Is Riboflavin the Photoreceptor in the Induction of Two-dimensional Growth in Fern Gametophytes?  

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Blue light controls the development of 2-dimensional form in the gametophyte phase of numerous ferns (2 and literature therein). Although the photoreceptor for this system is still unknown, it has been hypothesized to be a flavoprotein (7).

Yeoh and Raghavan (11) have presented data which they interpreted as evidence for the existence of riboflavin (or lumichrome) as the photoreceptor. This conclusion was based upon the observation that these compounds reverse the inhibitory effects of 2-thiouracil or 2-thioglycoside. Several problems are encountered with this interpretation: (1) riboflavin is very unstable in light (5, 10), and (2) riboflavin is a well known sensitizer for the photodestruction of various compounds (4, 5). Because of the importance placed upon the knowledge of the receptor in any physiophysiological reaction, the above experimental design was reinvestigated.

Spores of Pteridium aquilinum (L.) Kuhn were collected in September, 1963, in Pulaski County, Indiana, and stored under refrigeration. The spores were filtered through 4 to 6 layers of lens paper to remove sporangia and leaf fragments before inoculation onto the surface of a modified Bold's algal medium (3), 25 ml per 10 cm petri dish. After 65 to 70 hours (approx 60% germination, cell number of 1.1, 0.1% of the plants 2-dimensional), samples were transferred with a sterile bacteriological loop to 7.5 cm × 1.0 cm test tubes containing 0.5 ml of the basal medium plus supplements. Cultures were assayed 4 days after treatment, as compared to 28 days required for the gametophytes of Phymatodes nigrescens used by Yeoh and Raghavan (11). All materials were incubated at 25 ± 1° over 2 G. E. “Cool White” fluorescent tubes, approximately 200 ft-c. Ten cultures were assayed for each treatment. At least 200 gametophytes from each culture were examined microscopically for the percentage of 2-dimensional plants (plants with at least 1 oblique cell wall, relative to the long axis of the organism); at least 50 gametophytes per culture were assayed for the cell number per filamentous plant. Statistical differences were determined by the F test, using the complete random design; individual means were compared by the use of the “least significant difference” (9).

2-Thiouracil and uracil were purchased from Sigma Chemical Company, St. Louis; riboflavin from Matheson Coleman and Bell, East Rutherford, New Jersey. All salts were reagent grade.

The percentage of 2-dimensional gametophytes was reduced by 2-thiouracil at both 2.0 and 4.0 μg/ml; both treatments differed from the control at the 0.01 level (fig 1). When uracil (30 μg/ml) was coincubated with 2-thiouracil (4.0 μg/ml), the in-

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**Fig. 1.** Effects of 2-thiouracil, uracil, and riboflavin upon the percentage of 2-dimensional (2-D) plants per culture and the cell number per filamentous (1-D) plant. C, controls on basal medium; T₂, 2-thiouracil, 20 μg/ml; T₄, 2-thiouracil, 4.0 μg/ml; U, uracil, 30 μg/ml; R, riboflavin, 30 μg/ml.
Inhibitor action was nullified in that the plants were comparable to those grown on uracil alone. The observation that this level of uracil was slightly toxic (differed from the controls at the 0.01 level) may account for the statement by Yeoh and Raghavan (11) that uracil did not completely nullify the inhibitor action. In their paper no data were presented for a control containing uracil alone. Comparable to the findings of Yeoh and Raghavan, riboflavin (30 ngl/ml) reversed the inhibitory effects of 2-thiouracil (40 ngl/ml) in the Pteridium system, even though the incubation time was only 4 days. These results are comparable to those obtained by measuring the number of cells per filamentous plant. In this case the only mean that differed statistically from the control was that obtained by 4.0 ngl/ml 2-thiouracil.

The photolabile nature of riboflavin was examined by following the changes in absorption spectra during the 4 day incubation period. Riboflavin (30 ngl/ml) was dissolved in the culture medium, pH 6.5, or in Fisher certified buffer solutions of pH 4.0, 6.0, 7.0, or 10.0. In each case, 5 screwtop vials (6 cm × 1.5 cm) were illuminated as above and 5 were maintained in darkness. The absorption between 320 and 500 nm was determined by the use of a Beckman DB spectrophotometer equipped with a Vericord model 43 recorder.

The changes in absorption of riboflavin illuminated in the culture medium are given in figure 2. The initial absorption characteristics were typical for riboflavin (1). After 20 hour illumination, a pronounced shift in absorption maximum was observed, although a minor absorption at around 450 nm indicated that a portion of the riboflavin was still present. This absorption at 450 nm was absent after 2 days in white light. The absorption was constant from the second through the fourth day of illumination. This new absorption spectrum was comparable to that obtained when riboflavin was illuminated at pH 4.0 but not to that at pH 10.0. It can therefore be concluded that the breakdown in the modified Bold's medium was consistent with the known photodestruction to lumichrome in acid or neutral solutions (10). These changes were not influenced by the presence of 2-thiouracil (40 ngl/ml).

The breakdown of riboflavin was confirmed by paper electrophoresis. Twelve ml samples were spotted on Whatman No. 1 filter paper (7.5 cm × 30 cm), 6 to 7 cm from the cathode end. The paper was wetted with the borate buffer, 0.05 M pH 9.2 (8) and subjected in the dark to 250 volts (12 mA) for 2.5 hours in a Thomas model 21 electrophoresis assembly. The strips were then dried at 60°C in the dark and examined under ultraviolet light (“Black Raymaster” tube from Geo. W. Gates and Company, Franklin Square, New York). Under these conditions, riboflavin migrated 3 to 4 cm and could be identified by a bright yellow fluorescence. After 20 hours in white light, only a small riboflavin area could be observed. A second spot was now present. 1 to 2 cm from the origin, exhibiting the typical blue fluorescence of lumichrome (10). By the second day of illumination, only the blue spot was present. The breakdown was not due to microbial action since it occurred even when 1 drop of chloroform was added to each vial at the start of the experiment.

The next question analyzed was whether the lack of inhibition of 2-thiouracil in the presence of riboflavin would be due to the photodestruction of the analog with the riboflavin as the sensitizer. Screw top vials, each containing 5 ml of 2-thiouracil (4.0 ngl/ml), riboflavin (30 ngl/ml), or both were illuminated as above for 4 days. The absorption between 280 and 320 nm was determined daily with a Beckman DB spectrophotometer. A slow photodestruction was observed for 2-thiouracil alone (fig 3A), possibly due to the emission of small quantities of ultraviolet light (5). The photodestruction in the presence of riboflavin was even more rapid, as indicated when the absorption of riboflavin alone was subtracted from that of riboflavin plus 2-thiouracil (fig 3B). Little if any 2-thiouracil was present after 1 day of illumination; none was present after 2 days. The changes occurred in the presence of chloroform (1 drop per vial) and therefore did not represent microbial degredation. When fresh 2-thiouracil (final conc of 4.0 ngl/ml) was added to riboflavin samples that had been illuminated for 4 days, and the absorption compared to that of the illuminated riboflavin alone, the difference spectrum was equivalent to that found at the beginning of the experiment. The loss of absorption by 2-thiouracil was therefore not the result of changes induced by the concurrent change in absorption of riboflavin during its photodestruction.

One must now return to the original hypothesis stated by Yeoh and Raghavan (11). First, one cannot conclude that riboflavin is the photoadapter as it is only present in the illuminated solution for
approximately 1 day. The breakdown product, probably lumichrome, is more stable. It is of interest that when Yeoh and Raghavan used lumichrome they found the reversal of inhibition was even more pronounced than when they used riboflavin. The meaning of this difference cannot be resolved here.

Second, Yeoh and Raghavan described that riboflavin nullified the inhibitory effects of 2-thiouracil and 2-thiouracil on the gametophytes of Pteridium aquilinum. A comparable nullification was described here for gametophytes of Pteridium aquilinum, a system chosen because the incubation time was only 4 days rather than 28 days. However, when the absorption of 2-thiouracil plus riboflavin was compared to that of riboflavin alone, it was observed that the 2-thiouracil was completely destroyed after 1 to 2 days of illumination in the presence of riboflavin. It may therefore be concluded that the nullification was due to the in vitro photodestruction of the inhibitor with riboflavin as the sensitizer. Whether any of the 2-thiouracil also altered the incorporation of pyrimidines into the riboflavin structure in vivo (6) was not determined.

Yeoh and Raghavan (11) also described experiments in which an analog of riboflavin, phenazine, inhibited gametophyte development. This is not surprising because of the importance of the flavin nucleotides in biological oxidation reactions.

It is still probable that a flavoprotein is the photoreceptor for the development of the 2-dimensional form in fern gametophytes, and riboflavin might even be involved as part of the flavoprotein. However, this hypothesis cannot be verified by the above experimental design.

**Literature Cited**


