*INDUCTION OF FERN SPORE GERMINATION**

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Abstract.—Light-induced germination of *Anemia* spores can be inhibited by AMO-1618, a selective inhibitor of gibberellin biosynthesis. The inhibitor has no effect on gibberellin-induced dark germination and its inhibition of light-induced germination can be reversed by supplying gibberellin. Barley-endosperm bioassay of concentrates of medium in which spores are imbibed in light reveals the presence of substances with gibberellin-like activity; assay of medium from dark-imbibed spores does not. Simultaneous exposure of spores to suboptimal levels of light and gibberellin leads to additivity of effect on germination level. Uptake of labeled gibberellin by spores in light is similar to that in darkness. The implications of these findings for the light-dependent synthesis of a gibberellin-like germination substance are discussed. The bearing of the observations upon understanding the interaction of light and gibberellins in seeds of higher plants is considered.

Diverse developmental processes in plants are sensitive to the effects of light and gibberellins. Both the processes of seed germination and of flowering, for example, are stimulated by light exposure and by gibberellin treatment in a wide variety of flowering plants. Spores of various species of ferns have also been shown to germinate in response to red light and to gibberellin.[1–5] Most of those species which *form antheridia* in response to antheridogen-A or -B also *dark germinate* in response to the *same* hormone. *Both* responses occur with species specificity to *one* of these two antheridogens, but not to the other antheridogen, showing a striking phyletic parallel between the patterns of activity of the two antheridogens in these very different physiological events.[6] For several reasons, the fern spore constitutes a particularly favorable material with which to examine the relationships between light and gibberellin action. In contrast, for example, to the complex multicellular tissues of seeds, the spore is a single cell. In seeds, responses to light and hormones may occur in different cells or tissues; in spores both responses necessarily occur within one cell. Inasmuch as many of the physiological aspects of germination are common to both seeds and fern spores, spores provide a potentially significant and simple model for the more complex processes regulating germination of seeds. The studies reported below are based on the observations that the spores of *Anemia phyllitidis* do not germinate in the dark and that gibberellin,[5] antheridogen-B,[6,7] and light each can initiate germination of imbibed *Anemia* spores. A variety of observations led to a series of experiments designed to test the hypothesis that a germination substance, related to the gibberellins or antheridogens, is synthesized within the illuminated imbibed spore.

Materials and Methods.—Source of fern spores: Spore-producing plants of *Anemia phyllitidis* (L.) Swartz were grown in the greenhouses of the Rockefeller University. Spores were collected, sieved, and stored at 4° until use.[8]

Chemicals.—Potassium gibberellate (Calbiochem) was twice recrystallized in the acid form (dec. 210–211°). Other gibberellins were the kind gift of Dr. D. Broadbent of Imperial Chemical Industries, Ltd. AMO-1618 was purchased from the Rainbow Color and Chemical Company, Sepulveda, Calif.

Culture methods: Spores were surface sterilized in 100 mg (dry wt.) batches in 15% Chlorox for 10 min.[8] They were then centrifuged and resuspended in 10 ml sterile water aliquots for a series of three washes and finally resuspended in 10 ml of sterile water giving a spore concentration of approximately 10 mg/ml. Inoculations were made from these suspensions with sterile serological or Pasteur pipettes.

Moore's liquid culture medium supplemented with a trace element solution was used. The medium was buffered at pH 4.5 with 2-(*N*-morpholino) ethanesulfonic acid (MES) at a concentration of 0.05 *M*.[9, 10] Culture vessels were 15 × 100 mm test tubes each containing one ml of medium. All cultures were maintained at 21° ± 2. Those grown in light received approximately 7500 lux from banks of twelve 8-foot GE Cool White fluorescent bulbs and six 60-watt incandescent bulbs.

Assay of germination: The percentage of germination was determined by microscopically observing and counting a fixed number of spores from each of several culture vessels. If the spore wall was dehisced or open and the spore cell appeared to be intact, the spore was considered to be germinated.

Barley-endosperm assay for gibberellins: The method of Jones and Varner[11] was used to assay gibberellin-like substances. Barley seeds, var. Himalaya, were kindly provided by Dr. R. A. Nilan, Washington State University, Pullman. Half-seeds were preincubated for 2 to 3 days on moist sand at 4°. The assay was performed at 21° with an incubation time of 24 hr. After filtration, the culture medium to be bioassayed was concentrated to $^1/_{20\text{th}}$ its original volume, filter-sterilized through Millipore filters, and used in the bioassay. Cultures were checked for bacterial and fungal contamination and were not used if such contamination was found. In parallel with the assay of the concentrates, standard solutions were also assayed.

Thin-layer chromatography: Aliquots of the concentrates were spotted on silica gel sheets (Eastman Chromagram sheet 6061, without fluorescent indicator) and developed with di-isopropyl ether-acetic acid (95:5).[12] The R_f's of control gibberellins were determined by locating fluorescent spots which became visible after spraying the chromatograms with 5% sulfuric acid in ethanol and heating for several minutes at 80 to 85°. Chromatogram sections to be bioassayed were dried overnight to dispel solvents and placed in vessels prepared for the barley endosperm assay.

Preparation of ^{3}H-GA: The double bond within the A ring of gibberellic acid (= gibberellin or GA) was tritiated according to the method of Kende[13] by the New England Nuclear Corp. A sample of specific activity 1.83 mc/mg was obtained. The material was purified by twice chromatographing in the thin-layer system described above. Specific activity of the resulting sample was unknown inasmuch as the ratio of ^{3}H-GA to unlabeled GA, which was added as a carrier, was not determined.

Results.—Effect of pH on germination: The effect of various substances upon spore germination was found to be dependent upon the hydrogen-ion concentration of the culture medium, much as is the antheridogen-induced response leading to antheridium formation.[10] Although there is relatively little effect of hydrogen-ion concentration upon the light-induced process (Fig. 1.), the response of spores grown in darkness is markedly pH dependent when suboptimal concentrations of gibberellin are used. There is an evident resemblance between the curve plotted for GA-induced dark germination and the titration curve for a weak acid, such as gibberellic acid. Curves very similar to those in Figure 1 may also be plotted from data obtained when antheridogen-B is used to induce dark germination. The data in Figure 1 are most simply interpreted as

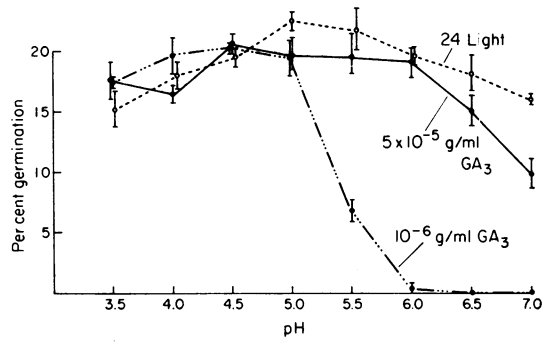

FIG. 1.—The effect of pH on light-induced and gibberellin-induced germination. Spores of *A. phyllitidis* were inoculated into 1.0 ml MES-Moore's medium buffered at the indicated pH values. Values represent the means of counts of 300 spores (3 replicates) ± the Standard Error.

evidence that the dark-grown spore is permeable to the *acid* form of gibberellin, but not to the salt form. As would be expected, by increasing the concentration of gibberellin in media of high pH, the germination level increases and the depression of germination is overcome.

Inhibition of germination with AMO-1618: The compound 2-isopropyl-4-dimethylamino-5-methylphenyl-1-piperidine carboxylate methyl chloride (AMO-1618) is a potent and selective inhibitor of gibberellin biosynthesis both in fungi[14] and in higher plants.[15–17] It has been shown to block the enzymatic conversion of geranylgeranyl phosphate to (-)-kaurene.[17] The inhibitory effects of AMO-1618 may commonly be overcome by supplying gibberellin exogenously. Selective inhibition of light-induced germination of *Anemia* spores by AMO-1618 would thus provide strong supporting evidence for the hypothesis that: (1) light stimulates synthesis within the spore of a substance which plays a regulatory role in germination, and, (2) the substance shares a distinctive segment of biosynthetic pathway with the gibberellins. In order to test the effect of AMO-1618 on light-induced germination, it is first necessary to show that the *action* (as distinct from the biosynthesis) of gibberellins in inducing germination is not impaired by the inhibitor. If GA action were not inhibited by nontoxic levels of AMO-1618, it would be possible to test for selective inhibition of the synthesis of a gibberellin-like substance. That GA action is not impaired is illustrated in Figure 2. When $10^{-3}M$ AMO-1618 (the highest concentration showing no inhibition of vegetative growth of *Anemia* gametophytes) is supplied to spores kept in darkness, germination in response to gibberellin occurs normally. The

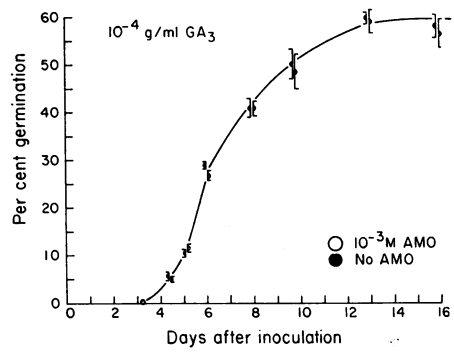

FIG. 2.—Effect of AMO-1618 on GA-induced dark germination. Spores cultured in 1.5 ml MES-Moore's medium (pH 4.5) containing 10^{-4} gm/ml GA_3. Experimental points are means of percentage of germination, ± Standard Error, based on counts of 200 spores from each of four replicates.

final germination levels at several gibberellin concentrations ranging from 10^{-4} to 10^{-6} gm/ml are not depressed by AMO-1618. It can therefore be assumed that the inhibitor does not alter the action of gibberellin in this system.

Preliminary results of AMO-1618-treated spores indicated that in continuous light the inhibitor delays attainment of maximum germination level to nearly twice the usual interval. Such treatment does not, however, decrease the eventual germination percentage. This suggests that at the concentration used, AMO-1618 depresses but does not eliminate synthesis of the germination substance. With partial inhibition by AMO-1618, a saturating concentration of the substance could eventually be reached within spores exposed to continuous light. However, if suboptimal light exposures were used, the synthesis of the hypothesized germination substance might be limited and the amounts of the substance kept from approaching the saturation concentration. The use of short light exposures might then provide a sensitive method for evaluating decreases in biosynthesis of the substance resulting from AMO-1618 treatment. Consequently, the effects of 10^{-3} *M* AMO-1618 were tested at four different light exposures (Fig. 3). At the end of the illumination period, the spores were transferred to the dark; germination counts were made at the indicated times. As can be seen in Figure 3, there was a decrease in the final germination level in each of the cases where light was limiting. With a light exposure of 48 hours, the AMO-1618-treated spores showed only 15 per cent inhibition of germination level relative to control spores. In the 24-hour light exposure group there was 50 per cent inhibition and in the 17-hour group there was 72 per cent inhibition.

Detection by bioassay of gibberellin-like substances produced by spores: Attempts to detect gibberellin-like substances after extracting them from 1-gm samples of germinating spores were unsuccessful. Because of the limited supply of *Anemia* spores, it has not yet been feasible to try to isolate gibberellins directly from larger quantities of spores. It was thought, however, that the substances might nevertheless diffuse from the spores and accumulate in the culture medium in measurable quantities. Thus, concentrates of medium from germinating

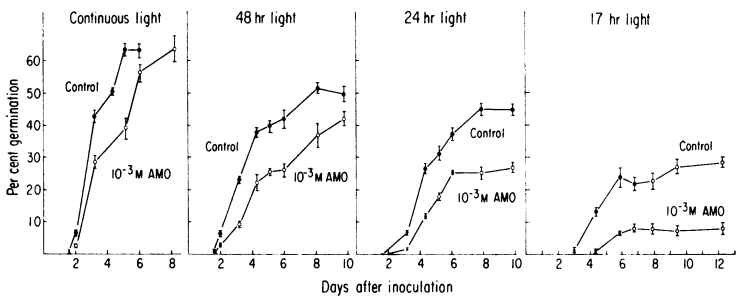

FIG. 3.—Effect of AMO-1618 on light-induced germination of *Anemia* spores. Same inoculum as in Fig. 2, experiment run simultaneously. Spores inoculated into 1.5 ml of MES-Moore's medium (pH 4.5) and placed in light. Left panel: cultures continuously illuminated. 48-hr panel: cultures transferred to dark 48 hr after inoculation. 24- and 17-hr panels similarly treated after the indicated time interval. Counts of 200 spores from each of four replicates were made. Means of the replicates ± the Standard Error are shown.

TABLE 1. *Production of gibberellin-like substances by Anemia spores.*

Conditions	n*	μg amylase†	Gram equivalent GA_3 per gm spores
Control	5	0.1 ± 0.08	
46 hr light	4	0.5 ± 0.1	—
92 hr light	5	18.5 ± 8.1	10^{-7}
92 hr dark	5	0.3 ± 0.3	—

* n = number of replicate flasks containing 10 half-seeds.
† means per 10 seeds ± Standard Error.

spores were tested in the barley endosperm bioassay for gibberellins. In our laboratory, this assay is sensitive to 5×10^{-11} gm/ml GA_3. With this method, concentrates of medium from spores imbibed while exposed to light consistently exhibited gibberellin-like activity. By contrast, concentrates of medium from dark-imbibed spores did not. A typical experiment is illustrated in Table 1. Activity could be detected only during the germination period. It is necessary to point out, however, that the precise time of appearance of biological activity varied somewhat from experiment to experiment. Amounts of biologically active material detected were equivalent to 6×10^{-9} to 10^{-7} gm of GA_3/gm of light-treated spores.

The biologically active substance(s) could be partially purified and conveniently chromatographed in systems commonly used for separating gibberellins and antheridogens.[8, 12] Data from a representative chromatogram are summarized in the activity profile shown in Figure 4. The predominant zone of gibberellin-like activity corresponds in position with that of GA_1 and GA_3 but differs in its R_{GA_3} from those of GA_{2-9}. A second zone of much lower biological activity was of more variable R_f, but sometimes occurred with an R_{GA_9} approximating 1.0. Experiments using additional solvent systems are in progress; through these it is hoped that the germination substance(s) can be resolved from or provisionally identified as GA_1, GA_3, or antheridogen-B. Additional types of bioassays are also being tested to this same end.

Combined gibberellin and light treatments: Another consequence of light induction of gibberellin biosynthesis can be tested. If suboptimal amounts of light were provided to spores, suboptimal quantities of gibberellin-like substance would be formed in them. By suitably combining light and gibberellin treatment of spores, the response to the paired stimuli should be equal to the *sum* of

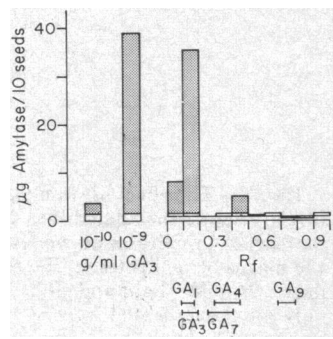

FIG. 4.—Chromatogram of medium from a light-exposed culture of *Anemia* spores. Chromatogram bioassayed by barley-endosperm method. Authentic samples of 5 gibberellins were chromatographed as standards. GA_3 at two concentrations served as a standard for determining the relative activities of the chromatogram segments. Six controls, run with 1 × 2 cm sections of fresh unchromatographed silica gel sheet, gave a mean of 1.20 μg amylase produced by 10 half-seeds. This value is indicated by the horizontal line extending across the chromatogram values. Shaded portions of the bars represent values significantly greater than the control ($p < 0.05$, Student's *t*-test).

their separate, suboptimal effects. Figure 5 demonstrates that in combination the two stimuli lead to final germination levels very close to this sum. When rates and time of earliest germination are investigated, relative to the response to either single stimulus, the onset of germination is slightly advanced in spores treated with continuous light and gibberellin. The rate of germination, however, appears to be unchanged. This result is obtained only at saturating conditions.

Effect of light on uptake of ^3H-GA$_1$: In order to determine whether light affected the permeability of the cell membrane to GA transport, the uptake of labeled GA$_1$ by imbibed spores was measured. Spores were inoculated into one milliliter volumes of MES-Moore's medium containing approximately 10^6 counts per minute ^{3}H-GA$_1$. The spores were then placed either in white light or darkness. At the times shown in Table 2, spores were collected and three times washed and centrifuged. They were then suspended in 0.5 ml water, added to 10 ml Bray's[18] solution, and counted in a Packard Tricarb scintillation spectrometer (model 3375).

Discussion.—The data obtained through use of the inhibitor AMO-1618 are consistent with the view that a (-)-kaurene-derived germination substance(s) is synthesized within illuminated germinating spores. Knowledge of the speci-

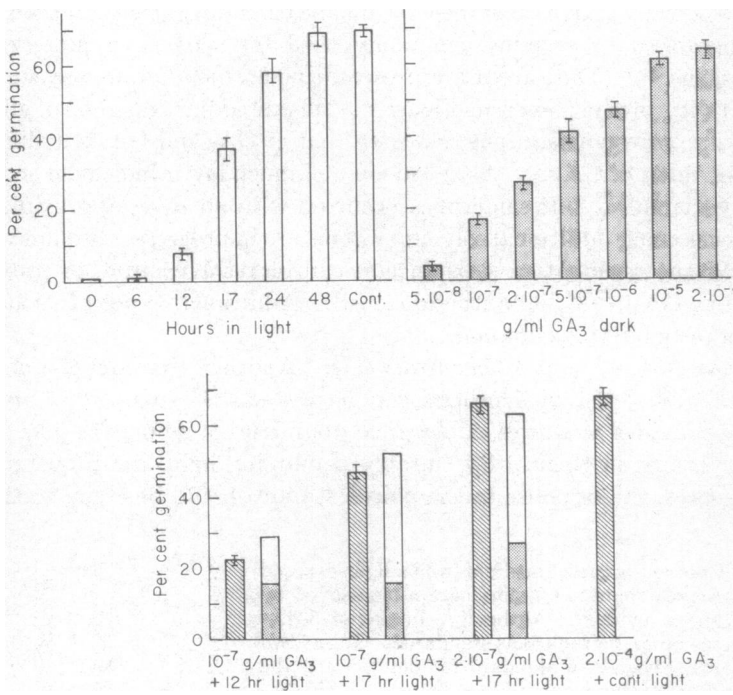

FIG. 5.—The effect of simultaneous treatment with light and gibberellin on final germination levels. Spores inoculated into 1 ml of MES-Moore's medium (pH 4.5). Counts of 300 spores for each of 4 replicates at each condition were made 14 days after inoculation. Values represent the means of replicates ± the Standard Error. *Top left:* light alone. *Top right:* GA$_3$ in the dark. *Bottom:* light and gibberellin. Gibberellin was present continuously, light exposure was only for the indicated interval directly following inoculation. Experimental values are represented by striped bars. Bars composed of white and stippled segments are the sums of the gibberellin and light treatments applied singly as determined in the upper figures.

TABLE 2. *Uptake of ^{3}H-GA$_1$ by Anemia spores.*

Hours after inoculation	Counts per Min	
	Light	Dark
0	74 ± 4	
12	1300 ± 200	1070 ± 200
24	1550 ± 130	1600 ± 200
36	1790 ± 60	2070 ± 110
48	2100 ± 210	1960 ± 280
72	2440 ± 200	2510 ± 700

A total incubation volume of 1.3 ml contained 0.3 ml of spore suspension (4 mg spores), 0.8 ml 0.08 *M* MES-Moore's medium, and 0.2 ml aqueous ^{3}H-GA$_1$. Three samples were taken at each experimental condition.

ficity of the inhibitor is derived, however, from studies with other groups of plants and should be confirmed with *Anemia*. Bioassay for gibberellins, a major group of (-)-kaurene derivatives, reveals the production of substances with gibberellin-like activity in culture filtrates of light-grown, germinating spores, and the absence of such activity in dark-grown spores.

The identification of gibberellin-like substances within seeds[11, 19] and the ability of gibberellin to overcome a light requirement for germination[20, 21] have been taken as evidence for the physiological role of gibberellins in seed germination. There is further support in the frequent observation of additivity of response to simultaneous application of GA and light to seeds responsive to the two individual stimuli.[20–22] Similar results were obtained with fern spores in the present report; it was found, moreover, that a gibberellin-like substance was detectable only in spores subjected to inductive light conditions. Very little information exists, however, on the influence of light on the gibberellin level of germinating seeds. Köhler has shown that the gibberellin content of lettuce seeds is enhanced in the light.[23] In seeds, cold treatments which are sufficient to induce germination in some species have been shown to increase endogenous gibberellin levels.[24–26]

There is other evidence, however, which appears to diminish the likelihood of the involvement of endogenous gibberellins in the light-induced germination of seeds. The effect of inductive red light and gibberellin upon germination could be experimentally separated in lettuce seeds by a high temperature treatment.[27] The time of initial germination in lettuce seeds treated with red light and with gibberellin can differ under appropriate conditions.[28] Most compelling are experiments showing synergism between short red light exposures and gibberellins in promoting lettuce seed germination.[29, 30]

It has been suggested that phytochrome effects may be mediated by changes in membrane permeability.[31] Short times of action for several phytochrome responses[32–35] as well as changes in transport of various substances in response to red or far red treatments[36–39] have led to this concept. The light-stimulated germination of spores of several ferns,[2–4] including *Anemia*,[40] are believed to be mediated by phytochrome. It thus seemed possible that through phytochrome control of permeability to gibberellin, spores or seeds simultaneously treated with light and exogenous gibberellin could reach the endogenous gibberellin concentration necessary for germination in advance of those treated singly with these stimuli. The present studies with spores of the fern *Anemia* suggest that light stimulates synthesis of gibberellin-like germination substances, but does not lead to increased transport of GA into the spore. The latter process could still,

however, account for the synergistic responses between GA and red light observed with lettuce seed germination. It would be of considerable interest to learn whether light-induced stimulation of GA permeability occurs in such seeds as those of lettuce.

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