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# STRUCTURAL, BIOCHEMICAL AND PHYSIOLOGICAL ASPECTS OF PHOTOMORPHOGENESIS IN FERN GAMETOPHYTES

By

Gary P. Howland

June, 1971

A Dissertation Presented to the Faculty of the Graduate School of Yale University in Candidacy for the Degree of Doctor of Philosophy

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# A B S T R A C T

The transition from filamentous to prothallial growth in gametophytes of the fern Dryopteris filix-mas can be controlled by the quality of incident light. Data from time-lapse photography indicate that the primary morphological response, tip-swelling, begins 2-3 hours after transfer from red to blue light. A difference in total-RNA base composition between filamentous and prothallial gametophytes appears to reside in a difference in the relative amounts of chloroplast ribosomal-RNA (rRNA) between these two growth types. Phosphorus-32 labelling shows that an increased rate of chloroplast rRNA is not detected until about 1 day after transfer from red to blue light; although cytoplasmic rRNA synthesis is stimulated much sooner. In addition, other experiments indicate that RNA of these gametophytes cannot readily be labelled with radioactive uridine. These data suggest that uridine incorporation is not a satisfactory assay for RNA synthesis in fern gametophytes. Exploratory experiments have revealed an apparently specific effect of several reagents known to interfere with the metabolism of microtubules, while no specific effect of U.V. irradiation or ethylene could be shown.


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# I. GENERAL INTRODUCTION

Ontogeny of multicellular organisms is characterized by changes in shape. These changes can be the result of cell divisions, cell expansion, cell movements and/or cell death. With rare exception, only cell divisions and cell expansion contribute to morphogenesis in vascular plants, where each cell is delimited by a more or less rigid cell wall.

Most earlier studies of plant morphogenesis have concentrated on multicellular organs. Ashby (1948) has examined the interactions of cell division and cell expansion in the two-dimensional development of leaf shape. He points out that these two general factors can each vary with regard to distribution, orientation and duration (cf. also Esau, 1965, pp. 467-470). Taking advantage of the symmetrical growth of some gourd fruit, Sinnott (1960) has assayed changes in volume, length, and girth. Measuring the orientation of cell divisions, he found that a very high proportion of the mitotic spindles were parallel to the longitudinal axis of an elongate type of fruit, while in an isodiametrically developing gourd mitotic spindle angles were randomly distributed. The correlation between orientation of cell division and morphogenesis is clear in this case, suggesting that the former is causally related to the latter. Studies on morphogenesis in the shoot apex have generally placed great emphasis on patterns formed by the planes of cell divisions (cf. Sinnott, 1960, pp. 59-79). Esau (1965, pp. 104-105) states that leaf primordia are initiated by cell divisions. However, morphogenesis can occur in the absence of cell division in wheat plants which have received doses of gamma-irradiation. Haber (1962) has demonstrated that concurrent oriented cell divisions are

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not required for morphogenesis of an existing wheat leaf primordium; and Foard (1971) found that initiation of the fourth leaf primordium (discernible as a bump on the meristem) could occur without the normal periclinal cell divisions in the epidermal cell layer.

Owing to the extreme complexity of morphogenetic processes in multicellular organs, many workers have turned their attention to experimental systems which can be manipulated more precisely. The experimental advantages offered by the fern gametophyte have been enumerated by Miller (1968a) and by Voeller (1971). The fern gametophyte is a multicellular eukaryote with morphologically well-defined differentiative stages. It is now well established that many of these differentiative steps can be experimentally controlled. The fern gametophyte's simple morphology permits direct measurement of cell division and expansion, in living material if desired. These organisms can be grown in axenic culture on a scale approximating microbial systems, allowing the application of many useful biochemical procedures. Finally, techniques which permit a rapid completion of the life cycle in culture (D. Stein and E. Klekowski, personal communication) and the selection of genetic mutants (Carlson, 1970) have opened an area which undoubtedly will prove fruitful in the near future.

Over the past 70 years the differentiation of the Polypodiaceous fern gametophyte from an initial filamentous growth phase to a planar phase (prothallus) has attracted the interest of many researchers (cf. Miller, 1968a). Even though it was soon discovered (Twiss, 1910; Klebs, 1916/17, cited in Miller, 1968a) that light quality could control this morphogenetic transition (red light permitting only filamentous growth; blue, allowing the differentiation into prothalli), little more is known

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about the mechanism controlling this morphogenetic transition.

Investigating the basis of this differentiation Hotta (1960) reported that changes in protein content and total-RNA base composition of Dryopteris erythrosora gametophytes were correlated with the transition from filamentous to prothallial growth, and that inhibition of protein or nucleic acid synthesis caused a reversion to the filamentous type of growth. Pursuing the suggestion of a control mechanism involving RNA and protein synthesis a number of other workers have used metabolic inhibitors in an attempt to specifically block the transition to prothallial growth, but interpretations of these results are unclear since those workers generally did not take into account the general inhibition of growth (cf. Burns and Ingle, 1968; Miller, 1968b).

Based primarily on direct measurements of protein and RNA content and synthesis in Dryopteris filix-mas gametophytes, Mohr and his associates have postulated that the transition from filamentous to prothallial growth involves "differential gene activation" leading to the synthesis of a "morphogenetic enzyme" (cf. Mohr, 1965). As a reflection of this "differential gene activation" they report that in gametophytes of Dryopteris filix-mas an increased rate of RNA synthesis is associated with the morphogenetic transition (Drumm and Mohr, 1967a).

The present study was undertaken 1) to define the kinetics of the transition from filamentous to prothallial growth in Dryopteris filix-mas gametophytes in terms of cell division and cell expansion, 2) to evaluate the report of a change in total-RNA base composition, and 3) to relate the kinetics of any changes in RNA metabolism to the kinetics of the morphogenetic transition. In addition, several types of exploratory experiments were carried out to evaluate the involvement of

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cytoplasmic microtubules and the plant hormone ethylene in the morphogenetic reaction.

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## II. GROWTH OF FERN GAMETOPHYTES IN CULTURE

### Introduction

Mohr and his associates have reported on the germination (Mohr, 1956a), growth and photomorphogenesis (Mohr, 1956b; Mohr and Barth, 1962; Mohr and Ohlenroth, 1962; Mohr and Holl, 1964), and the relationship of photomorphogenesis to protein (Ohlenroth and Mohr, 1963, 1964), amino acids (v. Deimling and Mohr, 1967), RNA (Drumm and Mohr, 1967a), and DNA (Drumm and Mohr, 1967b) levels in Dryopteris filix-mas gametophytes. The present study utilized this previous work as a basis. The plant material and culture conditions were as nearly identical to those employed by Mohr's group as possible.

### Materials and Methods

Dryopteris filix-mas spores were provided by Professor Hans Mohr from a supply collected at the Tübingen Botanical Garden in 1964. Spores of Dryopteris erythrosora and Osmunda cinnamomea were collected from plants in Marsh Botanical Gardens, Yale University and from plants in the Yale Bog, Bethany, Connecticut, respectively. The spores were kept at ca. 4°C until used.

The culture medium used was that of Mohr and Ohlenroth (1962):

| | |
|---|---|
| $(NH_4)_2HPO_4$ | 0.5g |
| $KH_2PO_4$ | 0.5g |
| $MgSO_4$ | 0.3g |
| $CaCl_2$ | 0.05g |

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| | |
|---|---|
| $FeSO_4 \cdot 7H_2O$ | "trace"* |
| $ddH_2O$ | to 1000ml |

pH adjusted to 6.1

*28mg as 5.0ml of $FeSO_4 \cdot 7H_2O$ (0.56%)--EDTA (0.75%) stock solution

Thirty ml of medium was dispensed into 125ml erlenmeyer flasks and sterilized by autoclaving for 15 min at 15 psi and 121°C. Of several methods tried, the preincubation procedure described by Voeller (1964) proved to be most efficient in providing good yields of sterilized spores. One hundred mg of spores were incubated in the dark at 20°C for 36-48 hrs while suspended in 10 ml of medium supplemented with 1% sucrose. A drop of "Tween-80" (sorbitan monooleate) was included to promote wetting of the spores. During this dark incubation period, the spores of contaminating fungi and bacteria were able to germinate while the light-requiring fern spores could not. Subsequent sterilization of the spore suspension was with a 5-10% "Clorox" solution (0.26-0.52% sodium hypochlorite). After several washings in fresh medium the sterilized spores were collected by centrifugation and suspended at a concentration of about 10mg/ml. Five ml of this suspension was inoculated into each culture flask.

Cultures were maintained on New Brunswick Scientific Co. (New Brunswick, N.J.) "Gyrotory" shakers (ca. 140 cycles/min) which had been fitted with light-tight enclosures having a removable cover of either red (Rhom & Haas #2444) or blue (#2045) plexiglass 3mm thick. Red light was obtained from a bank of Sylvania F40R fluorescent lamps, the ends of which were masked with black tape to reduce the far-red

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emissions from the filaments. Blue light was obtained from Sylvania F40B fluorescent lamps. Spectral data are presented in Fig. 1. Light intensities at the level of the cultures were adjusted (red=ca. 520 ergs/$cm^2$·sec; blue=ca. 760 ergs/$cm^2$·sec) such that the growth rate (dry wt) would be the same under both light conditions (cf. Drumm and Mohr, 1967a). Temperature was maintained at 20 ± 1°C for optimal germination (Mohr, 1956a).

Germination was synchronized by incubating the cultures for 2 da in darkness; followed by 2 da red light; and, finally, 2 da darkness (Drumm and Mohr, 1967a). After germination, the gametophyte cultures were either left in red light for an additional period of growth, or transferred immediately to blue light.

Results and Discussion

The spore sterilization procedure was determined to be sufficient to kill the vegetative forms of all the contaminants. When spores were inoculated into parallel cultures cupplemented with 1% sucrose there was no subsequent evidence of microbial growth. In addition, incubation of 1ml aliquots of medium (from 1 wk and 3 mo cultures) with standard bacteriological broth for 24 hrs at 37°C showed no signs of contamination. This sterilization procedure did reduce the germination rate by about 50% (Table I), but the reduction in viability was considered acceptable in order to insure the elimination of all microbial contaminents.

As has been shown by Mohr (1956b) in Dryopteris filix-mas the transition from filamentous to prothallial growth can be controlled by visible light, with red permitting only growth and cell division in

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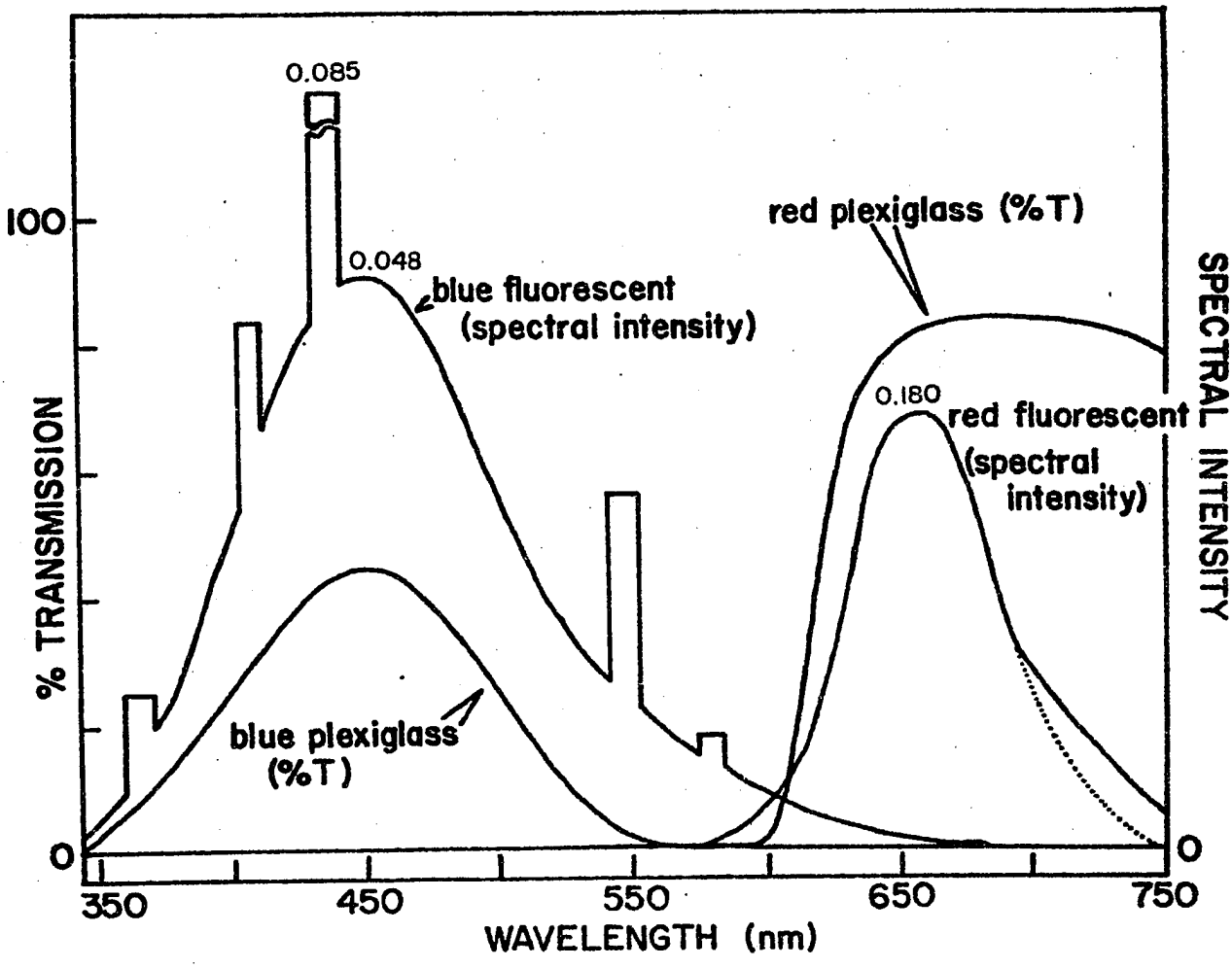


Figure 1. Spectral characteristics of plexiglass filters and fluorescent lamps.

Transmission data for blue and red plexiglass were measured (vs. air in the reference position) using a Beckman DB-G spectrophotometer. Spectral intensity data (uw/($cm^2$·nm·foot candle)) for 40 watt blue and red fluorescent lamps are from Iscotables (3rd edition, 1970. Instrumentation Specialties Company). The broken portion of the curve for the red fluorescent lamp is intended to represent the reduction of far red emissions achieved by masking the ends of the lamp tubes.

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one dimension. A minimum amount of blue light is required for the development of the two-dimensional prothallus (Mohr, 1956b). Ohlenroth and Mohr (1964) demonstrated that various mixtures of red and blue light were nearly as effective as blue light alone, and exposure to white light similarly allows this photomorphogenetic transition. Under the experimental conditions chosen for the present study, irradiation with red or blue light not only controls the photomorphogenetic process, but also provides the sole external energy source for growth of the gametophytes. Mohr and Ohlenroth (1962) have demonstrated that the photomorphogenetic control operates as well in gametophytes cultured on sucrose-containing medium, thereby excluding a nutritional effect.

| | GERMINATION RATE | |
|---|---|---|
| | STERILIZED | UNSTERILIZED |
| Dryopteris filix-mas | 33% | 76% |
| D. erythrosora | 26% | 36% |
| Osmunda cinnamomea | -- | 68% |

Table I. Effect of sterilization on rate of fern spore germination.

The choice of a liquid culture medium was based primarily on the ease with which the gametophytes could be collected for subsequent transfer to other media for an experimental incubation, or for biochemical or anatomical analysis. Growth in submerged culture has been reported to be slow and abnormal in that prothallial growth is retarded (Schindler, 1925, cited in Miller, 1968a); although periodic aeration

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relieved this inhibition of two-dimensional growth. In the present case, continuous mechanical shaking was employed to prevent the development of an anoxic condition within the cultures. Under these growth conditions the Dryopteris gametophytes were capable of both prothallial (blue light) and filamentous (red light) growth.

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# III. THE PHOTOMORPHOGENETIC RESPONSE--TRANSITION TO PROTHALLIAL GROWTH

## Introduction

Reports in the literature as early as 1910 (Twiss) indicated a blue-light requirement for the transition from filamentous to prothallial growth in fern gametophytes. Although this requirement may not operate in every species, (cf. Stephan, 1929; Sobota and Partanen, 1966) it is a controlling factor in the morphogenesis of many fern gametophytes, including those of Dryopteris filix-mas (Mohr, 1956b).

The first reports indicating that red light allows prolonged filamentous growth of fern gametophytes appeared over half a century ago (Twiss, 1910; Klebs, 1916/1917, cited in Miller, 1968a). Only under blue light were gametophytes of these species capable of prothallial growth (cf. also Mohr, 1956b; Mohr and Holl, 1964; Miller and Miller, 1964).

In Dryopteris filix-mas the photomorphogenetic transition is first evidenced by a swelling of the terminal portion of apical cell of the protonemal filament within several hours after transfer from red to blue light (Mohr and Holl, 1964; Mohr, 1965; Drumm and Mohr, 1967a; Payer and Mohr, 1969, also Pteridium aquilinum: Dill, 1969; Davis, 1969) coincident with a decrease in the rate of elongation. This "tip swelling" response has been used in the present study to assay the photomorphogenetic reaction. Further responses include an increased rate of DNA synthesis within about 12 hr after beginning of exposure to blue light, followed by an increase in the rate of cell

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division (Ito, 1970). In addition, there are gross changes in the mean size of chloroplasts, nuclei, and nucleoli (Mohr, 1956b; Bergfeld, 1963 ,b, 1967, 1970). (The photomorphogenetic effects on organelle size will be discussed in Chapter IV.)

The identity of the photoreceptor molecule is, as yet, unknown. Drumm and Mohr (1967a) have suggested a flavin or carotenoid as the blue-light receptor. Although phytochrome is physiologically demonstrable in these plants (eg. in spore germination, Mohr, 1956a), its action in the promotion of elongation (far red promotes more than red) is at odds with data from other systems known to be under phytochrome control (cf. Hillman, 1967). Also, Scharrenberger and Mohr (1967) have shown that the photomorphogenetic effect of blue light cannot be reversed even with light treatments which cause photoreversal of phytochrome. The Millers have presented evidence for a more complex action spectrum in the photomorphogenetic transition to prothallial growth (Miller and Miller, 1967a,b). These workers have argued that it is necessary to postulate the existence of a "yellow-light absorbing pigment ($P_{580}$)" in addition to phytochrome. Such a system is said to explain the spectral responses of fern gametophytes without requiring unique properties for phytochrome of fern gametophytes. More recently Sugai (1971) has argued, on the basis of careful dose-response action spectra, that the photoreceptor which mediates the inhibition of spore germination by blue light (Sugai and Furuya, 1967) cannot be phytochrome. It is not known at present if the same photoreceptor regulates the morphological transition in the gametophyte.

The present experiments are not focused on the problem of

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identifying the photoreceptor, but rather, are concerned with the kinetics of the response to blue light. How soon after exposure to blue light is morphogenesis detectable?

Materials and Methods

Gametophytes of Dryopteris filix-mas were grown as usual (cf. Chapter II).

At the beginning of an experiment 5 ml (medium + red-grown gametophytes) from a single culture (or several pooled cultures) were dispensed into 25 ml erlenmeyer flasks. After further incubation in red or blue light the gametophytes were fixed by the addition of 5ml 4% glutaraldehyde and stored at 4°C until assayed.

To assay the photomorphogenetic response 30-90 fixed gametophytes were photographed on 35mm film at a magnification of about 500x. These negatives were then projected, via a photographic enlarger, onto an X-Y plotting table, and the gametophyte dimensions (tip width, filament width, length and cell number) transcribed automatically onto magnetic tape (Edwin Industries, Silver Spring, Md.). The dimensions in microns, the morphogenetic index ratio (tip width/filament width=$B_1/B_2$; cf. Fig. 4), means and standard deviations, as well as histograms were produced from the raw data by computer programs.

Time-lapse cinematography was employed in order to observe the photomorphogenetic response continuously. While being photographed at 1 or 5 min intervals gametophytes were allowed to germinate and undergo a brief period of red-light elongation, followed by exposure to blue light. Any gametophytes which ceased growth in red, or failed to undergo morphogenesis in blue light within 8-12 hr were eliminated from

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Figures 2, 3, 4, and 5. Growth of Dryopteris and Osmunda gametophytes under red and blue light.

Gametophytes of Dryopteris grown for 1 wk under red (Fig. 2) or blue light (Fig. 3); or 18 hr after transfer from red to blue light (Fig. 4). Gametophytes of Osmunda grown for 2 wk under red light (Fig. 5). Scale bar in Fig. 2 equals 0.10mm. The ratio $B_1/B_2$ is used as a measure of the tip-swelling response (cf. Fig. 4).

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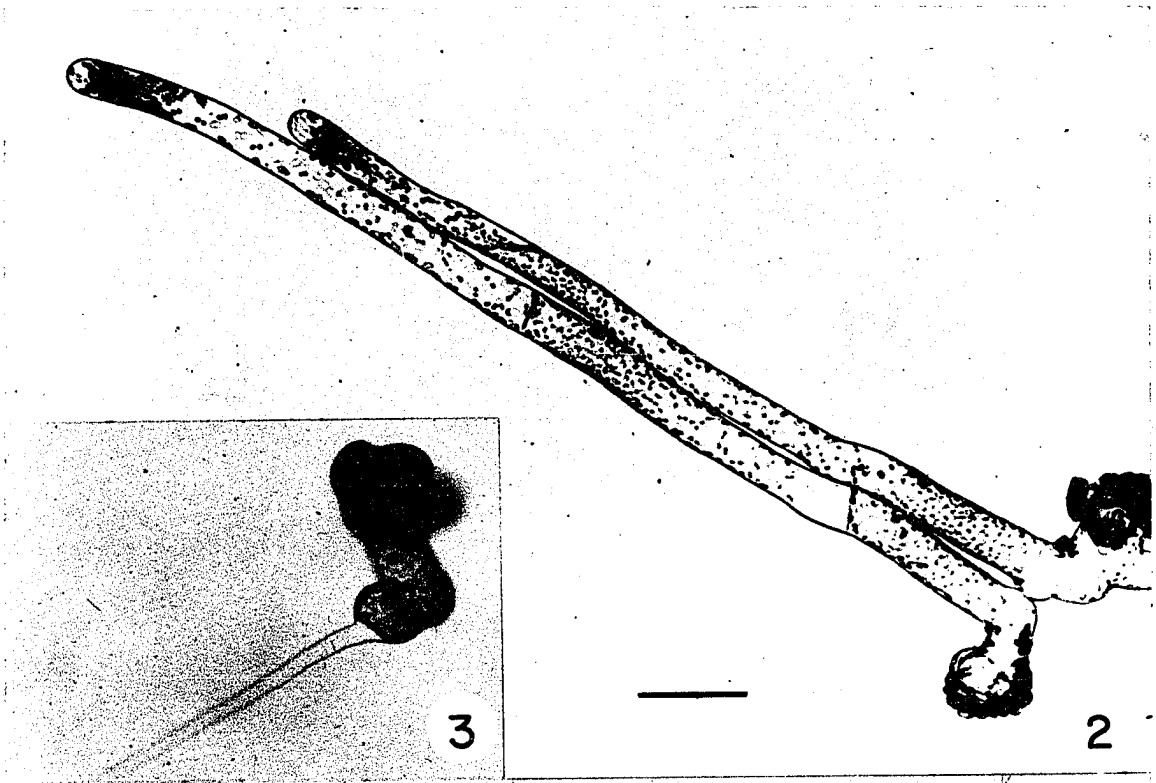


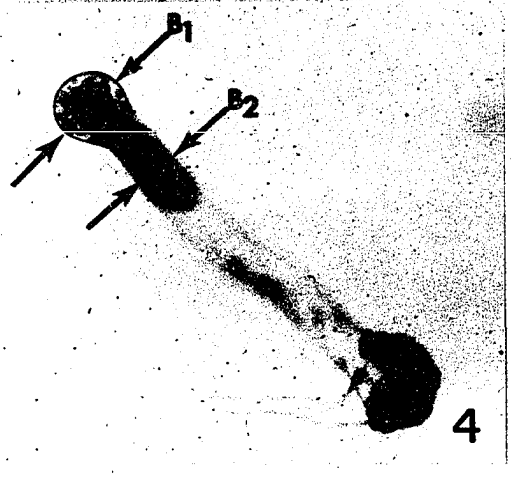


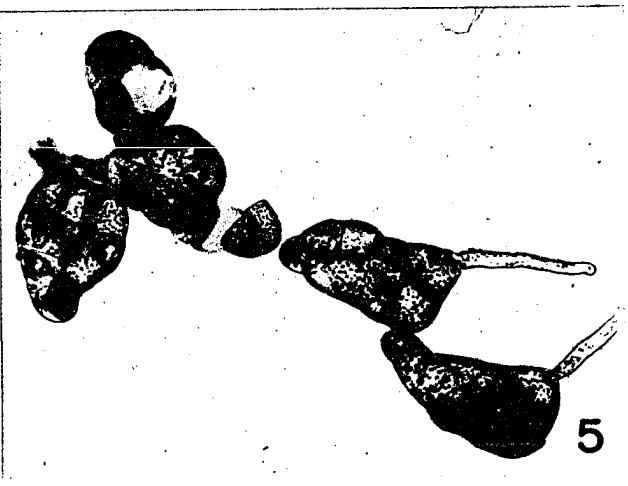


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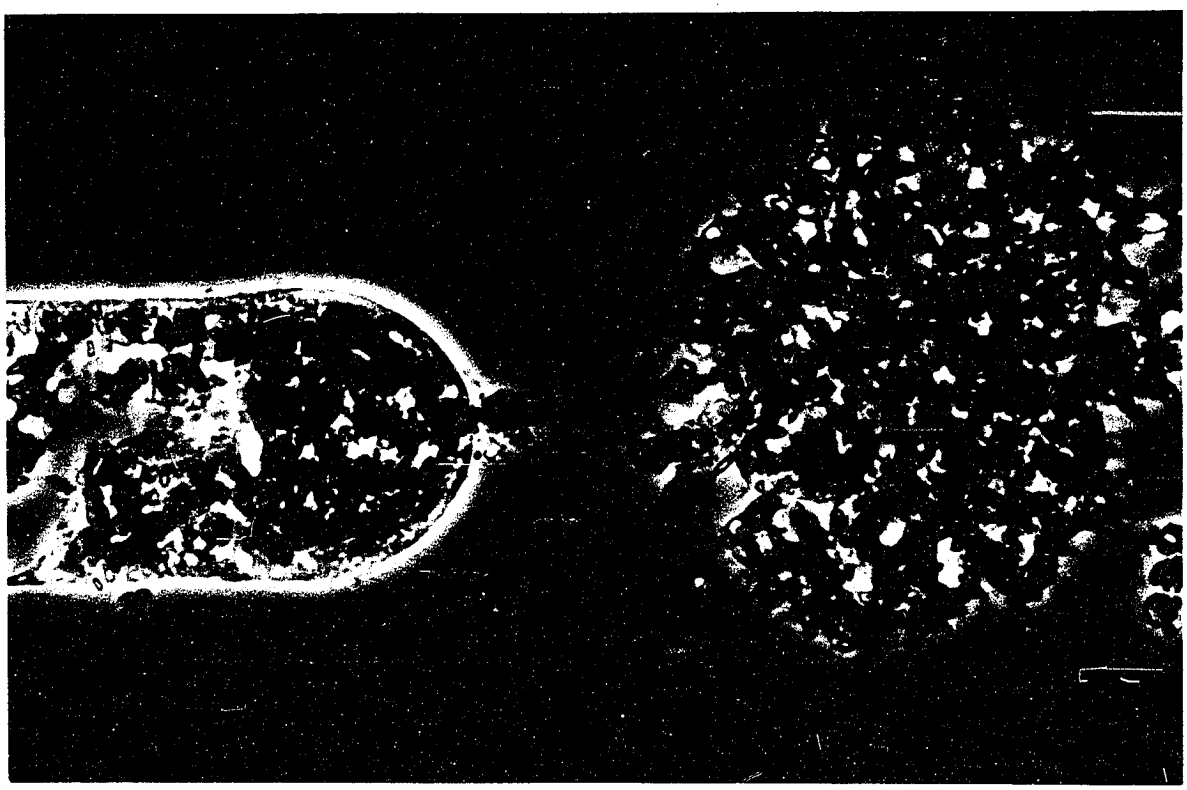

Figure 6. Extruding cytoplasm from weak spot in filament tip of *Dryopteris filix-mas* gametophyte.

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further consideration. Analyses of the elongation and tip swelling were made on 20x or 40x enlargements of the exposures on 16mm film at appropriate intervals.

Results and Discussion

As discussed earlier, Dryopteris filix-mas (and D. erythrosora) gametophytes will grow as uniserate filaments in red light, as two-dimensional prothalli under blue light, and will form a "tip swelling" in response to transfer from red to blue light (Fig. 2-4). On the other hand, gametophytes of Osmunda cinnamomea do not respond in the same way, instead developing into prothalli under either red or blue light (Fig. 5).

Careful examination of time-lapse sequences confirm Mohr's (1956b) observation that filaments of Dryopteris filix-mas elongate exclusively by tip-growth in red light. The narrowly defined region of the wall at which the tip-growth is focused can be seen as the weak point in the cell wall where the cytoplasm is extruded in gametophytes gently squeezed beneath a microscope coverglass (Fig. 6)(also, tip growth in fungal hyphae: Park and Robinson, 1966). Presumably this is the weakest point in the wall because microfibrils which are laid down there in random fashion have not yet undergone the reorientation into the stronger radial configuration (cf. Green, 1969). An alternative hypothesis would invoke the cross-linking of microfibrils (cf. Lamport, 1970) as the basis of wall-hardening. In these red light grown filamentous gametophytes, cell divisions occur only in the terminal cell; whereas after transfer to blue light subapical cells can be observed dividing (Fig. 7-9).

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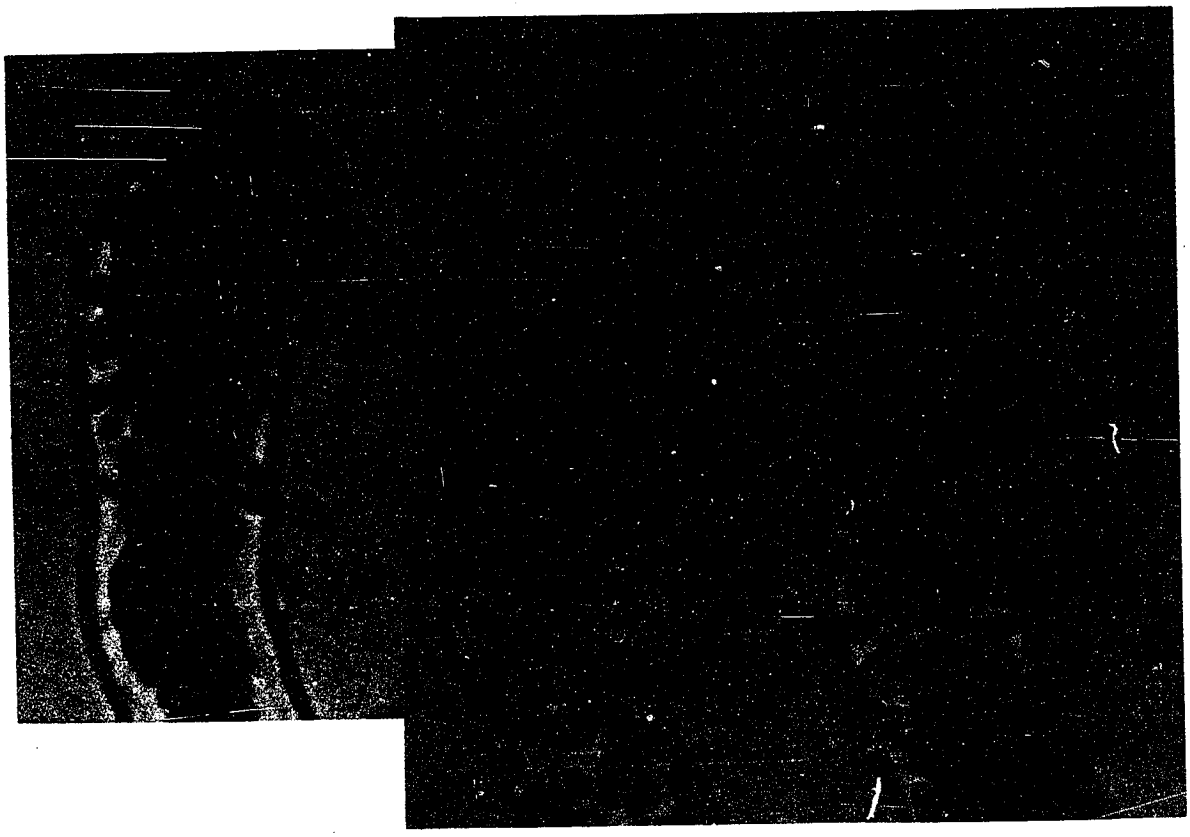

Figures 7, 8, and 9. Subapical cell division during photomorphogenetic transition in Dryopteris filix-mas gametophytes.

Enlargements from a time-lapse sequence at the beginning (Fig. 7), and 15 hr after (Fig. 8) and 20 hr after (Fig. 9) beginning exposure to blue light. Cross wall between apical and subapical cell (large arrows) and newly formed cross wall (small arrow) are indicated.

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The progress of the initial responses to blue light can be seen most clearly in time-lapse sequences (Fig. 10). The transition involves a change from tip growth to an isodiametric swelling. After transfer from red to blue light, the growing region of the cell wall enlarges to include the newly synthesized lateral wall as well as the extreme tip. The process(es) which lead to wall hardening in red light appear not to operate in blue light. It appears that "wall softening" is not involved because cell wall synthesized prior to blue light exposure is not involved in the tip swelling (Fig. 10), although a softening process must be involved in the production of lateral branches sometimes seen in a filamentous gametophyte.

Analyses of time-lapse data indicate a lag period of at least 2-3 hr, after which tip swelling begins (Fig. 11). The rate of filament elongation established in red light continues for a similar period in blue light. Increases in length which occur immediately after this time are the result of the isodiametric expansion of the apical cell tip. Cell division and subsequent enlargement begin contributing to the gametophyte length within about a day.

When data are averaged from population samples, rather than by following the morphogenesis of single gametophytes, the kinetics of increase in the mean ratio, tip width/filament width, suggest little or no lag period in the response to blue light (Fig. 12). However, Chi-square tests for homogeneity indicate the probability that the samples at 0, 0.75, 1 and 2 hr (Fig. 12, Expt. 2) were all drawn from the same population (Table II). Frequency distributions in the data (Fig. 13) allow subjective confirmation of these statistical results. The

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Figure 10. Time-lapse sequence of photomorphogenesis in a *Dryopteris filix-mas* gametophyte.

The gametophyte outline was traced on photographic enlargements made at 100 min intervals during growth in red light and after beginning exposure to blue light. Filament width is approximately 30 microns.

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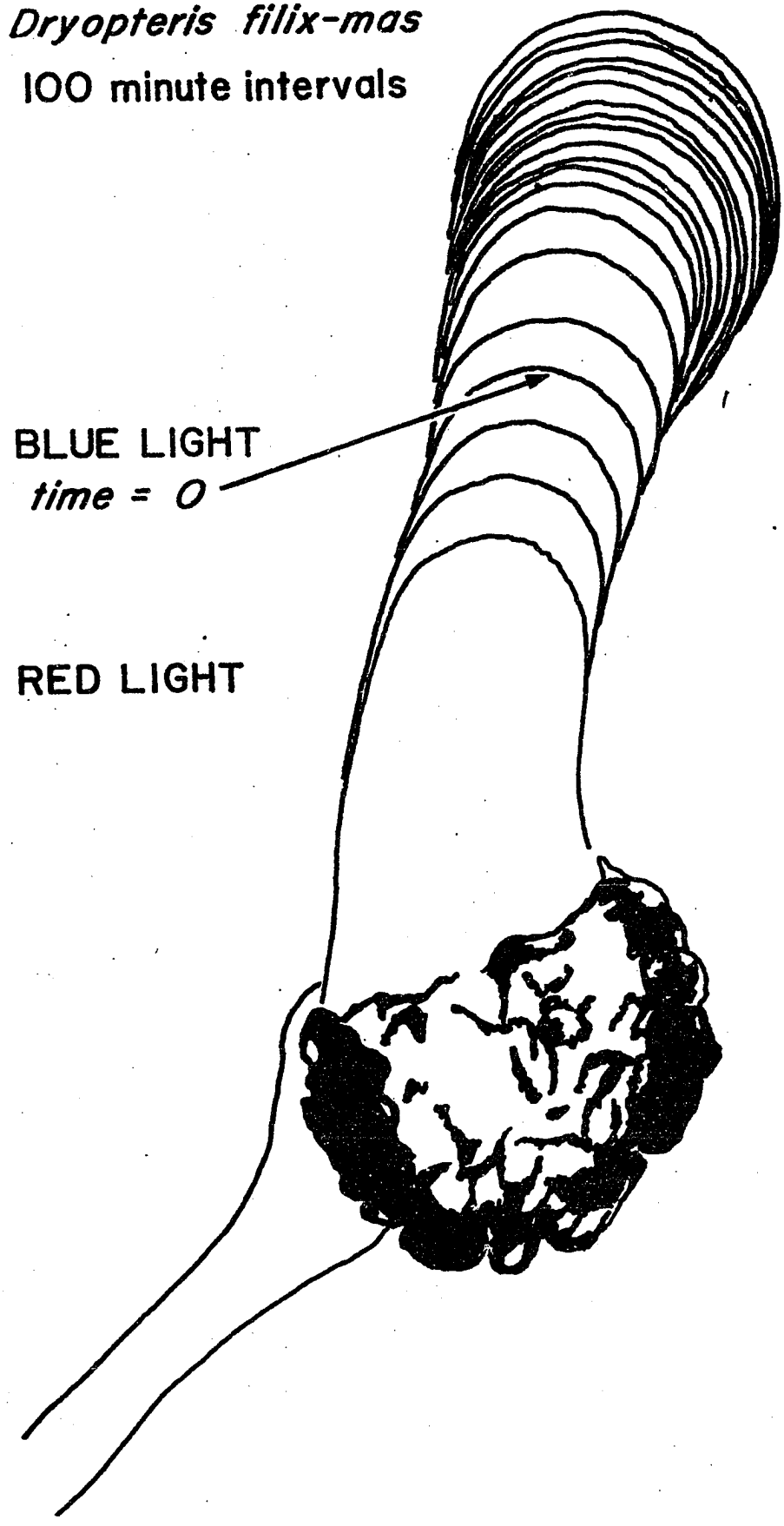


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Figure 11. Kinetics of the photomorphogenetic reaction: time-lapse data.

Data from eight separate time-lapse sequences. Measurements of $B_1$, $B_2$ and length were made on photographic enlargements of the original 16mm exposures. Morphogenetic index ($B_1/B_2$; circles) and length increase (squares) are shown for growth in red light (closed symbols) and after exposure to blue light (open symbols). Missing data points are due to out-of-focus image on original 16mm film.

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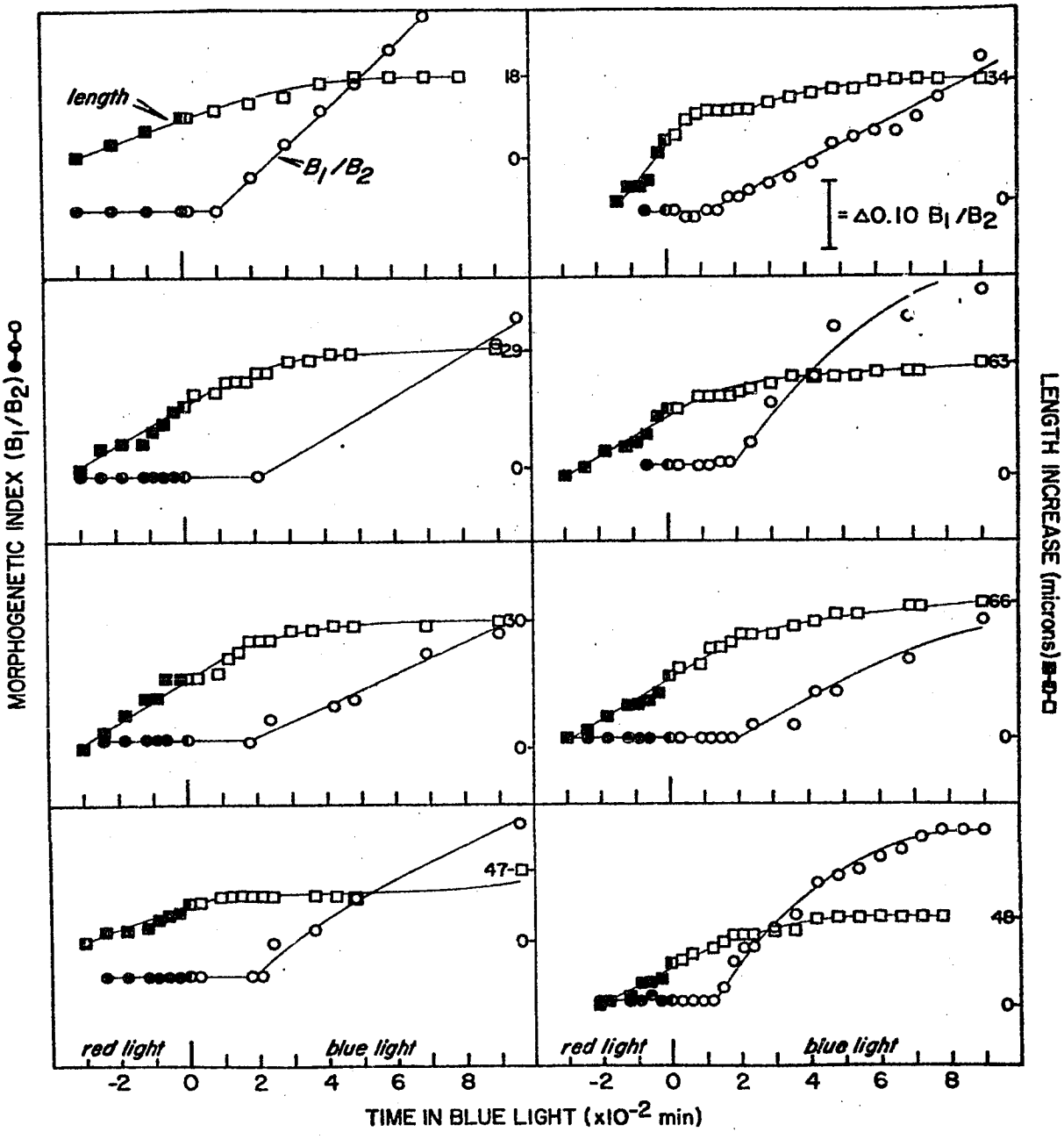


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Figure 12. Kinetics of the photomorphogenetic reaction: averaged data from population samples.

Two separate experiments (1 and 2) showing the increase in the morphogenetic index (mean $B_1/B_2$) in red light (closed circles) and at various times after transfer to blue light (open circles).

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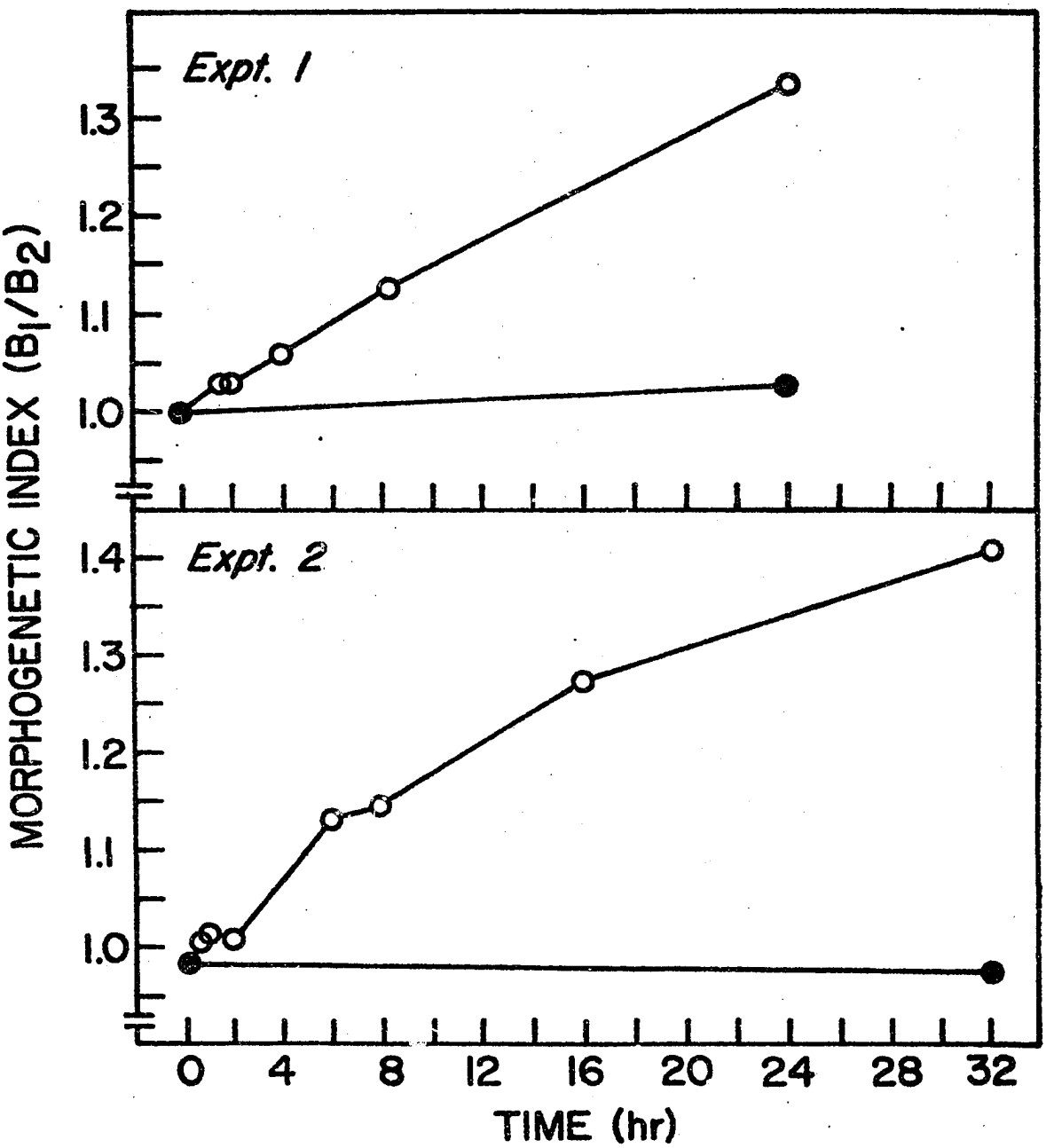


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| TREATMENT | PROBABILITY THAT SAMPLES COMPARED ARE DRAWN FROM THE SAME POPULATION ($B_1/B_2$) | | |
|---|---|---|---|
| Red t=0 | 0.50-0.75 | 0.50-0.75 | 0.50-0.75 |
| Blue t=0.75 hr | 0.25-0.50 | | |
| Blue t=1 hr | 0.05-0.10 | | |
| Blue t=2 hr | ≪0.005 | | |
| Blue t=6 hr | 0.1-0.25 | | |
| Blue t=8 hr | ≪0.005 | | |
| Blue t=16 hr | 0.01-0.025 | | |
| Blue t=32 hr | ≪0.005 | | |
| Red t=32 hr | | | |

Table II. Chi-square tests for homogeneity between time course samples.

Samples of Dryopteris filix-mas gametophytes are those presented in Figure 13. The null hypothesis assumes that the samples compared in each case have identical distributions within the $B_1/B_2$ classes indicated. Shown are the probabilities of obtaining the calculated Chi-square value by chance if, in fact, the samples compared were drawn from the same population (cf. Simpson et al., 1960. pp 324-326).

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Figure 13. Frequency distributions of gametophyte morphogenetic index.

Data are from Experiment 2, (Fig. 12). Values of n=39 (0 hr), 61 (0.75 hr BLUE), 41 (1 hr BLUE), 85 (2 hr BLUE), 43 (6 hr BLUE), 31 (8 hr BLUE), 35 (16 hr BLUE), 46 (32 hr BLUE), and 36 (32 hr RED). Morphogenetic index classes are those used for Chi-square tests of homogeneity (Table II).

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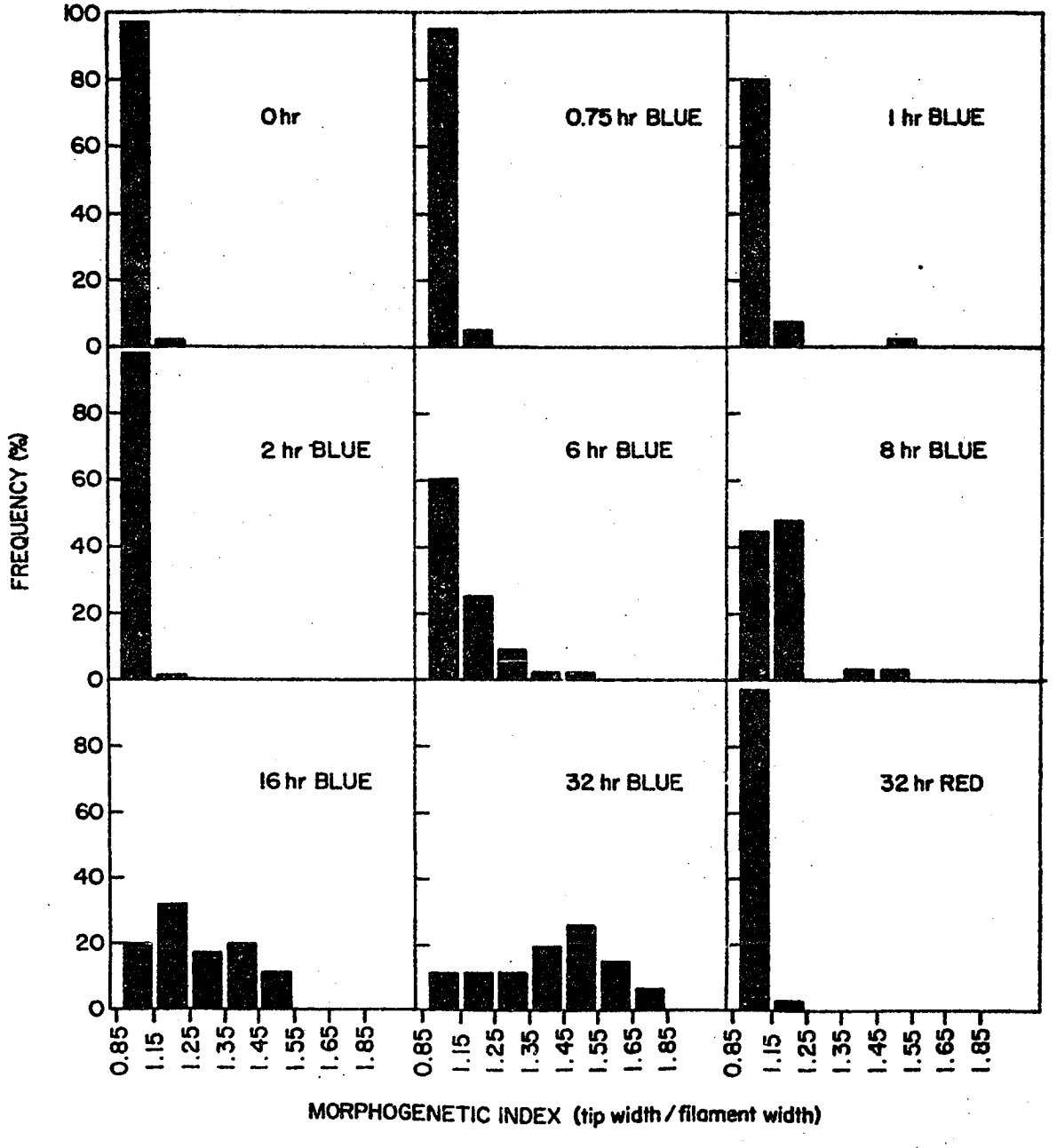


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histograms also show that about half of the gametophytes which will ultimately respond have done so by 8 hr. This suggests that at any particular time a certain fraction of the gametophytes are not compentent to respond to the photomorphogenetic stimulus, but that most of these acquire competence somewhat later. Nevertheless, the most rapid response is detected only after about 2-3 hr.

It is concluded from these experiments that any hormonal, enzymatic or macromolecular-synthetic process required to initiate the photomorphogenesis must begin during the first 2-3 hr of exposure to blue light.

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# IV. TECHNICAL ASPECTS OF RNA ISOLATION AND ANALYSIS

## Introduction

The object of examining RNA metabolism during the photomorphogenetic transition in fern gametophytes was to obtain both quantitative and qualitative data on transcriptional activity. For this reason it was necessary to apply techniques which would permit the isolation and purification of undegraded total-RNA. Other studies on RNA metabolism in fern gametophyte photomorphogenesis have utilized techniques which employed a hydrolysis as the basis for RNA extraction (Hotta, 1960; Drumm and Mohr, 1967a; Raghavan, 1968).

The standard procedure for the extraction and purification of high-molecular-weight RNAs involves the utilization of a phenol/buffer system to partition RNA into the aqueous phase, and lipids and pigments into the organic phase, while precipitating proteins at the interface (eg. Kirby, 1956). An important aspect of this procedure is the adverse effect of the phenol on the degradative activity of ribonuclease. More recently, a procedure employing diethyl pyrocarbonate to precipitate proteins directly from aqueous solution became available (Solymosy et al., 1968); and has proven, in the present system, to be a more effective inhibitor of nuclease activity, and a more efficient de-proteinizing agent.

Another potential source of technical problems involved the incorporation of radioisotopes into RNA. Microbial contamination has been shown to be at the source of spurious results obtained in various plant systems (eg. Lonberg-Holm, 1967; Sobota et al., 1968). The use of axenic cultures in the present work was intended to eliminate the possibility of this type of interference.

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By following the time-course of $^{14}$C-uridine uptake and incorporation into acid-extractable RNA, Drumm and Mohr (1967a) reported a higher rate of RNA synthesis in cultures of Dryopteris filix-mas gametophytes transferred to blue light compared to those which remained in red light over the same period. These workers present data indicating that RNA specific activity increases during the first 3 hr in both red and blue light, thereafter maintaining a more or less constant level. Their interpretation is that the RNA is completely labelled ("... die RNS ist soweit ‚durchmarkiert'") within 3 hr, and that, therefore, the total RNA of the gametophyte must turn over very quickly ("... die gesamte RNS der Vorkeime einem raschen ‚turnover' unterliegt"). Drumm and Mohr do not speculate as to how they were able to obtain from gametophytes in red and blue light, completely labelled RNAs with different specific activities even though the cultures in both treatments were incubated with the same levels of $^{14}$C-uridine, and there is little difference in the uptake of uridine in red vs. blue light (Fig. 7 in Drumm and Mohr, 1967a). However, their data on the uptake of $^{14}$C-uridine by the gametophytes do show kinetics which parallel their incorporation data. It is possible that the $^{14}$C-uridine incorporation results of Drumm and Mohr are primarily a reflection of $^{14}$C-uridine uptake into the internal precursor pool of the gametophytes. Since these workers used unsterilized cultures the data may be impossible to interpret.

More recently, Raghavan (1968) has published results showing similar kinetics of $^{3}$H-uridine incorporation with axenic cultures of Pteridium aquilinum gametophytes. Results presented in that report can be used to calculate that in a 4 hr labelling period the ratio "incorporation into total-RNA/uptake" is higher in gametophytes grown

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for 8 da in blue light than those grown in red light (14.3% vs. 7.6%). However, contrary to Raghavan's interpretation of his results, younger gametophytes (2 da post-germination) reveal the reverse situation (5.1% in red light; 3.5% in blue light). Similar calculations on the incorporation into RNA of the subcellular fractions show that in the older gametophytes every fraction reflects blue light stimulation, while in the younger gametophytes only the "ribosome-rich" fraction shows evidence of enhanced RNA synthesis in blue light. In both the study of Drumm and Mohr (1967a) and that of Raghavan (1968), there was no indication of the types of RNA molecules being labelled, although Raghavan (1968), did perform a crude fractionation of the subcellular organelles.

In the present experiments I was interested in determining whether specific RNAs were being synthesized in response to the blue-light exposure, and what the kinetics of this response were. As seen from the work of Drumm and Mohr (1967a) and Raghavan (1968) the nature of uptake mechanisms and internal RNA precursor pools might preclude adequate labelling of the RNA if a permeability barrier exists, or if the pools are not exchangeable with or expandable by labelled precursors in the outside medium. Therefore, experiments were performed to evaluate the possible influence of these factors.

## Materials and Methods

Materials--Phosphorus-32 as carrier-free $H_3{}^{32}PO_4$ in 0.92N HCl and $^3$H-5-uridine (28 c/mMole) were obtained from New England Nuclear. Carbon-14 (uniformly labelled) uridine (510 mc/mMole) was from Amersham-Searle. "Liquifluor" scintillation fluid concentrate was purchased from New England Nuclear. Diethyl pyrocarbonate (diethyl oxydiformate),

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acrylamide, N,N'-methylenebisacrylamide and N,N,N',N'-tetramethylethylenediamine were obtained from Eastman Organic Chemicals. Sodium dodecylsulfate (99% pure) was purchased from Sigma Chemical Company. Sephadex G-25 was from Pharmacia Chemicals. "Seakem" agarose was the product of Bausch and Lomb, Inc.

Gametophyte cultures--Spores of Dryopteris filix-mas, D. erythrosora and Osmunda cinnamomea were sterilized and cultured as described in Chapter II.

Labelling with radioisotopes--In the usual case, gametophytes were allowed 1-2 weeks' growth in red light. Under dim red light several cultures were combined for each treatment and the volume of culture medium reduced. Radioactive precursor was added at this point, and incubation in either red or blue light followed. Finally, the gametophytes were collected by suction filtration, rinsed extensively, and the RNA extracted as usual. Additional details are noted in the separate figure legends.

RNA extraction and analysis--A phenol/cresol/8-hydroxyquinolin extraction (Loening and Ingle, 1967) was originally employed, but sometimes did not provide pure RNA from these organisms (based on ultraviolet absorbance ratios of $\frac{A_{260}}{A_{230}} \geq 2.0$ and $\frac{A_{260}}{A_{280}} \geq 2.0$).

The use of diethyl pyrocarbonate (Solymosy et al., 1968) as a potent nuclease inhibitor and general precipitator of proteins has led to isolation of consistently pure RNA extracts. When necessary, the extracted RNA was stored at -20°C under ethanol. A conversion factor of 40ug RNA per absorbance unit at 260nm has been used.

For base composition analysis, RNA isolated by the phenol method

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was hydrolyzed and the nucleotides separated by high-voltage paper electrophoresis (Smith, 1955). The position of each of the nucleotide zones was located under an ultra-violet light, cut out and the $^{32}P$-nucleotides counted directly in a liquid scintillation counter. Incorporation of $^{3}H$- and $^{14}C$-uridine was assayed by the following procedure: Cultures were fixed in ice cold 0.3M perchloric acid for 30 min, homogenized, and the homogenate washed twice with cold 0.3M perchloric acid. The residue was then washed with 95% ethanol, 3:1 absolute ethanol/ether, and ether. RNA in the dried pellet was hydrolyzed by 0.3N KOH for 18 hr at 37°C. A portion of the hydrolysate was neutralized and assayed by scintillation spectrometry.

Acrylamide monomers were purified according to Loening (1967). The buffer of Bishop, Claybrook, and Spiegelman (1967) was used to prepare 2.4% gels to which 0.5% agarose was added (Peacock and Dingman, 1968). Gels were pre-run at 50 v (4-5 millamperes per gel) for 45 min. RNA samples were applied to gels, run at 50 v for 90 min or 3-4 hr and then rinsed in distilled water (Loening, 1968). The gels were scanned at 260nm with the linear transport accessory of a Gilford spectrophotometer (cf. Fig. 14).

After U.V. scanning the gels containing $^{3}H$ or $^{32}P$ RNAs were placed in an aluminum trough and frozen on dry ice. Using a modified Sorvall TC-2 tissue sectioner, 1.0 or 0.5 mm fractions were taken along the length of the gel. The slices were placed in sequence on a strip of filter paper, dried, and the fractions on the strip cut into individual vials containing 5ml of toluene scintillation fluid ($^{32}P$); or the fractions were solubilized with 0.1ml 30% hydrogen peroxide at 60°C overnight and counted in 10ml of toluene PPO-POPOP/Triton X-100 (2:1)

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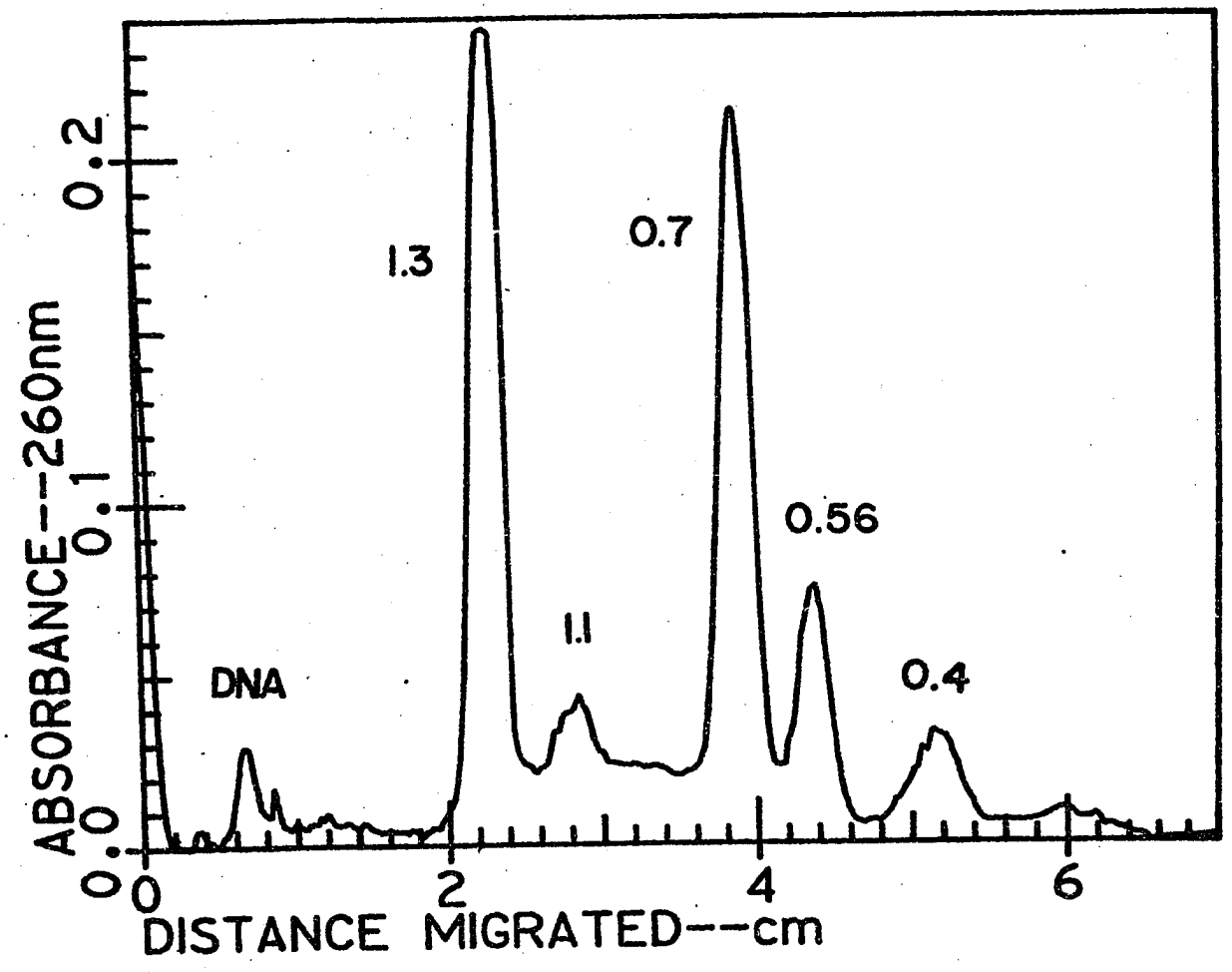


Figure 14. Resolution of rRNAs by acrylamide gel electrophoresis.

Molecular weights are approximately $1.3 \times 10^6$ (cytoplasmic heavy rRNA), $1.1 \times 10^6$ (chloroplast heavy), $0.7 \times 10^6$ (cytoplasmic light), $0.56 \times 10^6$ (chloroplast light), and $0.4 \times 10^6$ daltons (breakdown product of $1.1 \times 10^6$ dalton component). Electrophoresis was for 4 hr.

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scintillation mixture ($^{3}H$). A small amount of water (ca. 0.3ml) was added to the toluene/Triton X-100 samples to give a stable, homogeneous emulsion.

Scintillation counting was done in a Packard (Model 3375) or Intertechnique (Model SL-30) instrument. The standard method of internal channels ratio or automatic external standard channels ratio was employed to correct for quenching of tritium or $^{14}C$ activity when necessary. Counting data (on punched paper tape) for acrylamide gel fractions were usually handled via computer programs which performed quench correction, averaging of replicate counts, and plotting of data on-line with a cathode-ray-tube or a pen-and-ink plotter. Spectrophotometer scans of gels were transcribed onto magnetic tape using an X-Y plotting table (Edwin Industries, Silver Spring, Md.) and the absorbance data were plotted with the radioactivity data.

The amounts of rRNAs were measured directly by integrating the peak areas on the spectrophotometric scan of the gel, and by summing the radioactive incorporation into the rRNA peaks in the case of $^{32}P$ experiments. The values of the cytoplasmic heavy rRNA ($1.3 \times 10^6$ daltons, cf. Fig. 14) and the chloroplast light rRNA ($0.56 \times 10^6$ daltons) were multiplied by 1.5 and 3.0 respectively, to give total amounts of cytoplasmic and chloroplast rRNAs. This method to compute the amounts of cytoplasmic and chloroplast rRNAs is identical to that used by Smith (1970) and is based on the assumption that a 1:1 relationship exists between the light and heavy rRNA components for both the cytoplasm and chloroplasts. The formulae of Ingle (1968) were applied to evaluate the extent of heavy chloroplast rRNA breakdown.

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## Results and Discussion

RNA extraction--A major problem was encountered with the formation of tannins in the extraction of RNA. Apparently phenolic compounds in the gametophytes are oxidized to quinones, which, in turn, polymerize spontaneously among themselves and with cellular proteins and nucleic acids (cf. Loomis and Battaile, 1966). The most important factor in obtaining good RNA preparations after phenol/buffer extraction appears to be the speed with which the RNA could be separated from the phenolics of the gametophyte. Exclusion chromatography on Sephadex G-25 (0.02M $KH_2PO_4$, 4°C) was used to separate the RNAs ( and contaminating proteins and DNA) from the low-molecular weight components, including residual phenol from the extraction system.

Phenol/buffer extraction procedures yielded pure and generally undegraded RNA about 1/2 - 1/3 of the time. RNA purity was based on the following criteria:

a) the ethanol-precipitated RNA forms a homogeneous white grey-white pellet which dissolves readily upon addition of buffer and 0.1M salt.

b) from spectophotometric assay (cf. Fig. 15)

(i) the absorbance at 320nm is generally less than 2% of the absorbance at 260nm.

(ii) the absorbance maximum is at 260nm.

(iii) the absorbance ratios 260/280 and 260/230 are equal to or greater than 2.0.

c) acrylamide gel electrophoresis can resolve the RNAs into the four expected ribosomal-RNA components and transfer-RNA in the expected general proportions.

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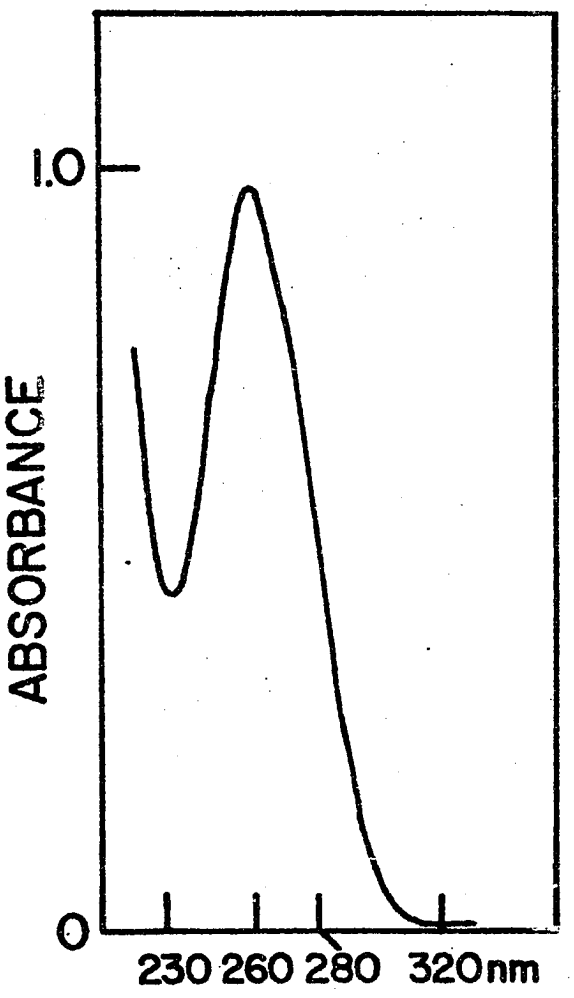


Figure 15. Absorbance spectrum of fern gametophyte RNA preparation.

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Modifications of the basic phenol/buffer procedure of Kirby (1956) including the use of various detergents (sodium dodecyl sulfate, Triton X-100, tri-isopropylnaphthalene sulfonate, naphthalene-1,5-disulfonate, p-aminosalicylate), RNase inactivating agents (polyvinyl sulfate, bentonite), mercaptoethanol, and insoluble polyvinyl pyrrolidone ("polyclar AT") did not yield RNA which was consistently pure. Ingle and Burns (1968) also encountered difficulties with phenol/buffer extraction of RNA from fern gametophytes. Fig. 16 lists the protocol which was finally chosen for phenol/buffer extractions.

Much more efficient than the phenol/buffer procedure is the RNA extraction technique employing diethyl pyrocarbonate (Fig. 17; Solymosy et al., 1968). This method avoids the use of phenol entirely, and also eliminates the problem of "tanning" encountered with the phenol/buffer system. A significant drawback to the use of diethyl pyrocarbonate is that this chemical is extremely reactive, and can apparently affect the structure of nitrogen bases in the RNA (Leonard et al., 1970). Such an effect imposes serious inhibitions on the use of this isolation method for RNA which is to be used in molecular hybridization experiments, or in systems to assay template activity for protein synthesis. On the other hand, acrylamide gel electrophoresis indicates that the degree of polymerization (molecular weight) is not affected. In addition, transfer-RNA amino acceptor activity is not significantly reduced by this procedure (Abadom and Elson, 1970).

Thus, for the present studies, the diethyl pyrocarbonate procedure offers the most reliable method of isolating pure, undegraded (i.e. degree of polymerization) RNA from fern gametophytes.

Acrylamide gel fractionation--To evaluate the reliability of the

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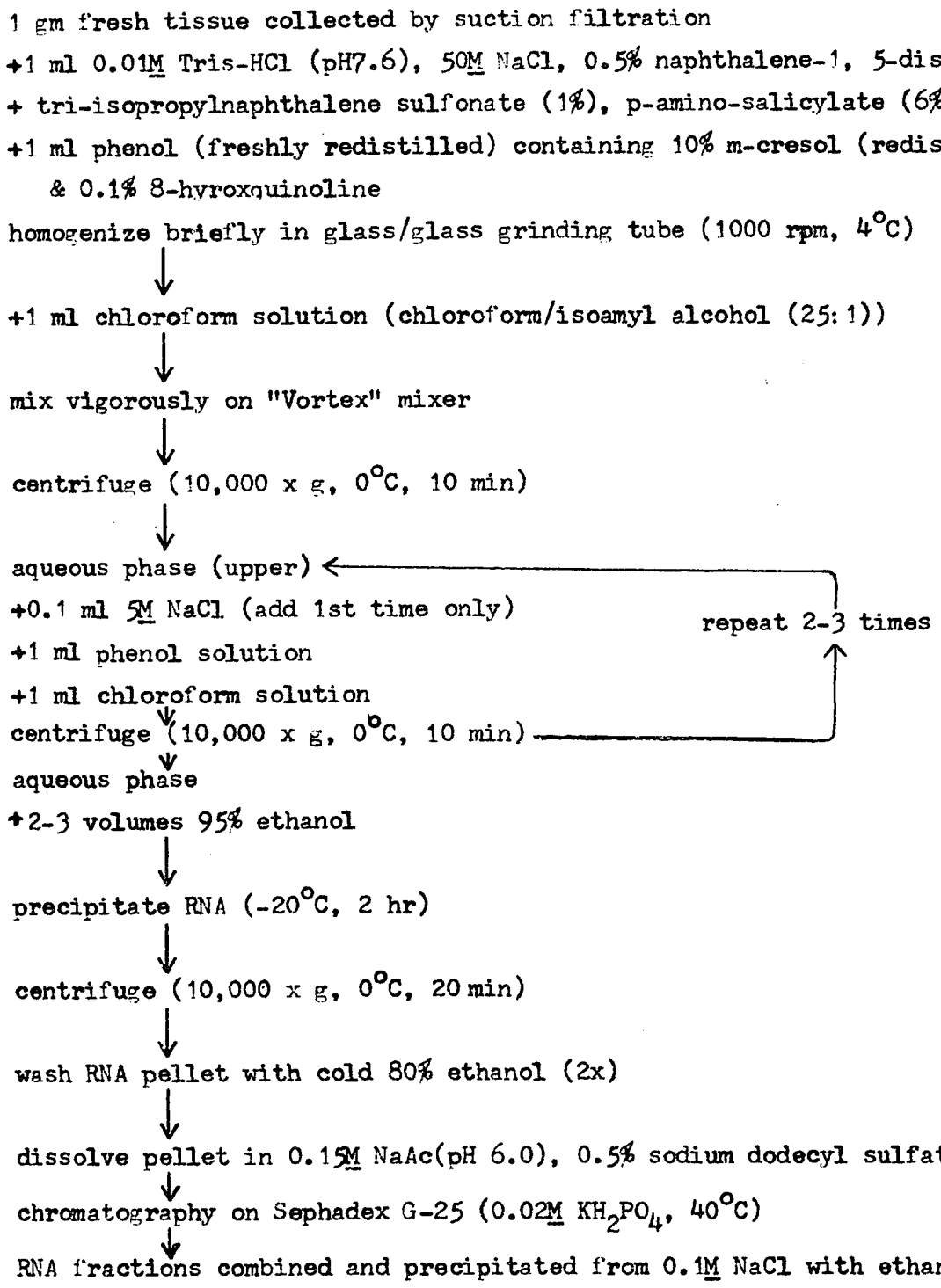


Figure 16. Phenol/buffer extraction procedure (Loening and Ingle, 1967).

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1 gm fresh tissue collected by suction filtration
+3 ml 0.05M Tris-HCl(pH 7.6), 1% SDS, 5mM $MgCl_2$
+0.1 ml diethyl pyrocarbonate
↓
homogenize briefly in glass/glass grinding tube (1000 rpm, 4°C)
↓
incubate (37°C, 5 min)
↓
rehomogenize
↓
centrifuge (10,000 x g, 0°, 15 min)
↓
supernatant
+0.3g NaCl
homogenize
↓
incubate (37°C, 5 min)
↓
centrifuge (10,000 x g, 0°, 20 min)
↓
precipitate RNA with 2-3 vol ethanol (-20°C, 2 hr)
↓
centrifuge (3,000 x g, 0°C, 10 min)
↓
wash RNA pellet with cold 80% ethanol (2x)
↓
dissolve pellet in 0.05 M phosphate (pH 7.4)
↓
centrifuge (10,000 x g, 0°, 20 min)
↓
RNA precipitated from supernatant with 0.1M NaCl and 2-3 vol ethanol

Figure 17. Diethyl pyrocarbonate extraction procedure (Solymosy et al., 1967).

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method used to fractionate gel cylinders containing radioactively labelled RNA, the following experiment was carried out: Phosphorus-32-labelled RNA was added to, and thoroughly mixed with a solution of acrylamide (2.5% final concentration) immediately before casting a gel cylinder in the usual manner. After allowing the acrylamide to polymerize completely the gel was removed from its plexiglass tube and placed on the stage of a specially modified Sorvall/TC-2 tissue sectioner (Howland, unpublished). Fractions were cut from the gel at various thicknesses, both frozen and unfrozen. In addition, a comparison was made between two scintillation counting situations; 1) fractions dried on pieces of filter paper, or 2) solubilized before scintillation counting. Table III shows that 1mm fractions can be cut on an unfrozen gel with a considerable degree of precision. However, freezing improved precision to about ±2% for 0.5mm fractions and allowed sectioning down to 0.25 and 0.125mm. The variation in observed counts per fraction can be reduced even further by solubilizing the gel slice. This variation is probably due to differences in self absorption and mild quenching within the gel slice dried on the filter paper. (It should be noted that the filter paper method permits recovery of the vial and scintillation fluid since all of the radioactivity can be removed with the piece of filter paper.)

Labelling with radioisotopes--The apparent difficulties encountered by Drumm and Mohr (1967a) and by Raghavan (1968)(cf. Introduction, this chapter) suggested that gametophyte RNA could not be readily labelled with radioactive uridine. Tests were carried out to determine the effect of exogenous uridine concentration on the rate of uridine incorporation into RNA. These experiments revealed a linear relationship

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| Fraction size | Percent Standard Error | | |
|---|---|---|---|
| | Cut unfrozen | Cut frozen | Cut frozen & solubilized |
| 1.00mm | ±2.64% (16) | ±2.24% (10) | ±1.41% (13) |
| 0.50mm | ±6.48% (10) | ±1.81% (10) | ±1.14% (10) |
| 0.25mm | -- | ±1.82% (20) | ±1.23% (10) |
| 0.125mm | -- | ±4.70% (10) | -- |

Table III. Precision of acrylamide gel slicing. Values of n are shown in parentheses.

between the concentration of uridine ($2x10^{-7}$ to $10^{-2}$M) and the amount of exogenous uridine incorporated into RNA (Fig. 18). The rates of incorporation were apparently independent of incubation time (2 vs. 12 hr), the mean hourly rates being the same at each concentration of uridine (Table IV). Only at the highest concentration of uridine ($5x10^{-1}$M) was there any indication that the uridine uptake mechanism had been saturated (cf. Reddy and Wyatt, 1967). In addition, there was no difference in incorporation between gametophytes labelled in red light or blue light except at $5x10^{-1}$M uridine. These results could be due to either a permeability barrier limiting entry of the uridine from the outside medium or an intracellular pool of uridine which is very large, unexpandable and/or unexchangable by uridine in the outside medium.

Dryopteris filix-mas gametophytes were incubated in $^{3}$H-uridine at a concentration of $1.0x10^{-1}$M in an attempt to saturate the uridine uptake system. The results (Fig. 19 show no evidence of differential incorporation between the red and blue treatments. However, the low level of incorporation in all treatments suggests that the internal

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Figure 18. The effect of exogenous uridine concentration on the incorporation of uridine in Dryopteris filix-mas gametophytes.

$^{14}$C-uridine incorporated during 12 hr in red light (curve A). $^{3}$H-uridine incorporated during: 2 hr immediately following transfer from red to blue light (B), 2 hr in a red-grown culture (C), 2 hr immediately following transfer from blue to red light (D), and 2 hr in a blue-grown culture (E).

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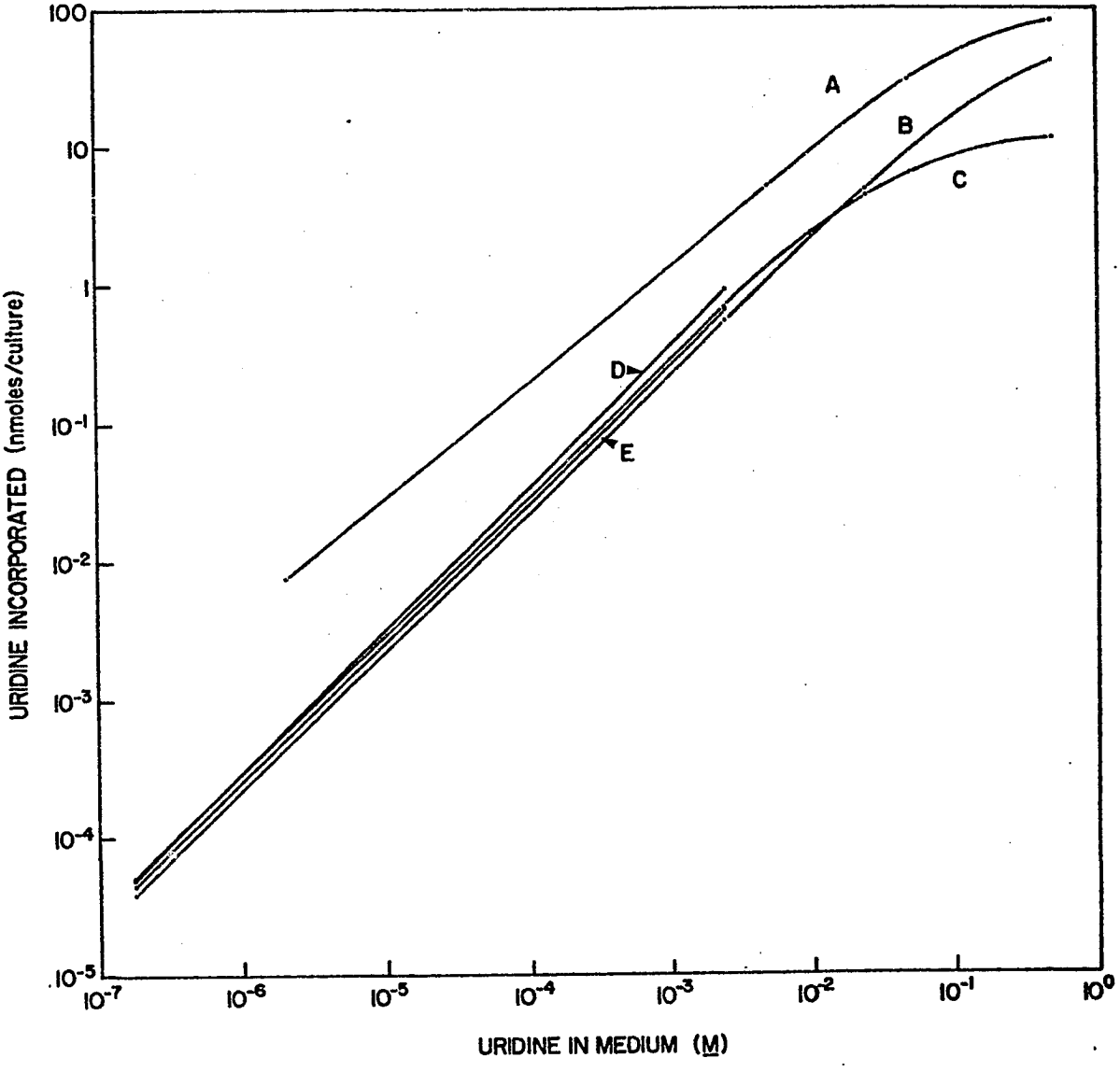


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| uridine(M) | isotope | labelling period (hr) | light treatment | nmoles uridine incorporated per culture per hr |
|---|---|---|---|---|
| $1.9 \times 10^{-7}$ | $^{3}H$ | 2 | R R | 0.000026 |
| " | " | " | R B | 0.000020 |
| " | " | " | B R | 0.000026 |
| " | " | " | B B | 0.000023 |
| $2.0 \times 10^{-6}$ | $^{14}C$ | 12 | R R | 0.00063 |
| $2.5 \times 10^{-3}$ | $^{3}H$ | 2 | R R | 0.35 |
| " | " | " | R B | 0.28 |
| " | " | " | B R | 0.46 |
| " | " | " | B B | 0.35 |
| $5.0 \times 10^{-3}$ | $^{14}C$ | 12 | R R | 0.43 |
| $2.5 \times 10^{-2}$ | $^{3}H$ | 2 | R R | 2.3 |
| " | " | " | R B | 2.4 |
| $5.0 \times 10^{-2}$ | $^{14}C$ | 12 | R R | 2.6 |
| $5.0 \times 10^{-1}$ | $^{3}H$ | 2 | R R | 5.8 |
| " | " | " | R B | 21.0 |
| " | $^{14}C$ | 12 | R R | 6.9 |

Table IV. Mean hourly incorporation of $^{3}H$- and $^{14}C$-uridine at various exogenous uridine concentrations.

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Figure 19. Gel electrophoresis of $^{3}H$-uridine incorporated into RNA of Dryopteris filix-mas gametophytes.

Shown is the amount of RNA in each fraction along the gels (ug; circles), and $^{3}H$ incorporated (dpm; squares). Uridine was supplied at a concentration of 0.1M and an activity of 100uc/ml. Counting rate was at least twice background for almost every fraction. Data are the means of three quench-corrected replicate counts. Gametophytes had grown for 10 da in red light prior to this experiment. Electrophoresis was for 90 min.

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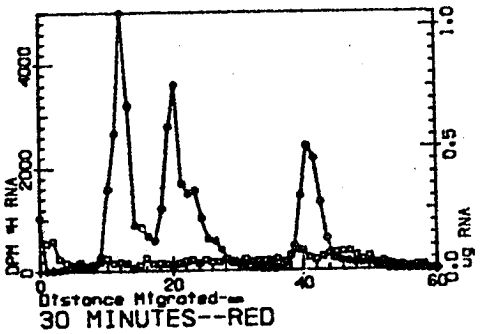


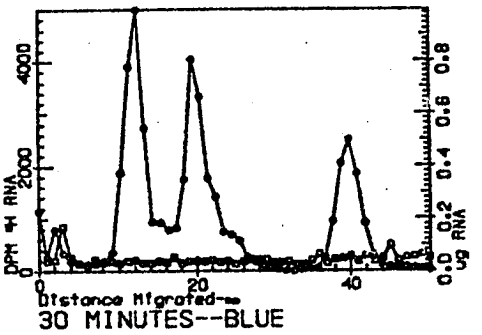


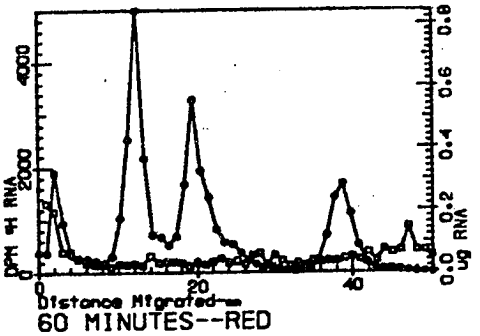


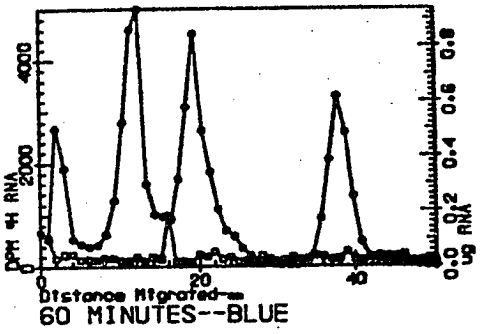


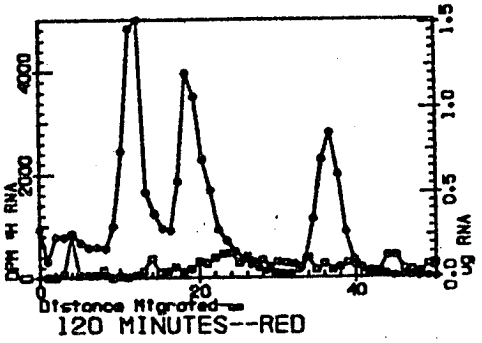


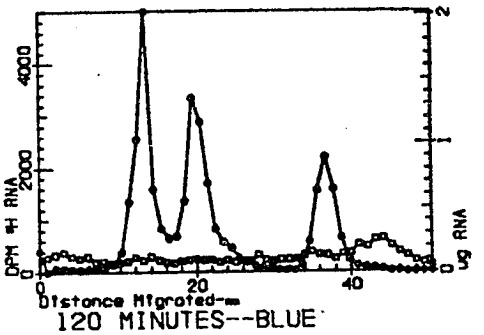


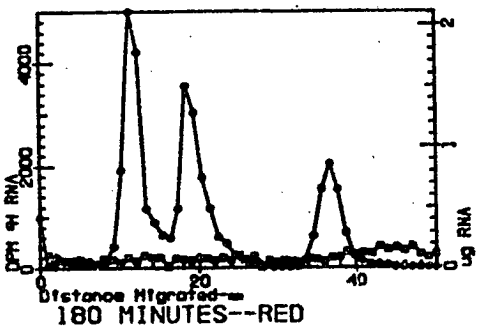


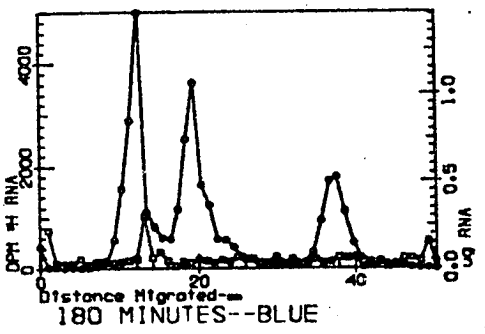


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pool of uridine is not readily exchangable with the outside medium, even at a uridine concentration which should saturate the uptake system of the gametophyte. Therefore, uridine incorporation is not a satisfactory assay for RNA synthesis in fern gametophytes, especially for short incorporation periods.

$^{32}$P-orthophosphate was subsequently used for RNA pulse-labelling experiments. By using up to 1.25 mc $^{32}$P/ml of phosphate-free medium incorporation was observed into all size classes of RNA (Fig. 20) with clear accumulation of activity in rRNAs at the end of 5 hr exposure to the isotope. Only by this time is there a discernible indication of incorporation into RNAs reported to be precursors of the cytoplasmic rRNAs (arrows, Fig. 20)(Loening, 1970; Leaver and Key, 1970). Later experiments indicated that a much lower concentration of phosphate was adequate to provide a significant amount of label in the fractions of rRNA resolved on acrylamide gels.

Finally, in order to completely label the gametophyte RNA synthesized under red or blue light (for base composition analysis; cf. Chapter V) sterilized spores were sown in previously autoclaved culture medium containing 10uc/ml $^{32}PO_4^{-3}$. The spores germinated as usual under red light, and, after a 6 da period of growth, showed the usual filamentous morphology in red light and prothallial morphology in blue light. Acrylamide gel electrophoresis of the phenol-extracted RNA revealed incorporation into all classes of RNA.

The incorporation of radioactive precursors has been a powerful tool in biochemical research, but a prime concern in the use of this technique must be the effect of the radiation on the normal growth and physiology of the organism in question. In the present experiments,

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Figure 20. Gel electrophoresis of $^{32}P$-orthophasphate-labelled ribosomal-RNA of Dryopteris filix-mas gametophytes.

Shown is the absorbance at 260nm along the length of the gel (continuous line) and the $^{32}P$ net cpm for each 1mm fraction (triangles). Phosphorus-32 was supplied at an activity of 1.25mc/ml in phosphate-free medium. Gametophytes had grown for 17 da in red light prior to this experiment. Arrows indicate the position of putative cytoplasmic rRNA precursor molecules. For ease of graphical interpretation each $^{32}P$ cpm scale (right vertical axis) was expanded to achieve a constant ratio to its corresponding absorbance scale (left vertical axis).

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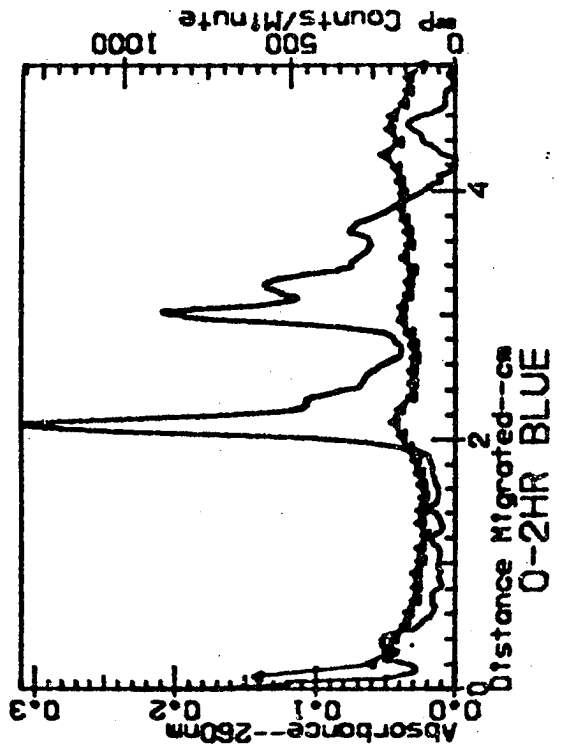


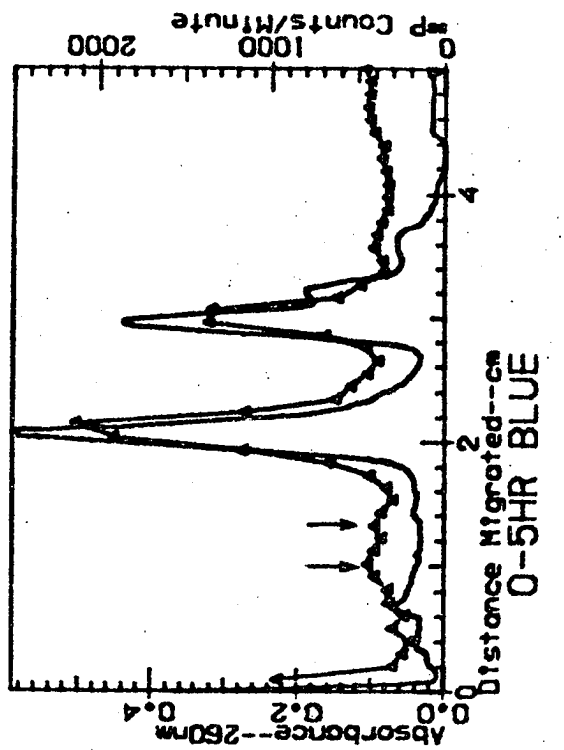


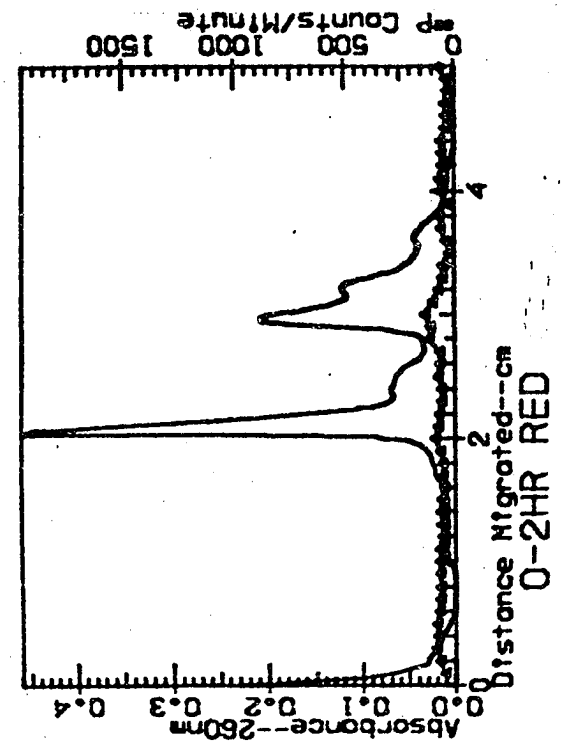


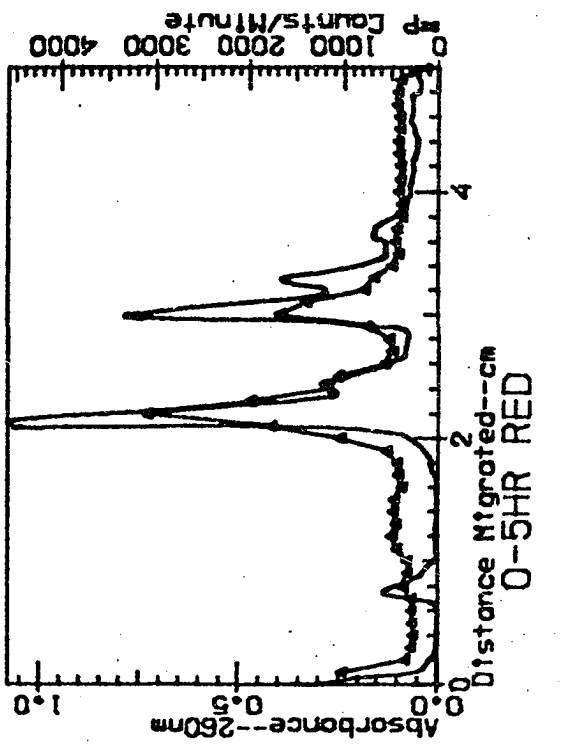


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the gametophytes are able to complete the entire sequence of development (eg. germination, filamentous growth, prothallial growth) in the presence of $^{32}P$ at 10uc/ml; and can complete the normal photomorphogenetic response after a 5 hr exposure to $^{32}P$ at 200uc/ml. On this basis it is assumed that radiation damage is inconsequential with regard to the morphogenesis of these gametophytes.

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# V. RIBOSOMAL-RNA METABOLISM DURING PHOTOMORPHOGENESIS

## Introduction

Hotta reported in 1960 that the transition from filamentous to prothallial growth in the gametophyte of the fern Dryopteris erythrosora is correlated with an increased protein content (per unit dry weight) and a shift in the total-RNA base composition to a higher percent guanine + cytosine level. A detectable change in total-RNA base composition would be interesting in that ribosomal-RNA generally comprises the overwhelming majority of total cellular RNA. Even though new "messenger-RNAs" might be synthesized, they would not be expected to be present in sufficient quantity to affect the overall RNA base composition. Such a change is suggestive of a shift in the ribosome population; an idea which has become generally acceptable only in light of more recent findings regarding the existence of ribosomes in cytoplasmic organelles. In addition, it is now known that ribosomes are heterogeneous with respect to their constituent proteins (Kurland, 1970) perhaps reflecting functional heterogeneity within physically indistinguishable classes of ribosomes.

Studying cytological changes which occur during the light-controlled morphogenesis in D. filix-mas, Mohr (1956b) found that the size of the chloroplasts adjusts to a specific value depending upon the quality of incident light. A closer analysis (Bergfeld, 1963) has shown that in red light the mean diameter is 4.8u, increasing to 7.8u upon exposure to blue light. Bergfeld (1967) has also reported that, after transfer to blue light, nucleolar volume was reduced by almost 70%, to about $200u^3$. The nucleoli were observed to increase to their former size of nearly $650u^3$ when the gametophytes were returned to red light or darkness.

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Recent studies have demonstrated the significance of nucleolar size as a manifestation of ribosomal-RNA synthesis. Brown (1966) and other workers have found that the reappearance of a visible nucleolus is correlated with the resumption of rRNA synthesis in developing animal embryos. Ohlenroth and Mohr (1963), using gametophytes of D. filix-mas, confirmed Hotta's observation regarding the increased protein content in prothallial gametophytes. Later, Mohr's group (Drumm and Mohr, 1967a) reported that this increase occurs only after the morphogenesis is detectable; although recent work from the same laboratory (Payer et al., 1969) indicates a more rapid effect. Drumm and Mohr (1967a) also reported an increased rate of RNA synthesis in blue light. This increased RNA synthesis occurs at least as fast as the transition from filamentous to prothallial growth as evidenced by the tip swelling reaction. Drumm and Mohr (1967a) inferred that the bulk of the increased RNA synthesis might be localized in the chloroplasts, and Mohr (1965) suggested that it may be chloroplast ribosomal-RNA. Although these workers state that the blue light-specific morphogenesis seems to be operating via the induction of a specific enzyme and that the blue light stimulation of RNA synthesis is indicative of this induction, it seems unlikely that a large change in the synthetic rate and overall level of RNA could be due to synthesis of new "messenger RNAs". Experiments were undertaken to evaluate the reported change in total-RNA base composition, and to identify the molecular species of RNA which are involved in this change. Finally, the kinetics of this change were related to the kinetics of the morphological transition which these gametophytes undergo.

Materials and Methods

The culture of gametophytes is deacribed in Chapter II. Procedures

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for RNA labelling with $^{32}P$-orthophosphate, RNA extraction, acrylamide gel electrophoresis and base composition analysis are discussed in Chapter IV.

For electron microscopy, samples were fixed overnight at room temperature in 6% glutaraldehyde in 0.2M sodium cacodylate, pH 6.8. After thorough rinsing, the tissue was post-fixed in 2% unbuffered $OsO_4$ for 1 hr; followed by dehydration in acetone and embedding in epoxy (hard mixture--Spurr, 1969). Sections were cut on a Reichert ultramicrotome; stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963); and examined in a Hitachi-7S electron microscope.

Results

Change in total RNA base composition--The total-RNA base compositions of sterile cultures of Dryopteris erythrosora were determined. Table V lists the data from phenol-extracted RNAs of gametophytes grown for 2 weeks after germination in either red or blue light. Acrylamide gel electrophoresis fractionation verified the presence of all major RNA species in these preparations. The base composition results essentially support Hotta's (1960) original observations on unsterilized cultures of D. erythrosora, in that the prothallial morphology is correlated with a shift in total-RNA base composition to a higher % G+C.

Steady-state amounts of rRNA--In order to evaluate the relative amounts of cytoplasmic and chloroplast rRNA, gametophytes were extracted by the diethyl pyrocarbonate method after growth in either red or blue light. All four major rRNA components were detected in every sample, except those from Dryopteris spores where the amount of

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Table V. Total-RNA base composition in filamentous (red light) and prothallial (blue light) gametophytes of Dryopteris erythrosora

| | %U | %G | %A | %C | %G+C |
|---|---|---|---|---|---|
| filamentous | 23.2 ± 0.1[a] | 26.6 ± 0.3 | 25.9 ± 0.2 | 24.3 ± 0.2 | 50.9 ± 0.3 |
| prothallial | 20.8 ± 0.2 | 29.8 ± 0.2 | 25.0 ± 0.3 | 24.4 ± 0.2 | 54.2 ± 0.3 |
| P | 0.001[b] | 0.001 | 0.02 - 0.05 | 0.6 - 0.7 | |

[a]Mean percent is shown with standard error (n=4).

[b]Probability, from Student's t-test, that observed differences between filamentous and prothallial RNA base composition is due to chance alone.

chloroplast rRNA was much reduced. Table VI shows that the percent chloroplast rRNA relative to the total rRNA changes significantly from filamentous to prothallial gametophytes of Dryopteris with the chloroplast rRNA increasing from 25% in red light to about 45% in blue. Osmunda gametophytes do not demonstrate a lower level of chloroplast rRNA in red light, and Fig. 5, Chapter II shows that Osmunda gametophytes do not grow as filaments in red light.

Also shown in Table VI are the relative chloroplast rRNA values for the spores of Dryopteris (8%) and Osmunda (39%), as well as the values for their sporophyte leaf tissues (82%). The exceptionally high value found in the sporophyte leaf tissue is supported by electron microscopic evidence (Fig. 21). The concentration of chloroplast ribosomes is obviously much higher in the sporophyte tissue than in the gametophytes. In addition, numerous polyribosomes (arrows, Fig. 21) are apparent in the sporophyte chloroplasts.

Kinetics of rRNA synthesis--Data presented and discussed in Chapter III indicate that morphogenesis is detectable within 2-3 hr after transfer from red to blue light. These kinetic data are consistent with similar data obtained for this species (Drumm and Mohr, 1967a; Payer and Mohr, 1969). If the correlation of an increase in the level of chloroplast rRNA is somehow related to the initiation of the morphogenesis, then increased chloroplast rRNA synthesis should be detectable by radioactive labelling within several hours after the start of illumination with blue light. The specific radioactivities of the chloroplast and cytoplasmic rRNA components, 5 hr pulse labelled, were determined after gel electrophoresis (Fig. 22), and the results are presented in Fig. 23, with the change in the ratio of these values at

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Table VI. Relative amounts of chloroplast rRNA[a]

| | | light | CHL rRNA / Total rRNA | n |
|---|---|---|---|---|
| Dryopteris filix-mas | spores (brown) | ------ | 8.1 ± 4.5% | 2 |
| | filaments | red | 25.0 ± 2.9% | 8 |
| | prothalli | blue | 44.1 ± 7.1% | 9 |
| | sporophyte leaf | daylight | 82.3% | 1 |
| Osmunda cinnamomea | spores (green) | ------ | 38.6 ± 0.3% | 2 |
| | prothalli | red | 58.6 ± 13.2% | 3 |
| | prothalli | blue | 48.3 ± 2.5% | 2 |
| | sporophyte leaf | daylight | 82.4 ± 7.8% | 2 |

[a]Mean percent is shown with standard deviation.

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Figure 21. Chloroplasts from gametophyte (a) and sporophyte (b) tissues of Dryopteris filix-mas.

Arrows in (b) indicate polysome profiles. Magnification is approximately 25,000x.

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Figure 22. Gel electrophoresis of ribosomal-RNA of Dryopteris filix-mas labelled with $^{32}P$-orthophosphate during photomorphogenesis.

Shown is the absorbance at 260nm along the length of the gel (continuous line) and the $^{32}P$ net cpm for each 0.5mm fraction (triangles). Phosphorus-32 was supplied at an activity of 200uc/ml in standard medium. Gametophytes had grown for 10 da in red light (a-d) or 3 da in red + 1 wk in blue light (e) prior to this experiment. For ease of graphical interpretation each $^{32}P$ cpm scale was expanded to achieve the same ratio to its corresponding absorbance scale.

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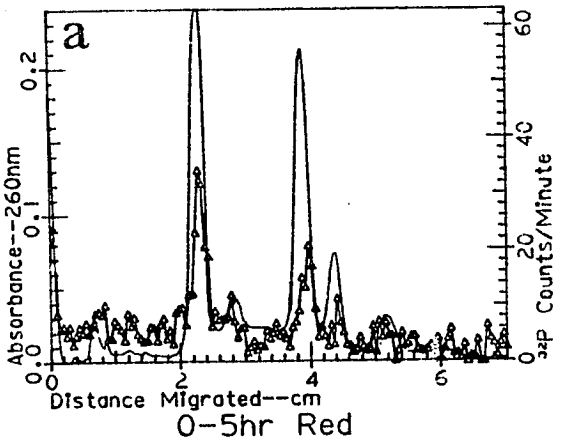
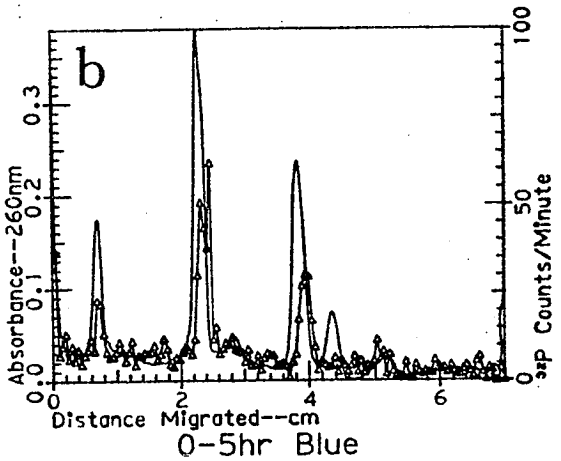
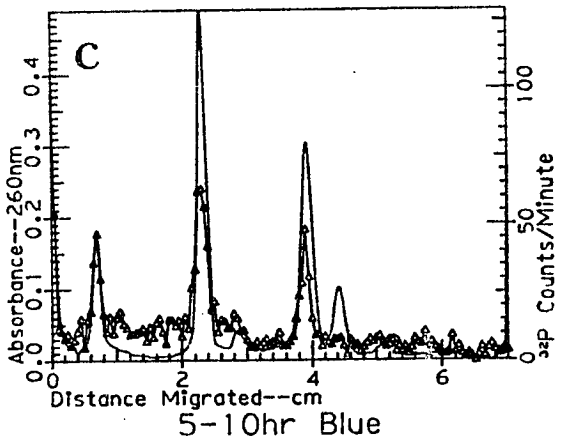


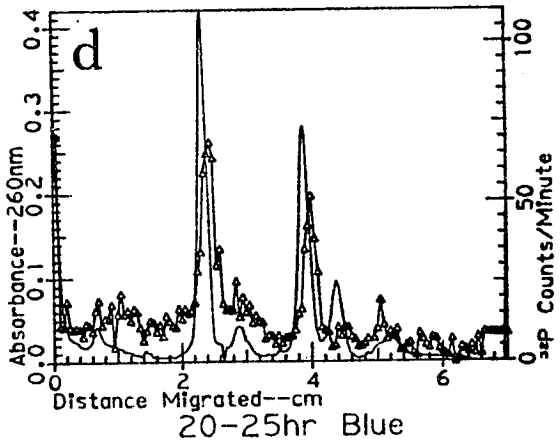

0-5hr Red



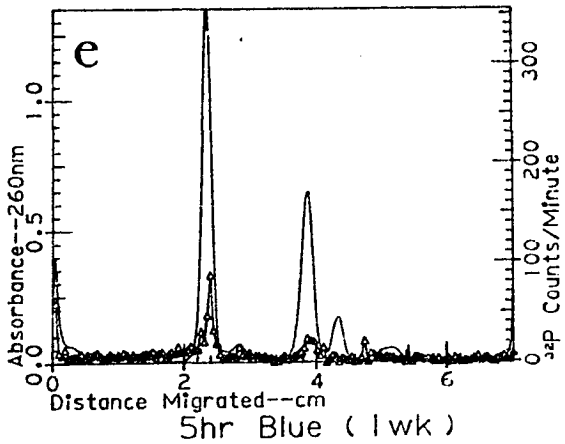

0-5hr Blue




5-10hr Blue




20-25hr Blue




5hr Blue ( 1wk )

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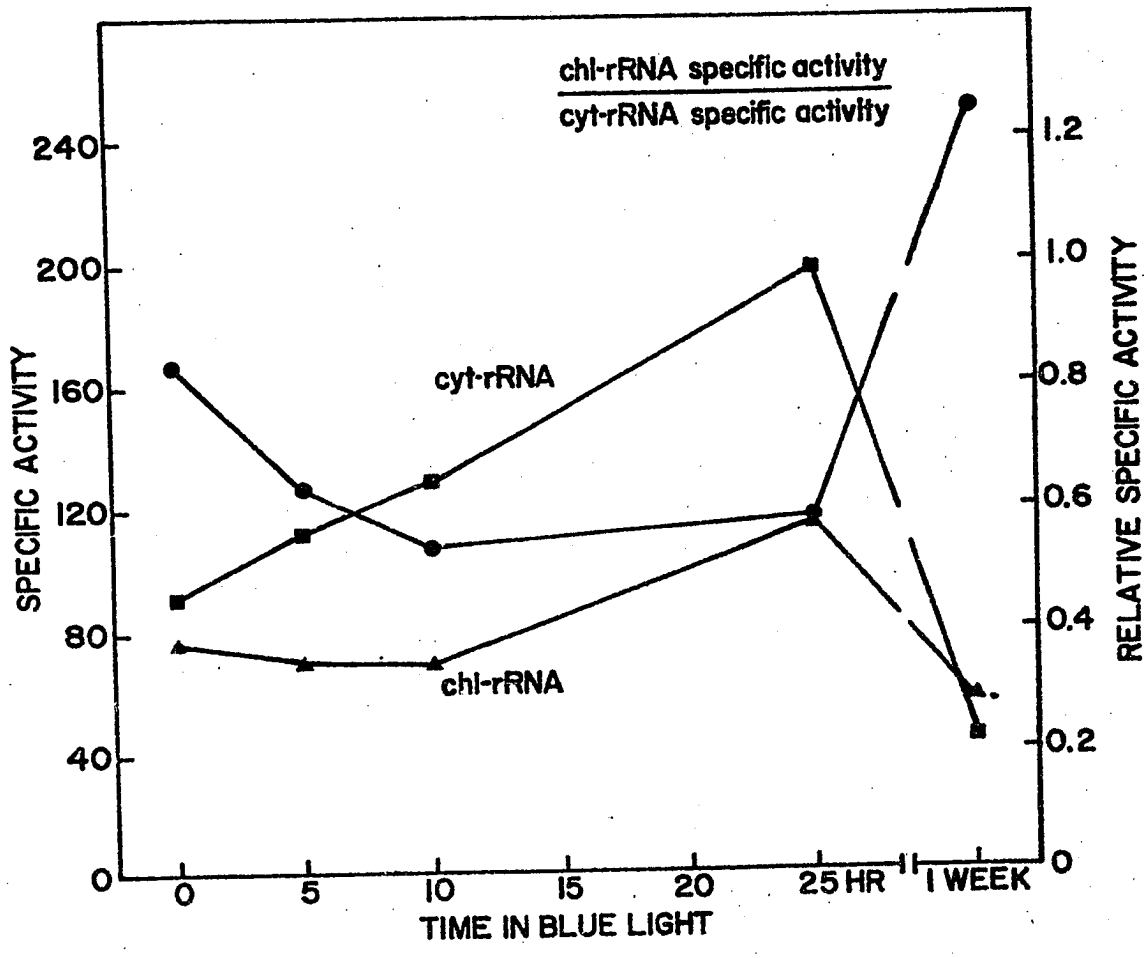


Figure 23. Chloroplast and cytoplasmic ribosomal-RNA synthesis in Dryopteris filix-mas during photomorphogenesis.

Data from gel electrophoretic analysis (Fig. 22). Chloroplast rRNA specific activity ($^{32}P$ net cpm/ug)(triangles) and cytoplasmic rRNA specific activity (squares) are presented. Also shown is the relative rate of synthesis ($\frac{\text{chl-rRNA}}{\text{cyt-rRNA}}$)(circles).

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various times after transfer to blue light. The data in Table VII reflect the fact that much of the chloroplast heavy rRNA ($1.1 \times 10^6$ daltons) had broken down (60-80%), but that about 85% of the newly synthesized ($^{32}$P-labelled) chloroplast rRNA remained intact. A similar difference in stability between newly synthesized and "bulk" chloroplast rRNA was reported by Ingle (1968). Correction formulae (Ingle, 1968) applied to the data generally resulted in values near the expected 2:1 ratios for the heavy:light components of the cytoplasmic and chloroplast rRNAs, indicating that the breakdown of the chloroplast heavy rRNA can be accounted for and corrections made in terms of Ingle's (1968) analysis of chloroplast rRNA breakdown. The data in Fig. 23 show that the relative rate of chloroplast rRNA synthesis is not immediately stimulated by transfer from red to blue light. The ratio decreases at first, due to an accelerated rate of cytoplasmic rRNA synthesis. This initial response is followed by a gradual increase in chloroplast rRNA synthesis beginning about 15-20 hr after transfer to blue light.

In 5-hr pulse labelled Dryopteris prothallial gametophytes (grown for 1 wk in blue light) the relative specific activity of chloroplast rRNA is almost 50% higher than that for filamentous gametophytes labelled in red light (Fig. 23), indicating that the shift to the higher proportion of chloroplast rRNA in prothallial gametophytes takes place over a much longer time than the morphological transition. The lower rRNA specific activities observed in the prothallial gametophytes is apparently due to a reduced rate of RNA synthesis in the non-meristematic cells.

## Discussion

A difference in total-RNA base composition which is associated with

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Table VII. Ratios of rRNAs from Dryopteris filix-mas gametophytes pulsed with $^{32}P$ for 5 hr periods.

| | Cytoplasmic rRNA (1.3/0.7) | | | | Chloroplast rRNA (1.1/0.56) | | | | % Breakdown of 1.1 rRNA | |
|---|---|---|---|---|---|---|---|---|---|---|
| | RNA-ug[a] | | RNA-cpm[b] | | RNA-ug[a] | | RNA-cpm[b] | | RNA | RNA |
| | Observed | Corrected[c] | Observed | Corrected[c] | Observed | Corrected[c] | Observed | Corrected[c] | ug | cpm |
| 0-5hr Red | 1.10 | 1.86 | 1.76 | 3.00 | 0.76 | 2.54 | 1.20 | 2.21 | 70 | 40 |
| 0-5hr Blue | 1.51 | 2.13 | 2.00 | ---- | 0.98 | 2.44 | 1.88 | ---- | 59 | 0 |
| 0-10hr Blue | 1.25 | 1.96 | 1.79 | 2.22 | 0.42 | 2.00 | 1.58 | 2.54 | 79 | 38 |
| 20-25hr Blue | 1.23 | 1.92 | 1.64 | ---- | 0.53 | 2.00 | 2.08 | ---- | 74 | 0 |
| 1wk + 5hr Blue | 1.73 | 2.15 | 1.95 | ---- | 0.52 | 1.99 | 2.01 | ---- | 74 | 0 |

[a]Data from area measurements on gel scans.

[b]Data from radioactivity determinations on fractionated gels.

[c]Corrections made according to Ingle (1968).

the filamentous or prothallial growth of Dryopteris gametophytes (Table V and Hotta, 1960), and observations on size changes of nucleoli and chloroplasts (Bergfeld, 1962, 1967) strongly suggested a shift in the relative amounts of cytoplasmic and chloroplast rRNA present in the two growth phases. The magnitude of the net difference in G+C content between the filamentous and prothallial Dryopteris gametophytes can be evaluated as follows: In the blue light-grown prothallial gametophytes there is an additional RNA component present (quantitatively or qualitatively distinct). The G+C content of this hypothetical "blue-induced RNA" must relate to the fraction of the total RNA which it comprises such that the %G+C of the total RNA is elevated by 3.3%. Figure 24 illustrates the range of possible combinations which could result in the observed change in base composition. If one assumes that the %G+C for chloroplast rRNA is near 60, the change in base composition could be entirely accounted for by an increase of some 20-25% in the relative amount of chloroplast rRNA.

This inference has been verified by direct measurements of the relative amounts of cytoplasmic and chloroplast rRNA in 1-dimensional and 2-dimensional gametophytes (Table VI). In filamentous gametophytes of Dryopteris the relative amount of chloroplast rRNA is 25%, whereas it is about 45% in the prothallial gametophytes. The magnitude of this difference is sufficient to account for the observed shift in total-RNA base composition. In contrast to the situation in Dryopteris, there is no indication of a lower level of chloroplast rRNA in Osmunda gametophytes grown in red light. Osmunda gametophytes failing to grow as filaments in red light gave additional support to the possibility of a meaningful correlation between the fluctuations in rRNA levels and

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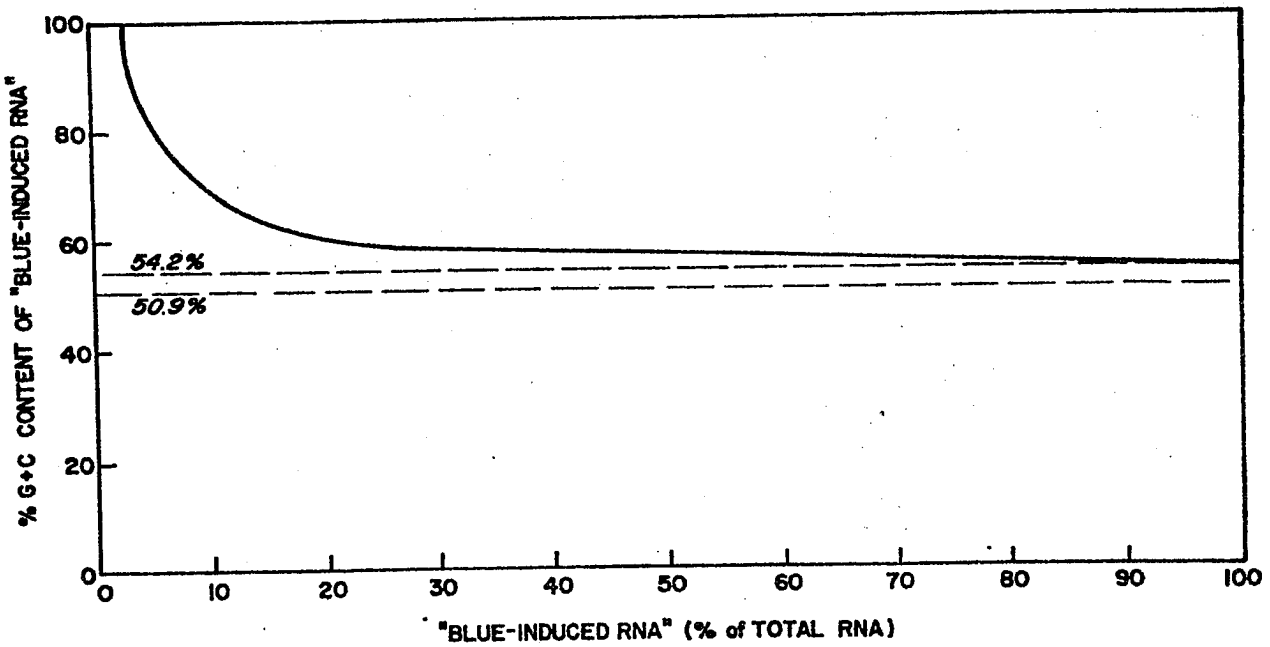


Figure 24. Theoretical %G+C content of "blue-induced RNA".

Theoretical curve based on the measured difference in base composition between filamentous and prothallial gametophytes (Table V).

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the morphogenetic transition. The findings of yet higher relative levels of chloroplast rRNA in the leaf tissue of both Dryopteris and Osmunda sporophytes (Table VI; Fig. 21) contributed further to the correlation between differences in rRNA levels and morphology.

Raghavan (1968) has reported that a chloroplast-rich fraction (and presumably chloroplast ribosomes) are labelled much sooner and much more extensively than cytoplasmic ribosomes when gametophytes of Pteridium aquilinum are transferred from red to blue light. His interpretation of the data (that blue light enhances RNA synthesis in the various particulate fractions of Pteridium gametophytes) ignores the difference in $^{3}$H-uridine uptake between red and blue light treatments (Table 2 in Raghavan, 1968). Recalculating from Raghavan's data as ($^{3}$H-uridine in RNA)/($^{3}$H-uridine uptake) indicates the reverse: in every fraction except the "ribosome-rich" fraction blue light treatment has resulted in a lower rate of RNA synthesis. Interpretation of the results is further complicated by the degree of cross-contamination between the subcellular fractions, and by the difficulty of labelling the RNA-precursor pools with $^{3}$H-uridine in these organisms (cf. Chapter IV). These complications have been overcome in the present study by utilizing 1) gel electrophoresis to resolve the chloroplast and cytoplasmic rRNAs, and 2) $^{32}$P-orthophosphate in 5 hr pulses to allow adequate labelling of the RNA precursor pools.

If the observed increased synthesis of Dryopteris chloroplast rRNA were a primary event in the photomorphogenetic transition it should occur at least as rapidly as the swelling of the gametophyte filament tip. Pulse labelling shows that no increased synthesis of chloroplast rRNA is detectable during the early part of the 1- to 2-dimensional

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transition, although such an increase is detected when the labelling is carried out at a somewhat later time (Fig. 23). This is consistent with the observation that chloramphenicol, presumed to have its primary effect on protein synthesis in the chloroplasts, did not prevent the photomorphogenetic transition (Bergfeld, 1968). However, a stimulation of cytoplasmic rRNA synthesis is detected soon after transfer from red to blue light. The rate of cytoplasmic rRNA synthesis continues to increase for at least the first day. These data are consistent with the observations of Bergfeld (1967) on nucleolar size changes: He found that the reduction in nucleolar size that occurs in blue light was preceded by a transient increase in nucleolar size. The transient stimulation of cytoplasmic rRNA synthesis may be a reflection of the induced synthesis in the cytoplasm of the "morphogenetic enzyme" postulated by Mohr's group (cf. Payer et al., 1969).

Burns and Ingle (1970) have recently reported that there is no increase in the relative amount of chloroplast rRNA during photomorphogenesis in unsterilized cultures of Dryopteris borreri, a result consistent with the present findings. But these workers found that the chloroplast rRNA level did not change even in gametophytes more than a week after transfer from red to blue light. The difference between their results and mine are likely due to the morphological and biochemical differences between D. borreri and D. filix-mas which these workers have reported. These differences can be summerized as follows:

a) In Dryopteris borreri the photomorphogenetic response is first evidenced by a lateral cell division, two cells removed from the apex of the gametophyte filament. This reaction is observed after 200 hr (Burns and Ingle, 1970). In the present

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species (D. filix-mas) the photomorphogenetic response (tip swelling) is detectable within 2-3 hr (Chapter III).

b) Ribosomal-RNA cannot be efficiently phenol-extracted from D. borreri gametophytes unless chloroform is included in the procedure, although Ingle and Burns (1968) found that this is not a requirement with D. filix-mas gametophytes.

c) D. borreri is an obligately apogamous species, whereas both D. filix-mas and D. erythrosora are sexually reproducing (Manton, 1950, p. 55).

Since a change in the total-RNA base composition has been shown for D. erythrosora (Table V and Hotta, 1960); and since a decrease in nucleolar size (Bergfeld, 1967) and an increase in chloroplast size (Bergfeld, 1962) have been shown to accompany the transition from filamentous to prothallial growth in D. filix-mas, it would be interesting to have such data from D. borreri.

The present study shows that an increased level of chloroplast rRNA is correlated with prothallial morphology in gametophytes of Dryopteris filix-mas. However, the increase in chloroplast rRNA does not occur as rapidly as the swelling of apical cell; and, therefore, this increase in rRNA cannot be a primary event in the transition to 2-dimensional growth. However, since specific changes in ribosomal-RNA levels are clearly correlated with the different morphological stages (spore, protonemal filament, prothallus, sporophyte leaf) it is possible that rRNA differences reflect critical physiological differences regulating growth of this fern. These experiments do answer the questions raised by Hotta's early observation on total-RNA base composition changes, and also provide a molecular correlate (i.e. rRNA synthesis) for the

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cytological observations of Bergfeld on the changes in chloroplast and nucleolar size.

Even though attempts to detect the blue-light induced synthesis of "messenger-type" RNA have yielded negative results, one cannot rule this out as a possible mechanism. The stimulation of cytoplasmic rRNA synthesis may be a reflection of specific activation of genes which are controlling the morphogenetic transition. But rather than pursue this line of investigation further (with its likelihood of continued negative results and severe technical limitations) I began to explore several other possible mechanisms which might be responsible for triggering the photomorphogenetic response. These are discussed in the following chapter.

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# VI. EXPLORATORY EXPERIMENTS

## Introduction

As discussed in the previous chapter, it has not as yet been possible to detect a specific effect on RNA synthesis which could be associated with the initial morphogenetic response. The differential stimulation of cytoplasmic rRNAs might be a reflection of the synthesis of a developmentally distinct class of cytoplasmic ribosomes, in the sense of evidence (Kurland, 1970) for heterogeneous populations of ribosomes; or possibly the induction of specific m-RNAs whose synthesis is coordinated with rRNA synthesis. But such suggestions are so speculative in the absence of any supporting evidence from the present system that they will not be pursued further.

Ultraviolet irradiation--Ultraviolet light (UV) has been used in a number of developing systems to inactivate unidentified "morphogenetic substances" which apparently are required for normal differentiation (Rana pipiens germ plasm, Smith, 1966; cap formation in Acetabularia, Hammerling, 1956; semi-cell regeneration in Micrasterias, Kallio, 1959; differentiation of gonidia in Volvox, Kochert and Yates, 1970). If a similar type of UV-labile "morphogenetic substance" is responsible for the filamentous-to-prothallial transition in Dryopteris gametophytes, UV treatment should block the tip swelling response; and if the UV effect is specific for a "morphogenetic substance" there should be no reduction in the rate of cell division (a parameter which would indicate any gross lethal effect of the UV exposure).

Cytoplasmic Microtubules--Dill (1969), in a preliminary electron microscopic examination of the response to blue light in Pteridium aquilinum gametophytes, observed that axially oriented cortical micro-

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tubules near the growing tip (cf. review by Newcomb, 1969) present in red light grown filaments were missing after a 2 or 4 hr exposure to blue light. Dill provided support for the idea that oriented cortical microtubules might be involved in directing filamentous growth in red light. By adding colchicine to cultures of gametophytes in red light Dill was able to induce tip swelling (also Nakazawa, 1959, 1968), the primary photomorphogenetic response in this system. Colchicine has long been recognized as a reagent which can disrupt the integrity of various types of microtubules (eg. flagellar, spindle, cortical, axopodial). From these experiments it seems that there is a positive correlation between the disappearance (disruption) of cortical microtubules and the onset of the photomorphogenetic transition.

Several other treatments known to affect microtubules were tested with regard to their effect on the tip swelling response in the absence of blue light.

Ethylene--In some plant systems ethylene can cause reduced cell elongation and isodiametric swelling (Burg and Burg, 1968). That this swelling involves the expected loss of microfibrillar orientation was also demonstrated. Considering that the correlation between oriented microfibrils and oriented microtubules is without known exception (Newcomb, 1969), it is suggested here that ethylene may be exerting an effect on cortical microtubules. In addition, ethylene can inhibit cell elongation (cf. Pratt and Goeschl, 1969)(but recall that gametophyte filaments elongate by tip growth). Ethylene has also been shown to exert an effect on nucleolar size in Phaseolus petiole explants (and presumably rRNA synthesis)(Webster, 1968). With regard to the morphogenetic transition in Dryopteris filix-mas gametophytes: Can exogenously

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applied ethylene replace the requirement for blue light? Several experiments were conducted to evaluate a possible role for ethylene in the photomorphogenetic response presently under consideration.

Materials and Methods

Gametophytes were cultured as described in Chapter II. Although cultures were initially sterile, no rigorous attempt was made to maintain this condition over the period of the present experiments (less than 2 da ). The tip-swelling assay procedure was discussed in Chapter III (second method).

Colchicine (U.S.P. grade; Nutritional Biochemicals Crop.), vinblastine sulfate ("Velban"; Eli Lilly & Co.), and water-$D_2$ (99.7% D; New England Nuclear Corp.) were used. Ethylene (98%) was purchased from Fisher Chemicals. "Ethrel" (2-chloroethylphosphonic acid) was the product of Amchem Products, Inc. Ultraviolet light was obtained from a Westinghouse "Sterilamp" (782L-30) at a distance of 40cm. Gametophytes in a shallow layer of medium were exposed directly to the UV source for periods of 2-64 min.

Results and Discussion

Ultraviolet light--After UV exposure, gametophyte samples were placed in blue light for an additional 48 hr growth. In the untreated gametophytes about 80% exhibited the tip swelling response, and 3/4 of these had undergone at least one cell division in this region (Fig. 25). With increasing UV dosage there is a reduction in the number of gametophytes which exhibit the tip swelling phenomenon. But there is also a concomitant inhibition of cell division, so that the proportion of gametophytes with both swollen tips and no additional cell divisions actually shows a modest increase with increasing UV dose. From these

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Figure 25. Ultraviolet dose curve for tip swelling and cell division in Dryopteris filix-mas.

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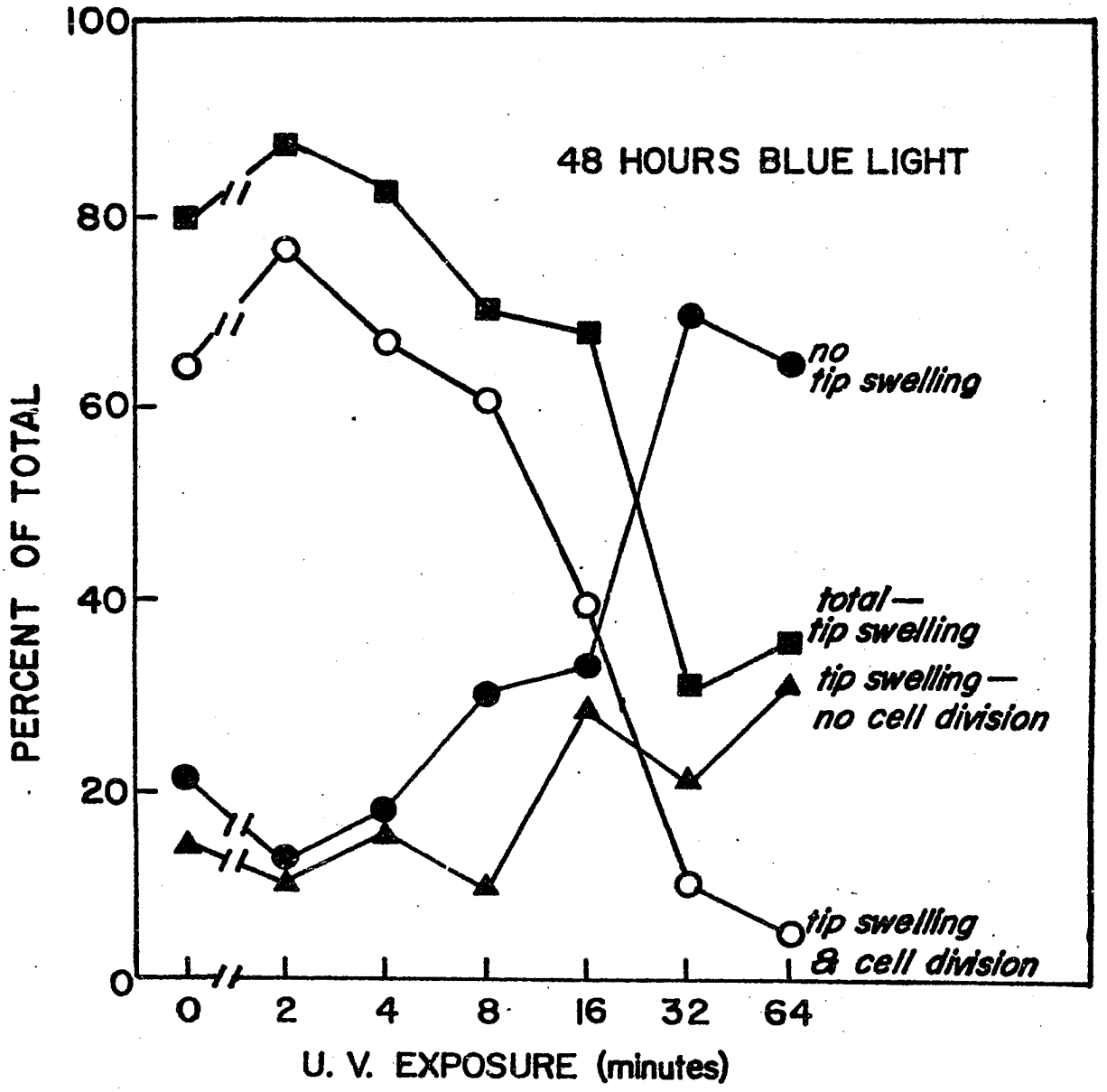


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data it appears that photomorphogenesis in fern gametophytes is not mediated through a UV-labile morphogenetic substance as has been reported for other differentiating systems. The inhibition of tip swelling at high UV doses is probably a non-specific effect, similar to that observed in fern gametophytes exposed to high levels of antimetabolites (Miller, 1968b; Burns and Ingle, 1968; Dill, 1969).

Microtubules--The effect of colchicine on inducing morphogenetic tip swelling (Fig. 26; also Dill, 1969) argues in favor of the involvement of cytoplasmic microtubules in the maintenance of filamentous growth in these organisms. Experiments with vinblastine (Fig. 27) and $D_2O$ (Fig. 28) provide two additional and separate lines of evidence in support of this view. Each of these treatments has been shown to have a detrimental effect on the normal metabolism of microtubules in other systems (colchicine, Inoue and Sato, 1967; vinblastine, cf. Huebner and Anderson, 1970; $D_2O$, Inoue and Sato, 1967).

Note that with colchicine and $D_2O$ the maximal tip swelling response was induced in red light at a concentration less than that which caused a reduced response in blue light (Fig. 26 and 28). With vinblastine all concentrations tested had an inhibitory effect on the normal blue-light response; but at 0.03% vinblastine the cultures which remained in red light had achieved nearly the same level of response as its counterpart in blue light. Vinblastine is known to inhibit RNA and protein synthesis (Creasey, 1968), as well as causing disruption of microtubules. The results obtained with vinblastine in blue light are most probably due again to non-specific effects (Miller, 1968b; Burns and Ingle, 1968; Dill, 1969), especially since all of the gametophytes appeared very unhealthy (i.e. plastids pale and rounded up) in the highest concentra-

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Figure 26. Colchicine dose curve for tip swelling in Dryopteris filix-mas.

Figure 27. Vinblastine dose curve for tip swelling in Dryoptiers filix-mas.

Figure 28. Heavy water ($D_2O$) dose curve for tip swelling in Dryopteris filix-mas.

Experiments were terminated after 32 hr.

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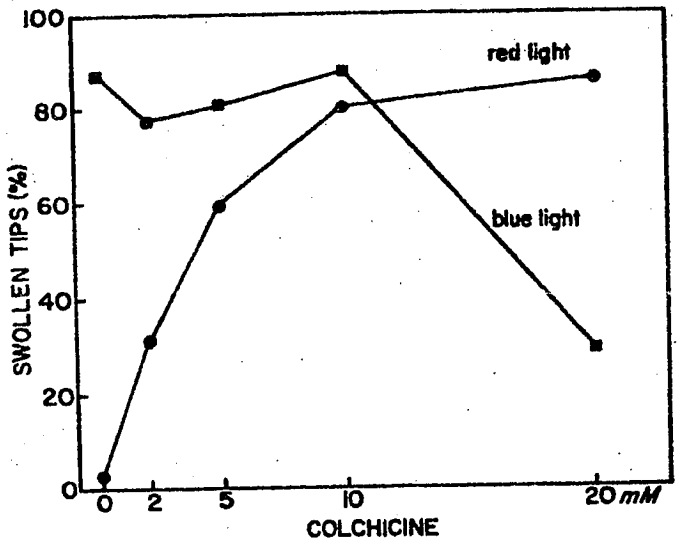


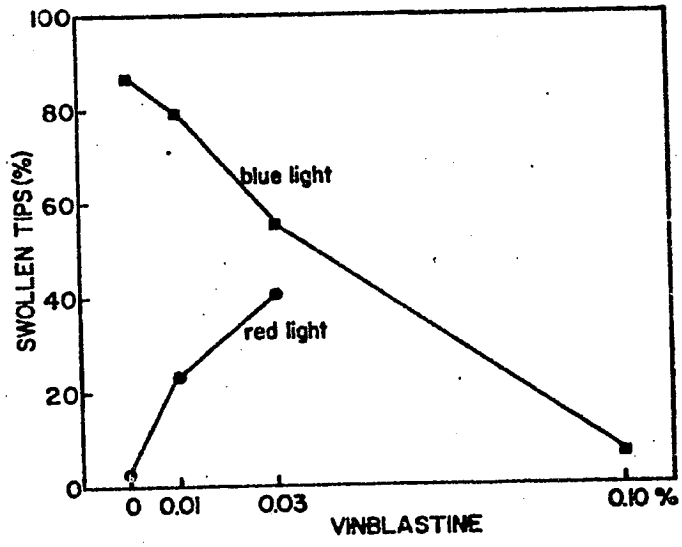


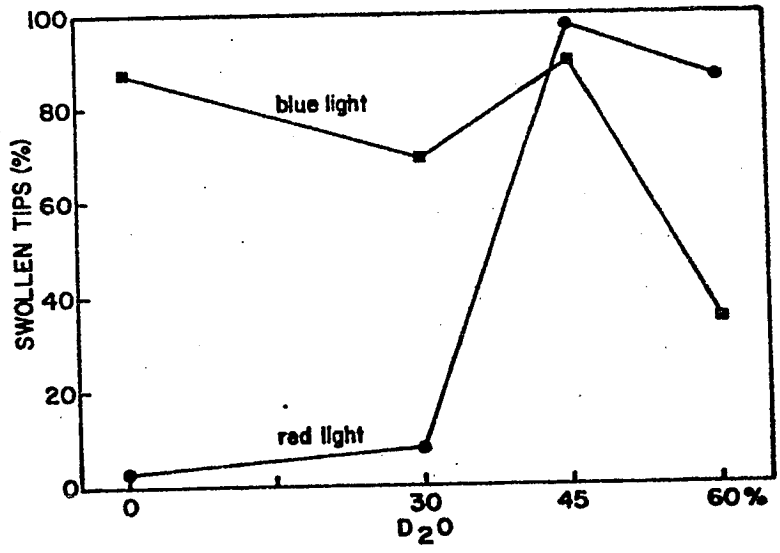


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tion tested.

Based on these experiments with colchicine, vinblastine, and $D_2O$ it may be concluded that cytoplasmic microtubules play an important role in the maintenance of filamentous growth. These treatments which affect microtubules in different ways can each induce the morphogenetic reaction without need of exposing the gametophytes to blue light. The additional evidence from electron microscopy (Dill, 1969), though only preliminary, makes the argument even more compelling.

Since treatments which disrupt microtubules (colchicine: Green, 1969; Pickett-Heaps, 1967; vinblastine: cf. Huebner and Anderson, 1970; have the same effect as one which stabilizes microtubules ($D_2O$, Inoue and Sato, 1967; Gross and Spindel, 1960) the observed effect of these treatments is probably one of interference with normal microtubule metabolism. Further, one could speculate that the tip swelling response is under "negative control". That is, the gametophyte appears to exert active control in maintaining filamentous growth; while the transition to prothallial growth can be achieved by interference with (the present experiments) or by relaxation of control over (in the normal blue light effect) cytoplasmic microtubules. It should be noted that the presumed interference with microtubule metabolism is a specific effect on the morphogenesis of these organisms, not observed with other metabolic inhibitors (eg. actinomycin-D: Mohr, 1965; cycloheximide: Dill, 1969).

Ethylene--Experiments with ethylene were based on the observations in other plant systems that ethylene treatment results in the cessation of elongation and the initiation of isodiamentric swelling. The data discussed in Chapter III show that the photomorphogenetic transition from filamentous to prothallial growth in gametophytes of Dryopteris

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filix-mas is first detected as a cessation of filament elongation and the initiation of isodiametric swelling in the apical cell of the gametophyte. Incubation of Dryopteris filix-mas gametophytes with a range of ethylene concentration under red or blue light or in darkness has yielded inconclusive results regarding the possible involvement of ethylene in the tip swelling reaction. Table VIII lists the results of one such experiment. Mercuric perchlorate solutions were used to quanitatively trap atmospheric ethylene (Burg, 1962). After 40 hr incubation with a mercuric perchlorate solution there is no significant difference in the proportion of gametophytes having swollen tips in blue light (76% vs. 73% with $Hg(ClO_4)_2$), while there appears to have been an enhancement of the tip swelling response in red light (19% vs. 39%) and in darkness (9% vs. 21%). This result is in direct contradiction with the hypothesis put forth above. However, added ethylene cannot elicit the tip swelling response in red light or in darkness, as might have been suspected from the results with mercuric perchlorate. Adding to the complication is the fact that none of the treatments gave a complete response (re. the controls). It is not clear why these experiments have not yielded conclusive results, but a possible experimental difficulty may be incomplete absorption of ethylene by the mercuric perchlorate solution. The use of the ethylene releasing chemical "Ethrel" (cf. Pratt and Goeschl, 1969) reduced the tip swelling response in blue light, although again the tip swelling response of the ethrel-treated cultures in red light is elevated above that of the red light control. Recent studies with gametophytes of Onoclea (P. Miller et al., 1970; Miller and Miller, 1970) have pointed to a natural role for ethylene in maintaining filamentous growth (the reverse of the situation predicted on the basis

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Table VIII. The effect of ethylene and Ethrel on the tip swelling response.

| | PERCENT SWOLLEN TIPS | | |
|---|---|---|---|
| TREATMENT (40hr) | RED | BLUE | DARK |
| control (unsealed) | 19% | 76% | 9% |
| $Hg(ClO_4)_2$* | 39% | 73% | 21% |
| 0.01 ppm Ethylene | 25% | 82% | 18% |
| 0.1 ppm " | 31% | 80% | 23% |
| 1.0 ppm " | 39% | 78% | 38% |
| 10. ppm " | 27% | 88% | 31% |
| 100. ppm " | 23% | | |
| 1000. ppm " | 27% | 70% | 16% |
| 0.1 ppm Ethrel | 38% | 20% | |
| 1.0 ppm " | 42% | 32% | 23% |
| 10. ppm " | 51% | | 28% |

*$Hg(ClO_4)_2$ solution (5% mercuric oxide in 2.5M perchloric acid) contained in an open tube and sealed inside flask with gametophytes. This is defined as 0 ppm ethylene.

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of ethylene effects in other plant systems). In assaying for the transition to prothallial growth the Millers have used the criterion of the formation of an oblique crosswall, while the present study was concentrated on the initial response of tip swelling. It may be that in Onoclea the transition to prothallial growth in the absence of ethylene results directly from a reduction in the rate of elongation, bypassing the tip-swelling stage.

Conclusions

The present experiments have not clearly demonstrated a role for ethylene in the morphogenesis of Dryopteris filix-mas gametophytes. Further, there is no specific effect of UV light on inhibition of morphogenesis, as has been reported in many other systems. However, experiments utilizing chemicals which specifically interfere with microtubule metabolism indicate that such treatments can lead to a tip swelling response in the absence of blue light. Based on the mode of action of these treatments on microtubules it is proposed that morphogenesis (i.e. tip swelling) is under "negative control", and that in the absence of blue light the gametophyte is actively directing filamentous growth through the agency of cytoplasmic microtubules.

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## VII. SUMMARY

The transition from filamentous to prothallial growth in gametophytes of the fern Dryopteris filix-mas is under photomorphogenetic control. Morphogenesis in this system is first detected as a swelling of the terminal portion of the apical cell. In the present study, time-lapse cinematography was used to analyze this response. Under red light, growth is strictly filamentous, proceeding exclusively by tip growth as in fungal hyphae. A weak point in the cell wall located at the apex of the filament is interpreted as the region of wall synthesis. As the filamentous gametophyte grows, wall hardening apparently takes place along the flanks of the hemispherical tip so that at a diameter of about 30u there is no further lateral expansion. When exposed to short wavelengths of light (i.e. blue light) the gametophyte first responds by producing a bulbous swelling at the growing tip. The beginning of this swelling is noted after 2-3 hr, and appears to result from a relaxation of the wall hardening process. Wall synthesized prior to blue light exposure does not expand, suggesting that wall softening is not involved in the tip swelling response. Experiments with agents known to disrupt cytoplasmic microtubules (colchicine, vinblastine) and to stabilize them (heavy water-$D_2O$) have shown that both types of specific interference with microtubule metabolism results in tip swelling (in the absence of blue light). These results indicate that this photomorphogenetic response is under "negative control"; an active wall hardening process probably being responsible for continued filamentous growth.

Earlier reports have indicated that changes in protein and nucleic acid metabolism as well as organelle size changes are correlated with the photomorphogenetic response in gametophytes of Dryopteris filix-mas.

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In the present study an examination of RNA metabolism has confirmed a difference in total-RNA base composition correlated with filamentous or prothallial growth. Analyses of the relative amounts of chloroplast and cytoplasmic rRNAs show about 20% more chloroplast rRNA in the prothallial gametophytes. It is calculated that this difference is of sufficient magnitude to be wholly responsible for the observed differences in total-RNA base composition. Analysis of the effect of transfer from red to blue light on rRNA synthesis has revealed a rapid stimulation of cytoplasmic rRNA synthesis, but chloroplast rRNA synthesis is unaffected during the first 10-15 hr of exposure to blue light. This is much slower than the manifestation of the photomorphogenetic response (tip swelling). Later, the rate of chloroplast rRNA synthesis surpasses that for the cytoplasmic rRNA eventually establishing the higher relative level of chloroplast rRNA in steady-state prothallial gametophytes. These observations (i.e. the early transient increase in cytoplasmic rRNA synthesis and the delayed increase in chloroplast rRNA synthesis) provide a molecular correlate for published observations regarding changes in nucleolar and chloroplast sizes during the photomorphogenetic transition.

Results from exploratory experiments have indicated that it is not possible to selectively inhibit the tip-swelling response by ultraviolet irradiation, suggesting that the photomorphogenetic response is not mediated through a U.V.-labile "morphogenetic substance" (eg. stable mRNA) in the cell. The inhibition of tip swelling at high UV doses is apparently a non-specific effect, as has been observed by others using various antimetabolites. In addition, it has not been possible to demonstrate a role for ethylene in the photomorphogenetic response in

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Dryopteris filix-mas gametophytes, in spite of reported ethylene effects with Onoclea gametophytes.

It is apparent from this study and others that events specifically related to the transition from filamentous to prothallial growth in Dryopteris filix-mas gametophytes are accompanied by a spectrum of correlated but unrelated responses. The continuing aim of this study is to evaluate the relationship of such correlated responses to the photomorphogenetic response in order to approach an understanding of the photomorphogenetic process.

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