitive phase. In the context of this hypothesis only prothalli developed from spores shed late would form antheridia in nature.

Materials and Methods

The prothalli were grown from steriley inoculated spores on a mineral, agar-solidified medium. Erlenmeyer flasks containing 30 ml of medium or petri dishes containing 60 ml of medium were used. Antheridium-inducing activity was assayed against young prothalli of O. sensibilis L. which were cultured in 50 ml-Erlenmeyer flasks containing 10 ml of medium. The procedure used in sterilizing and inoculating the spores as well as the composition of the medium and other conditions of culture and assay have been described (5, 6). The sporophylls of O. sensibilis were collected in December 1963 in Grassy Sprain, Westchester County, New York.

When the medium of Onoclea cultures was harvested for assay, the prothalli were carefully scraped off the agar surface by means of a glass spatula. The medium was then frozen in a freezer cabinet and, following thawing, the liquid filtered off. It was stored in the frozen state and is termed full strength Onoclea medium below.

Results

The hypothesis formulated above was tested by inoculating spores of O. sensibilis among 10-day-old and in other experiments among 15-day-old prothalli of this same species. This was done in the expectation that the prothalli developing from the second batch of spores would form antheridia in response to antheridial factor elaborated, and secreted into the medium, by the older prothalli. Not a single one of the younger prothalli formed antheridia even though antheridium-inducing activity could be detected in the harvested medium while these younger prothalli passed through their sensitive phase.

This finding led to the hypothesis that the antheridium-inducing activity demonstrated in the medium of Onoclea cultures was not present in the undisturbed medium but arose as a result of the procedure used in harvesting and preparing the Onoclea medium for assay. In the preparation of the assay the harvested Onoclea medium was autoclaved because earlier studies had shown the factor elaborated by P. aquilinum to be stable to such treatment (4).

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The possibility that the antheridium-inducing activity in the harvested *Onoclea* medium arose as a result of autoclaving was, therefore, subjected to an experimental test.

*Onoclea* prothalli were grown in 125 ml-Erlenmeyer flasks containing 30 ml of the agar-solidified medium. A dilution factor of approximately 3 was used. If autoclaved for a period of 10 minutes, the medium from 16-day-old cultures was active to a dilution of one-tenth, i.e., to a dilution 3 times higher than with the earlier used spore sample. Following filter sterilization no activity was detected even at one-third full strength *Onoclea* medium.

With *Onoclea* medium harvested 20 days after inoculation, antheridia were induced in the assay prothalli to a dilution of one-thirtieth upon autoclaving. Upon filter sterilization, no activity was detected in some experiments even at one-third full strength, but in most experiments the filter-sterilized *Onoclea* medium induced antheridia to a dilution of one-third full strength. Thus autoclaving leads to an approximately 10-fold increase in the antheridium inducing activity of medium from 20-day-old *Onoclea* cultures. The aqueous extract from 20-day-old prothalli was active to one-thirtieth full strength if autoclaved and mostly to one-third if filter-sterilized.

Attempts were then made to increase the titer of the activity. The medium of 16-day-old *Onoclea* cultures was active to a dilution of one-thirtieth rather than one-tenth upon autoclaving if the spores were inoculated more densely. Petri dishes containing 60 ml of medium were used as culture vessels in these experiments. Autoclaving for 20 minutes instead of 10 minutes increased the activity by less than a factor of 3. More success was achieved when the effects of autoclaving and boiling at pH 2, 4 and 6 were compared. Both autoclaving and boiling occurred for a period of 10 minutes. The acidity was adjusted by means of NaOH and HCl. Following return to normal temperature, the pH of the media was adjusted to 5.1, the pH of freshly made up medium. All batches of *Onoclea* medium were then added to the assay flasks by filter-sterilization. The results are recorded in Table I.

Maximal activity was obtained upon boiling or autoclaving at pH 2. Exposure to pH 2 for a period of 25 minutes at room temperature did not detectably increase the activity of the *Onoclea* medium.

The possibility may be considered that the nonactivated medium contains a heat-labile inhibitor of antheridium formation. Medium from 14-day-old *Onoclea* cultures was used to obtain pertinent information. (In the nonactivated state, this batch of *Onoclea* medium was inactive even at one-third full strength but following activation it was active to a dilution of one-thirtieth full strength.) No inhibition of antheridium-formation could be detected even if the nonactivated *Onoclea* medium was added (by filter-sterilization) at one-third full strength, i.e., at a high concentration and the activated medium at one-thirtieth full strength, i.e., the lowest effective concentration.

The active factor is destroyed if boiled for 10 minutes at pH 12 but is stable to boiling for the same length of time at pH 2. Both the active factor and the unknown one (the heat-labile inhibitor or the substance which upon heating is converted into an active one) can be extracted from the medium by ethylacetate and are adsorbed on charcoal but are only partially eluted from it by 2 washings with methanol. The properties of the antheridium-inducing activity in *Onoclea* medium are similar to those described for the antheridium-inducing factor elaborated by *Pteridium aquilinum* (4, 11, 12).

**Discussion**

The results show that the bulk of the antheridium-inducing activity previously demonstrated in medium and prothallial extracts from maturing cultures of *O. sensibilis* does not exist in the undisturbed culture but arises as a result of the autoclaving used in preparing the medium for assay.

Filter-sterilized *Onoclea* medium either failed to induce antheridia or was much less active than autoclaved medium. It was of interest that young prothalli which grew intermingled with older ones failed

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**Table 1.** The Yield of Antheridium-Inducing Activity was Determined in Medium Harvested from 16-Day-Old *Onoclea* Cultures Following Boiling or Autoclaving for 10 minutes at pH 2, 4 or 6

<table>
<thead>
<tr>
<th>Treatments of harvested</th>
<th>Dilutions of assayed <em>Onoclea</em> medium</th>
<th>1/3</th>
<th>1/10</th>
<th>1/30</th>
<th>1/100</th>
<th>1/300</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boiled</td>
<td>pH 2</td>
<td>49</td>
<td>49</td>
<td>50</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>pH 4</td>
<td>50</td>
<td>28</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>pH 6</td>
<td>48</td>
<td>25</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Autoclaved</td>
<td>pH 2</td>
<td>49</td>
<td>48</td>
<td>48</td>
<td>19</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>pH 4</td>
<td>48</td>
<td>50</td>
<td>48</td>
<td>19</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>pH 6</td>
<td>50</td>
<td>46</td>
<td>13</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Filter-sterilized</td>
<td>pH 4</td>
<td>39</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

 Recorded are numbers of assay prothalli (out of 50 counted) which responded with antheridium formation.
to form antheridia even when activity was demonstrated in filter-sterilized medium that was harvested while these young prothalli were competent to form antheridia. It seems likely, therefore, that even the activity demonstrated in filter-sterilized medium arises during the preparation of the medium for assay.

It should be pointed out that the medium obtained from cultures of another fern species, *P. aquilinum*, was active to the same concentration upon filter-sterilization and upon autoclaving (5). It seems quite certain, therefore, that the activities reported for *Pteridium* medium correspond more closely to those found in the undisturbed culture medium.

The finding that most of the activity previously demonstrated in *Onoclea* medium arises upon heating allows for 2 alternative interpretations: Either heat activation leads to the destruction of an inhibitor of antheridium formation or it results in the formation of an active substance from an inactive one. The second interpretation is favored by the finding that nonactivated *Onoclea* medium failed to counteract antheridium formation in response to activated medium. However, an ultimate decision between the 2 interpretations must be based on the physical separation of the 2 factors.

**Summary**

It was earlier reported that medium harvested from prothallial cultures of the fern species *Onoclea sensibilis* contains antheridium-inducing activity. Recent results show that the bulk of the activity does not exist in the undisturbed culture medium but arises instead as a result of the autoclaving during the preparation of the harvested medium for assay. The increase in activity upon autoclaving allows for 2 alternative interpretations: Either it leads to the activation of an inactive compound or to the destruction of a heat-labile inhibitor. The results favor the first interpretation but do not rigorously exclude the second.

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**Literature Cited**