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Photocontrol of the Germination of *Onoclea* Spores

II. ANALYSIS OF GERMINATION PROCESSES BY MEANS OF ANAEROBIOSIS

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ABSTRACT

The oxygen requirements during the three phases of photoinduced germination of *Onoclea sensibilis* L. spores were analyzed by temporarily applying nitrogen atmosphere. The dark preinduction phase, during which the spores imbibe water and establish sensitivity to irradiation, involves an oxidative process which can be reversibly inhibited and stimulated by nitrogen and air, respectively. The induction phase of germination is characterized by a pure photochemical reaction, independent of temperature and oxygen. The postinduction phase, when the photoproduct triggers dark processes eventually leading to the protrusion of the rhizoidal or protonematal cells, involves an oxidative process which occurs within the first 10 hours of this phase. This oxidative process differs in kinetic characteristics from that in the preinduction phase. The oxidative process is inhibited by nitrogen treatment, but following nitrogen inhibition the ability of the spores to germinate can be reinstated by a long period of air intervening between the nitrogen treatment and a second irradiation. This suggests that enzymes or reactants which are needed in the postinduction process decay under anaerobic conditions and are resynthesized when the spores are transferred to air. Spores take up acetocarmine stain towards the latter part of the postinduction phase. Stain uptake is apparently succeeded very closely by cell division, and some time later by protrusion of the germling cells.

Light induces or enhances germination of spores of many fern species (13, 14, 16, 18). Photocontrolled fern spore germination may operationally be divided into at least three sequential phases: (*a*) a dark preinduction phase in which the spores imbibe water and establish sensitivity to irradiation; (*b*) an induction phase, when light induces germination maximally; and (*c*) the postinduction phase, in which the photoproduct triggers dark processes that ultimately terminate in the protrusion of the rhizoid and protonemata. Although many investigations have dealt with characterization of the quality and quantity of light required for the induction phase of germination, the nature of the preinduction and postinduction phases of germination in fern spores has been little investigated (6, 9, 15, 20).

The action spectrum and the general characteristics of photoinduced germination in *Onoclea sensibilis* spores have been reported previously (19). Spores of *Onoclea* require at least 6 hr of dark imbibition before maximum sensitivity to irradiation is established, and this sensitivity is maintained for at least 48 hr. Spores are stimulated to germinate by all wavelengths of visible light, but are most sensitive to red light. Visible protrusion of the germling cells occurs about 50 hr after photoinduction.

In a series of preliminary experiments, *Onoclea* spore germination was found to be inhibited by N_2 atmosphere; the spores, however, could recover the ability to germinate once they were transferred to air and irradiated with visible light. Anaerobiosis thus seemed to be a useful tool for the investigation of oxidative reactions involved in the germination processes. The present paper is, therefore, aimed at analyzing the oxidative requirements of the three phases of germination by temporary application of a N_2 atmosphere.

MATERIALS AND METHODS

Plant Material and Germination Procedure. *Onoclea sensibilis* L. spores were collected, harvested, and stored as described earlier (19). The batch of spores used in the present investigation had a somewhat lower final percentage of germination (65.1 ± 2.8) and a slightly slower time course of germination than the batch of spores used in the earlier experiments (19). This was ascribed to aging of the spores after collection. The general characteristics of the light requirements remained the same, however.

Germination was scored 6 days after treatment by the protrusion method described earlier, unless stated otherwise. Counts of 400 spores from each replicate treatment were averaged to give the percentage of germination for a given treatment.

Nitrogen Gas Experiments. Experiments requiring a N_2 atmosphere were carried out using air-tight, glass, baby food jars (5-cm diameter). A 6-mm hole was drilled in the center of the lid of each jar, and a short piece of copper tubing was soldered to the hole. The jar was connected to a vacuum pump (Cenco-HYVAC 2, General Scientific Co., Chicago) and to a tank of prepurified gas (Matheson Scientific Co., Inc., Detroit) by means of a three way connector.

A small spatulaful of spores was sown on the surface of 3 ml of distilled H_2O in each baby food jar. Distilled H_2O was boiled, then cooled to room temperature shortly before use. The jar was evacuated with a vacuum pump and refilled with N_2 at least twice. The time of application of the N_2 and the duration of the N_2 treatment varied with experiments (see "Results"). If irradiation was done in N_2, the jars were tipped on their sides and irradiated from above. To account for the possible effects of evacuation on germination, controls for the N_2 experiments were also evacuated twice and refilled with

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air. After incubation in N_2, the spores were decanted into 5-cm Petri dishes and distilled H_2O was added to make 10 ml. Spores were then subjected to further treatments or allowed to germinate. Each treatment was duplicated in each experiment, and experiments were repeated at least twice.

Light Sources. Experimental irradiation was carried out with a broad band red filter which transmitted light above 590 nm with 50% transmission at 660 nm (incident energy 600–800 ergs cm^{-2} sec^{-1}). The source of the filter was a bank of four 15-w cool white fluorescent lamps filtered through red Plexiglas. Although less than 30 sec were required to attain maximum germination, 5 min of red irradiation were given to insure maximal effects.

For the dose response curves taken, the red filter was modified to reduce incident energy by covering the red Plexiglas with four double layers of cheesecloth and two layers of plastic screening. The incident energy achieved by these modifications was 200 to 300 ergs cm^{-2} sec^{-1}.

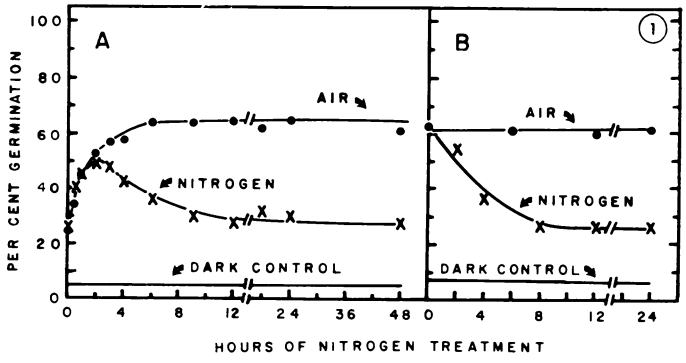
The dim green safelight used in these experiments was the same as described previously (19).

RESULTS

Preinduction. The normal development of photosensitivity during preinduction in air-treated spores of *Onoclea* is altered when a N_2 atmosphere is given throughout the preinduction period. This was shown by irradiating spores with 5 min of red light immediately after transfer to air from various periods of N_2 treatment. After irradiation, spores were incubated in air in the dark until germination was scored. Figure 1A shows the results of such an experiment. Air-treated spores develop maximum sensitivity to irradiation after 6 hr of dark imbibition. The development of sensitivity in N_2-treated spores follows that of the air controls during the first 2 hr of dark presoaking, but declines thereafter in 8 to 10 hr to the level of sensitivity of spores irradiated immediately after soaking.

Because the anaerobic treatment inhibited the development of maximum sensitivity a second series of experiments was designed to test whether the N_2 atmosphere would decrease the sensitivity to irradiation once full sensitivity had been developed. Spores were thus soaked in air for 12 hr before varying periods of N_2 treatment in the dark, and were irradiated immediately after release from N_2. The results (Fig. 1B) indicate that anaerobiosis causes an immediate loss of sensitivity to irradiation with a rate of about 6% per hr. The half-maximal and maximal inhibition is attained at 3.5 and 8 hr, respectively, in N_2. It should again be noted that the germination percentage declines to a level equivalent to that of spores irradiated immediately after being sown.

Inasmuch as anaerobic treatments were unable to cause complete inhibition when 5-min irradiation was carried out immediately after the transfer of spores to air, it is possible that some spores are induced to germinate by 5 min of red light after a few min of exposure to air. This possibility becomes highly likely if we consider the fact that these spores can be fully induced by less than 1 min of irradiation with red light (Fig. 3). Possible reversal of N_2 inhibition by air before red irradiation was thus tested as follows: spores were incubated in a N_2 atmosphere for 24 hr and then transferred to air for varying periods before irradiation. The results (Fig. 2) indicate that the above possibility is indeed true. The spores recover from N_2 inhibition quickly with an initial rate of about 40% per hr, and a half-maximal sensitivity to irradiation is attained at 1 hr in air. Furthermore, the inhibition by N_2 treatment and subsequent reversal by air of the spore sensitivity to irradiation can be demonstrated for at least two cycles of N_2 and air treatments (Table I).



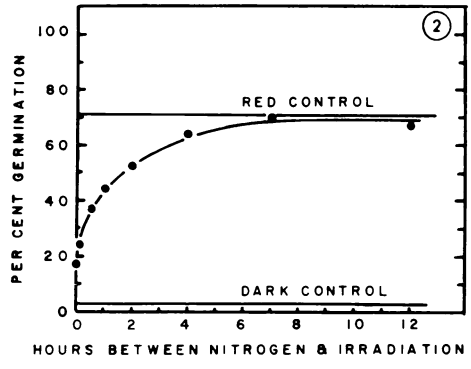


FIG. 1. Effect of N_2 treatment on the development (A) and maintenance (B) of photosensitivity for *Onoclea* spore germination. A: Spores were placed in N_2 atmosphere from the beginning of soaking and irradiated with 5 min of red light (180 kergs cm^{-2}) immediately after transfer from N_2 to air. B: Spores were placed for 12 hr in air from the beginning of imbibition, transferred to varying periods of N_2 atmosphere, and irradiated with 5 min of red light immediately after removal from N_2 atmosphere. The germination of spores treated with air throughout the preinduction phase and the value of dark control spores are given in the figure for comparison.

FIG. 2. Recovery of photosensitivity of *Onoclea* spores after N_2 inhibition. Spores were incubated for 24 hr in N_2 from the beginning of soaking and were transferred to air for varying periods (abscissa) before 5 min of red irradiation (180 kergs cm^{-2}). Red control is the germination of spores irradiated with 5 min of red light after 24 hr of dark presoaking in air.

Table I. *Inhibition of Photosensitivity by Nitrogen Atmosphere and Subsequent Reversal by Air*

Onoclea spores were irradiated with 5 min of red light (180 kergs cm^{-2}) immediately after various presoaking treatments (the first column), and were incubated in the dark until germination was scored six days later. Each value represents the average percentage of germination for four slides of 200 spores per slide.

Presoaking Treatment	Germination ± SE	Inhibition of Germination
	%	%
12 hr air	73.8 ± 1.8	0
12 hr N_2	40.5 ± 3.6	45.3
24 hr air	78.3 ± 1.5	0
12 hr N_2 → 12 hr air	67.7 ± 1.8	13.4
36 hr air	77.2 ± 0.9	0
12 hr N_2 → 12 hr air → 12 hr N_2	44.4 ± 2.5	42.5
48 hr air	65.7 ± 2.0	0
12 hr N_2 → 12 hr air → 12 hr N_2 → 12 hr air	56.4 ± 4.4	15.6

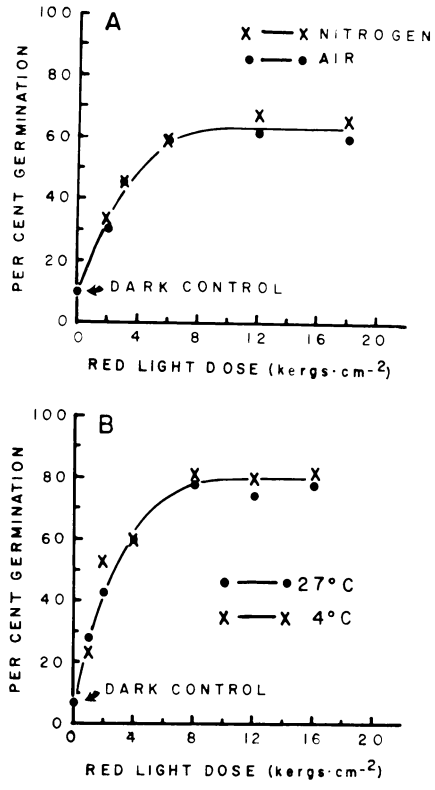


FIG. 3. Dose response curves for promotion of germination of *Onoclea* spores held in N_2 (A) or at 4 C (B). Spores were dark presoaked in air at 27 C for 12 hr, incubated in N_2 for 15 min or at 4 C for 45 min in the dark, irradiated with red light (incident energy, 200 ergs cm^{-2} sec^{-1}) in N_2 or at 4 C, and transferred 15 min after irradiation to air at 27 C for germination.

The results from these anaerobiosis experiments indicate that (*a*) spores are not injured by the anaerobic treatment; and (*b*) an oxidative process is required for the development and the maintenance of spore sensitivity to irradiation.

Induction Phase. During the induction phase of germination the pigment absorbs the radiation and triggers the germination process. To determine the nature of this phase, the dose response curves of spores held in N_2 or at 4 C were compared with those of spores irradiated in air at 27 C. Spores were soaked in the dark in air for 12 hr at 27 ± 1 C, placed either at 4 C for 45 min or in a N_2 atmosphere for 15 min, irradiated with various dosages of red light at 4 C or in N_2, and transferred to air at 27 C 15 min after the termination of irradiation (total time in N_2, 30 min; and at 4 C, 1 hr). The results shown in Figure 3, A and B, indicate that there is no difference between the dose response curve in N_2 or at 4 C and their respective controls. The variability in extent of germination between the two experiments was ascribed to change in response of the spores with age (19) and with adverse effects of evacuation. From the above observations it can be concluded that the induction phase of germination is a pure photochemical reaction, independent of temperature and O_2.

Postinduction. In *Onoclea* spore germination, we noted earlier that a long dark phase intervenes between photoinduction and the protrusion of the germling cells (19). Edwards and Miller (6) observed that a nuclear-cytoplasmic stain, acetocarmine-chloral hydrate, permeated light-induced *Onoclea* spores but failed to permeate noninduced spores, and that stain permeation temporally preceded the protrusion of the germling cells. To compare acetocarmine uptake with protrusion, spores were presoaked for 12 hr in the dark, irradiated with 5 min red light, and were then scored for stain uptake and protrusion at various times after irradiation. The results are shown in Figure 4. The acetocarmine stain begins to permeate the spores 16 to 20 hr after irradiation, and maximum percentages of stain uptake were obtained by 45 hr. In contrast, the protrusion of the primordial cells does not begin until 30 to 35 hr after irradiation and the maximum percentages of protrusion are not reached until 120 hr. The half-times of stain uptake and protrusion are 28 and 70 hr, respectively, after irradiation, and the rate of permeation and the rate of protrusion are 5 and 0.85%, respectively. It should be noted that the extent of stain uptake and protrusion is identical (6). These results indicate that a later portion of the postinduction phase is composed of two kinetically separable periods; (*a*) an earlier, rapid period of acetocarmine-chloral hydrate stain uptake; and (*b*) a later and slower period of protrusion of the rhizoidal or protonematal cells.

In order to analyze whether or not all periods of the postinduction dark phase are susceptible to N_2 inhibition, spores were placed in an anaerobic atmosphere for varying durations at three different times: (*a*) 15 min before irradiation (essentially the same as the application of N_2 atmosphere at zero time of postinduction, Fig. 3B); (*b*) 5 hr after irradiation; and (*c*) 10 hr after irradiation. After various periods in N_2, the spores were returned to air for germination. A diagram of the N_2 treatments and the data plotted on the basis of the duration of N_2 treatment are presented in Figure 5.

When the N_2 atmosphere is applied at zero time after irradiation (curve A), inhibition of germination begins immediately after irradiation and increases with time. Maximal inhibition is reached after 6 to 8 hr in N_2, at which time germination is reduced to the level of the dark controls. Curves B and C indicate that (*a*) no noticeable lag precedes inhibition; and (*b*) inhibition becomes less complete as the time of N_2 application is delayed. It should be noted that the spores regain their ability to germinate after a few days in diffuse laboratory light. These results suggest that an oxidative process is required for the postinduction dark processes, and that the photoproduct becomes unusable in N_2 for the postinduction germination process unless subsequent light treatment is given.

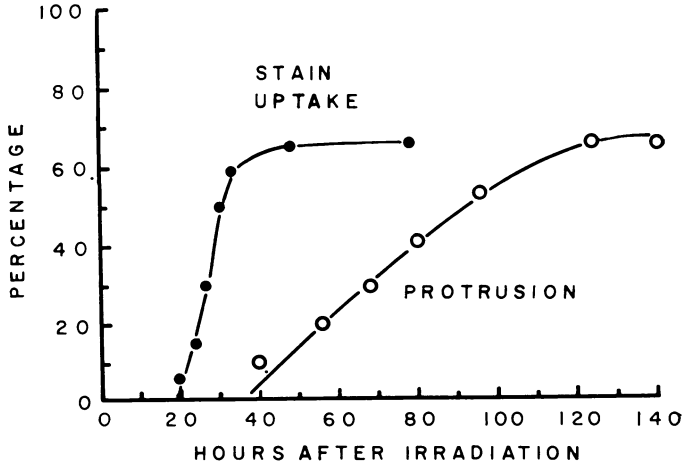


FIG. 4. Time course of acetocarmine-chloral hydrate stain uptake and protrusion of the rhizoidal or protonematal cells of *Onoclea* spores. Spores were presoaked in the dark for 12 hr, irradiated with 5 min of red light (180 kergs cm^{-2}), and dark incubated thereafter. Spore samples were removed under dim green light at given intervals after irradiation (abscissa) to score for stain uptake and protrusion. Each point represents an average percentage of germination for four slides of 200 spores per slide.

As the time of application of anaerobiosis after irradiation is delayed, the spores whose germination process has presumably passed through the oxidative process escape the inhibitory effects of N_2.

Because 12-hr treatments with N_2 effectively cause maximal inhibition in the above experiments, the N_2 escape curve was next examined by giving 12-hr N_2 treatments to spores at various times in air after irradiation. The results from two sets of experiments (Fig. 6) indicate that the spores have maximally escaped inhibition when the time of N_2 application is delayed for 10 or more hr after irradiation. The rate of escape is about 5% per hr, and the time for half-maximal escape is 4.5 hr. It is interesting to note that this rate of escape is about the same as the rate at which the spores take up the acetocarmine stain (Fig. 4), but the spores escape from N_2 inhibition about 24 hr before they take up the acetocarmine stain.

It was shown earlier that an insertion of air between N_2 treatment and red irradiation restored the spore sensitivity to irradiation (Fig. 2, Table I). This raises a possibility that the germination inhibited by N_2 treatments in the postinduction phase could be restored by transferring the N_2-treated spores to air and irradiating a second time with red light. Spores were thus placed in N_2 for 24 hr immediately after irradiation and transferred to air in the dark for varying periods before a second 5-min irradiation. The results (Fig. 7) indicate that postinduction recovery from N_2 treatment proceeds slowly in air with half-maximal time of about 26 hr. This slow recovery in postinduction is clearly different from the fast recovery in preinduction and suggests that resynthesis of a substance or pigment is needed for germination.

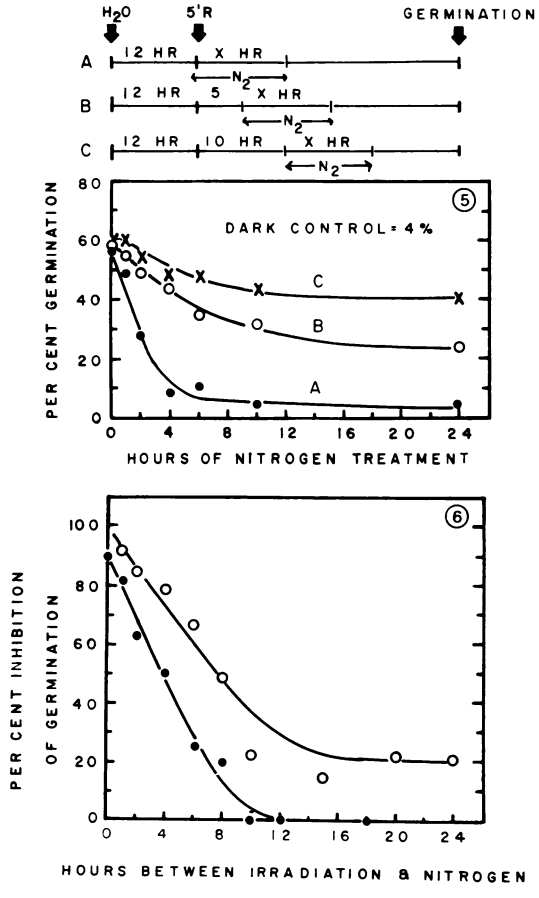


FIG. 5. Effect of timing and duration of N_2 treatment on the germination of *Onoclea* spores. Experimental procedure is diagrammed above.

FIG. 6. Escape of *Onoclea* spores from the inhibitory effect of N_2 treatment. Spores were dark presoaked in air for 12 hr, irradiated with 5 min of red light (180 kergs cm^{-2}), and transferred to N_2 for 12 hr at various times after irradiation (abscissa). The results from two separate experiments are plotted in terms of percentage of inhibition of germination.

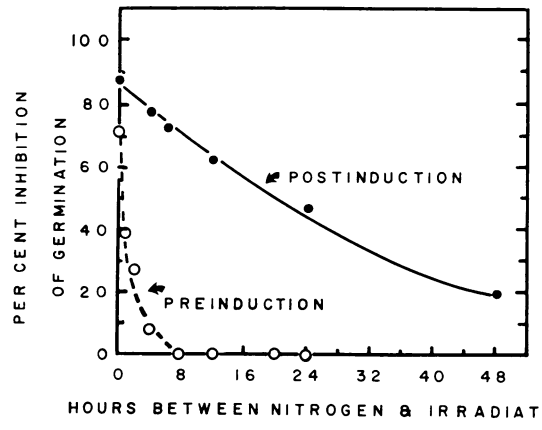


FIG. 7. Recovery of the ability of *Onoclea* spores to germinate after postinduction N_2 treatment. Spores were presoaked for 12 hr in air, placed immediately after 5 min of red light into 24 hr of N_2, and transferred to air for varying periods (abscissa) before a second red irradiation. The results presented in terms of percentage of inhibition of germination are compared with the preinduction recovery data of Figure 2.

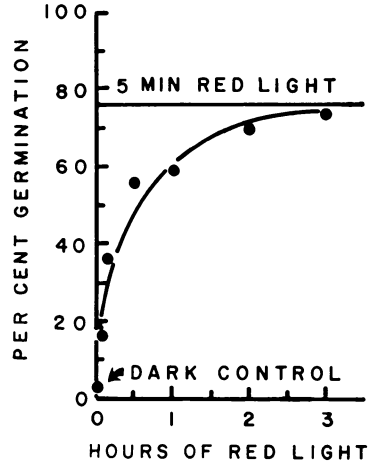


FIG. 8. Duration of second red irradiation required to induce *Onoclea* spore germination after postinduction N_2 treatment. Spores were presoaked for 12 hr in air, given 5 min of red irradiation, placed immediately in N_2 for 24 hr, and transferred to air for 12 hr before varying periods of red light (incident energy, 600 ergs cm^{-2} sec^{-1}).

To examine the possibility of synthesis, spores were reirradiated with varying doses of red light given 12 hr after the transfer from 24-hr N_2 treatment to air. The dose response curve (Fig. 8) shows that more than 3-hr irradiation (total energy $\geq 6.5 \times 10^6$ ergs cm^{-2}) is required to induce maximum germination. This is to be compared with less than 60 sec irradiation for spores that have not been treated with N_2. In view of the slow time course, the data suggest that resynthesis of a substance or of the photoreceptor pigment during postinduction recovery period is required for germination.

It can be concluded from the above studies of the postinduction phase that (*a*) the anaerobic treatment blocks the oxidative process which occurs within 10 hr of the postinduction

phase; (*b*) a substance or substances become unusable for the postinduction germination processes in N_2; (*c*) resynthesis of that substance occurs during postinduction recovery period in air; and (*d*) the visible protrusion of germling cells is preceded by a rapid uptake of acetocarmine stain in a later stage of the postinduction phase.

DISCUSSION

Our experiments show that anaerobiosis inhibits pre- and postinduction phases of germination in spores of *Onoclea sensibilis*. The inhibition can be interpreted as the blockage of germination by an inhibitor produced in the anaerobic atmosphere. Ethanol accumulates in many plant tissues as a result of anaerobic metabolism (1) and at high concentration may act as an inhibitor (12). On the other hand, ethanol has been shown to reverse blue light inhibition of germination in *Pteris vittata* (17) and light-inhibited elongation of excised wheat roots (10). It should be pointed out that the recovery kinetics are different for pre- and postinduction phases, necessitating the postulation of two specific inhibitors being produced in an anaerobic environment. This observation makes it difficult to assume that a compound such as ethanol causes the inhibition of both preinduction and postinduction phases of germination.

Another explanation for N_2 inhibition of germination is that two separate oxidative processes involved in the pre- and postinduction phases of germination are somehow inhibited by N_2 atmosphere. The rapid recovery observed after preinduction anaerobiosis (Fig. 2) and repeated reversibility of sensitivity by air and N_2 (Table I) imply that molecular oxygen may participate directly in a reaction required to develop and maintain photosensitivity. The inability of the spores to germinate when irradiated with a low dose of the second red light after postinduction N_2 treatment (Fig. 7 and 8), the slow recovery of photosensitivity (Fig. 7), and the requirement for a high dose of irradiation after removal from N_2 (Fig. 8) suggest that (*a*) molecular oxygen is not directly involved in the postinduction oxidative process; and (*b*) a substance required for germination decays under anaerobic conditions and is resynthesized when placed back into air. Thus, the characteristics of the oxidative processes in pre- and postinduction are clearly different.

What is then responsible for the inhibition by anaerobiosis of the postinduction process? Does the photoproduct itself decay in the nitrogen atmosphere? The Pfr form of phytochrome has been shown to undergo dark destruction *in vivo* (3, 4, 7), and to be less stable than Pr both *in vivo* (4) and *in vitro* (2, 5). In dark-grown seedlings, the destruction of Pfr is inhibited by reduced O_2 concentration, in the presence of respiratory inhibitors such as KCN, CO, and NaN_3 (3, 8). Pfr reverts to Pr in lettuce seeds when a N_2 atmosphere is applied during the postinduction period (11). In the case of *Onoclea* spore germination the involvement of phytochrome has not been clearly demonstrated (19). In view of practically no reinduction of germination immediately after a 24-hr N_2 treatment (Fig. 7), the reversion in N_2 of the metabolically active pigment to an inactive form is unlikely. Instead, a substance or substances responsible for germination decay in the postinduction anaerobiosis with a half-time of 2 to 4 hr (Fig. 5) and are resynthesized slowly in air after N_2 treatment with a half-time of about 26 hr (Fig. 7). Thus, under our standard conditions of air and 27 C, the responsible substance(s) must be turning over but kept at a high level by a somewhat faster rate of synthesis than decay. Since the earlier the application of N_2 in postinduction, the quicker the inhibition by N_2 (Fig. 5), the responsible substance(s) must be present in the initial period of postinduction. Unfortunately, with the physiological experiments described in this paper, it is difficult to pinpoint whether the pigment itself or other reactants in the early period of postinduction are decaying and being resynthesized.

Following the anaerobiosis sensitive period, the next observable step is the permeation of acetocarmine stain which occurs about 28 hr after photoinduction. Interestingly, the rate of N_2 escape (Fig. 6) and the rate of stain permeation (Fig. 4) are practically identical, suggesting that the postinduction processes proceed in a population of spores at the same rate up through stain permeation. Very shortly after the start of stain uptake, spores are observed to be composed of two cells. The observation that the rate of protrusion is much slower that the rate of stain permeation suggests that the postinduction processes change the rate between the stain uptake and the protrusion of the germling cells. It may be speculated that germination processes *per se* are terminated by changes in membrane permeability and probably by subsequent cell division, whereas protrusion represents a measure of elongation of the germling cells only.

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