Analysis of Germination Processes of Lettuce Seed by Means of Temperature and Anaerobiosis [1, 2]

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Although the seed of Grand Rapids lettuce has long been regarded as classical material for the study of photocontrolled germination (5, 6, 14, 15), our knowledge of the nature and mechanism of its germination is scanty (cf 11, 12, 32, 36). This condition is partly due to the complex nature of the processes, but is also in part due to the difficulty of separating the germination of the seed from the growth of the embryo (22, 27). After the seed has been moistened with water, its photosensitivity increases sharply at first, reaches a maximum, then gradually decreases (6, 24, 26). The seeds photoinduced at the time of maximum sensitivity take at least 5 hours before visible protrusion of their radicles (22, 27), and 50 % of them have not protruded for about 9 hours. Thus the imbibition of water and the photoreaction are widely separated from any visible germination. Our studies suggest that the germination comprises 4 phases: A) preinduction phase, when the seed takes up water and prepares for induction by red light; B) induction phase, when red light brings about the maximum induction, and reversal by far-red is also maximal; C) postinduction phase, when the seed undergoes a dark process; and D) phase of visible germination, when the radicle breaks through the surrounding coats. The existence of these 4 phases becomes clear when an attempt is made to analyze the photoinduced germination processes per se as distinguished from the growth of the embryo (cf 12, 36).

In a preliminary series of experiments it was found that germination did not take place under complete anaerobiosis, either in light or in darkness, but it did occur when the seeds were returned to air. Furthermore, the extent to which red light promoted germination depended upon the duration of the anaerobiosis beyond photoinduction. These studies suggested that the application of an anaerobic atmosphere would be a promising tool for the study of oxidative reactions during germination. The following experiments were, therefore, carried out to analyze each of the germination processes by temporarily applying a N_2 atmosphere and also a range of temperatures to the seeds. Phase 4 has already been discussed in connection with the role of the seed-coats in germination (27).

Materials and Methods

Seeds. Seeds of *Lactuca sativa* L. var. Grand Rapids obtained from Breck's of Boston, Mass. in November 1959, were used. They were kept in the cold room at 4°, taken out only as needed and showed an average germination of 25 ± 10 % in darkness at 25°. These seeds were from the same batch as those used in previous work (22, 25, 27).

Experiments with Nitrogen Gas. Nitrogen from a tank (Air Reduction Co., Inc.) was passed through 3 washing tubes of alkaline pyrogallol (20 g pyrogallol/100 ml 5 M KOH) and one of distilled water. The pyrogallol solution was prepared fresh every 3 days and the distilled water was boiled for 10 minutes before use. The gas stream was bubbled gently through the washing system for at least 30 minutes before experimentation. Later when prepurified N_2 was used the gas was passed only through distilled water.

Fifty seeds were introduced into a Thunberg tube and 1 ml of freshly boiled distilled water was added to the side arm. The tube was evacuated (< 2 mm Hg) and refilled with N_2 at least 4 times within 10 minutes before being finally filled with N_2. Distilled water was then tipped in from the side arm. In most of the experiments the seeds were thus allowed to imbibe water under N_2 for 1.5 hours and irradiated with red light from outside the tube. Care was taken that the seeds did not shade one another during irradiation; i.e., the Thunberg tube was laid horizontal. At a specified time, ranging from 0 to 24 hours after irradiation, the seeds were transferred to air for germination in a 5-cm petri dish with about 2 ml of distilled water.

In some cases 50 seeds were first soaked in 2 ml of distilled water in the air. At 1.5 hours after the beginning of imbibition they were exposed to red light for 5 minutes, then brought back to darkness again for 0.5, 2, or 5 hours. At each period the seeds were quickly dried between 2 sheets of filter paper, then introduced into a Thunberg tube. The tube was connected to the N_2 stream, the gas flushed through, and the seeds rewetted as before. After a

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given period of nitrogen treatment (0–24 hours), the seeds were transferred back to air for germination.

Air controls were prepared by shaking 50 seeds in 2 ml of distilled water in a 5-cm petri dish in the dark, irradiating at a given time after the beginning of imbibition, and examining for germination 2 days later. Whenever seeds were being soaked in water in presence of air, whether before or after the nitrogen atmosphere was applied, they were shaken in 2 ml of water in 5-cm petri dishes on a gently rotating shaker. Shaking was, however, not applied during the treatments with nitrogen or with light.

Experiments with Temperature. The effects of temperature were examined in a room precisely adjusted to the required temperature, ± 1°. Germination was not significantly affected in the presence of filter paper if sufficient water was added. In all experiments on temperature, therefore, 100 seeds were placed in a 10-cm petri dish on double layers of filter paper wetted with 10 ml of distilled water.

Light Sources. The red light source was a 100 w incandescent lamp filtered through a Corning Signal Red glass filter (50 % transmission at 630 mμ, 80 % transmission from about 665 to 750 mμ), and the source for far-red light was a General Electric 150 w reflector flood lamp filtered through Corning glass filter No. 7-69 (2600) (50 % transmission at 742 mμ). The lamps were placed 25 cm above the level of the seeds, at which the light intensities were about 60 ft-candles for red light and about 30 ft-candles for far-red. The red light transmits far-red, but the light intensity and the seed sensitivity are both enough higher in the red so that its effect predominates (cf 6). Although the saturating dose of both lights was reached at 1 minute exposure, most of the experiments were carried out with 5 minutes irradiation in order to secure the full effects. All manipulations were carried out under a dim green light, comprising a 20 w fluorescent lamp wrapped around by double layers each of yellow and dark green cellophane.

Results

The period when the seeds take up water and prepare for the maximum induction of germination by red light is considered as the preinduction phase. At 25° it takes about 1.5 hours in the dark (24, 26). At this time water uptake by the whole seed is not yet complete, but that by the embryonic axis is (24).

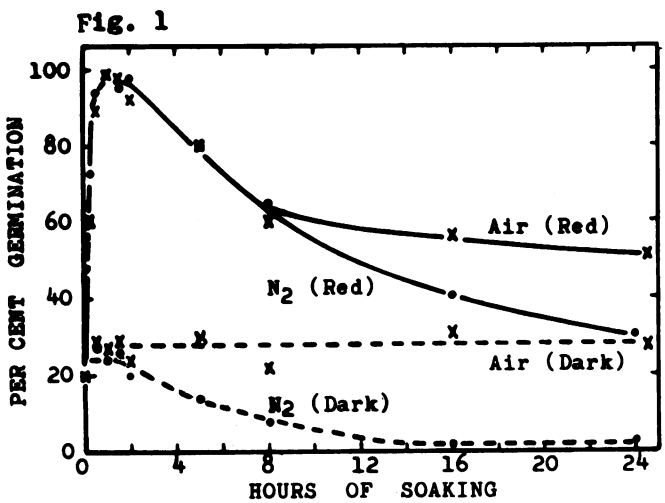
Effects of Nitrogen Atmosphere on the Preinduction Phase. If the seeds are placed in N_2 only before irradiating with red light, they germinate just like air controls. This was shown as follows: seeds were soaked under N_2 for various periods from the beginning, irradiated immediately after the transfer to air, then kept in the dark for 2 days. The sensitivity curves thus obtained are shown in figure 1, in which the control curve in air is drawn for comparison. The same sensitivity curve with a single peak at about 1.5 hours of soaking is obtained for seeds treated with N_2 and for air controls. However, the germination of dark-imbibed seeds is inhibited to an increasing extent as the period in N_2 increases beyond 2 hours, and this occurs also with red-irradiated seeds when the presoaking period before irradiation becomes very long. Essentially identical results were obtained both when 5 minutes red light was used and when the N_2 treatment was given through the red exposure.

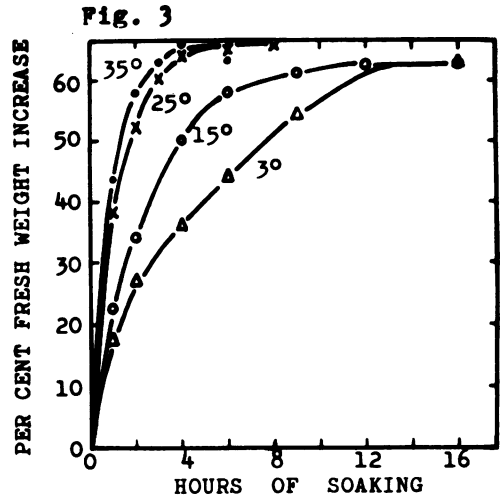
Since the preinduction phase is regarded as the period during which the sensitivity to red light is built up to its maximum, the above observations indicate that the N_2 atmosphere, if supplied before the red irradiation, does not affect the initial rise of sensitivity. Hence, this phase does not involve an oxidation process.

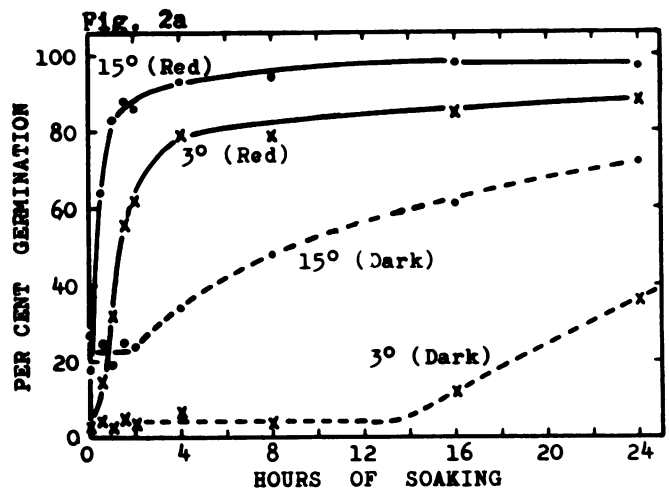
Effects of Temperature on the Preinduction Phase. Germination of Grand Rapids lettuce seeds is promoted at low temperatures but inhibited at 35°, if the seeds are maintained at the same temperature throughout (22). In the following experiments, however, temperature treatment was restricted to the period before exposure to light, the irradiation and germination being carried out at 25°. Seeds were allowed to imbibe at 3°, 15°, or 35° for different periods in the dark, then transferred to 25°, given 1 minute red light immediately, and scored for germination 2 days later. Figures 2a (3° and 15°) and 2b (25° and 35°) summarize the results.

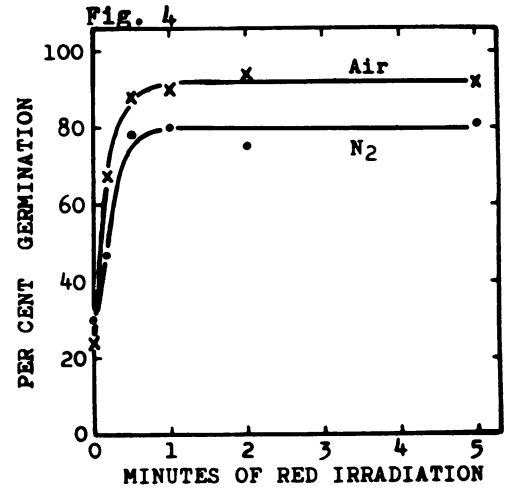
Pretreatments at 3° and 15° have generally similar effects (fig 2a); no decrease in sensitivity is observed after reaching the maximum (unlike 25°, where there is a steady decrease), and some dark germination is induced by the pretreatment. Indeed, after 24 hours at 15°, germination in the dark exceeds 70 %. The initial rise in sensitivity in seeds irradiated after 15° is almost as sharp as that of 25° controls, and the germination in the dark is induced much faster at 15° (as early as 2 hours of soaking) than at 3° (14 hr), without noticeable initial inhibition. Pretreatment at 35° does not promote dark germination, but decreases it steadily after 4 hours of imbibition (fig 2b). After 16 hours at 35°, irradiation causes no better germination than that which occurs at 25° in the dark. The general pattern of germination of red-irradiated seeds at 35°, however, is similar to that at 25°; the maximum sensitivity lies between 1.5 and 2 hours of soaking at 35° with a decline thereafter. The subsequent loss of sensitivity appears to parallel the increasing inhibition of dark germination.

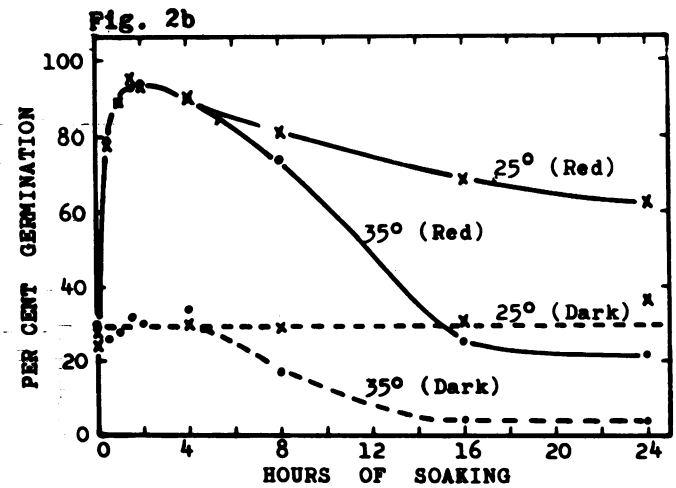
The initial rise in sensitivity, therefore, slows down as the temperature is lowered. Since one of the characteristics of the preinduction phase is the rapid uptake of water by the seeds (24), the effect of temperature on water uptake was studied directly. Seeds were allowed to imbibe at 3°, 15°, 25°, or 35° for various periods of time, and the increase of fresh weight was measured. The results (fig 3) show that the rate of water uptake does increase with temperature. Between 3° and 25° the Q_{10} approaches 2. The maximum uptake at any temperature lies between











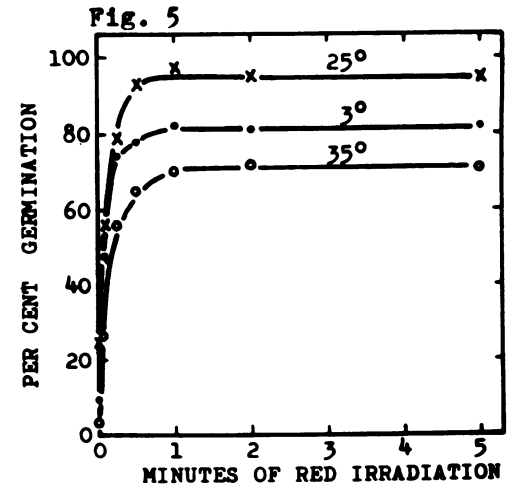


FIG. 1 (*upper left*). Effect of N_2 atmosphere on sensitivity curve. Seeds placed in N_2 from the beginning of soaking to immediately before red irradiation (1 min). Abscissa: hours of soaking before exposure to red light. Germination examined 2 days after light treatment. Solid line, red treated seeds; dotted line, dark control.

FIG. 2 (*middle left and lower left*). Effects of 4 different temperatures on sensitivity curve. Seeds treated at the given temperature only during the period before exposure to 1 minute of red light. Abscissa: hours of soaking before irradiation; germination examined 2 days later. Solid line, red treated seeds; dotted line, dark controls. Figure 2a, treatments at 3° and 15°; figure 2b, treatments at 25° and 35°.

60 and 65 %, which agrees well with previous results (24). Sensitivity to red light was shown above to reach its maximum after about 4 hours of soaking at 3°, after 1.5 hours at 15°, and after 1 hour at 25° and 35° (fig 2). These timings correspond to a water uptake of 35 % at 3°, 30 % at 15°, 38 % at 25°, and 45 % at 35°. In general, therefore, red light induces maximum germination when the water content of the seed has reached about 40 %.

Induction Phase. In this phase the pigment which has been converted to the far-red absorbing form (P_{fr}) by red irradiation can be reversed to the red absorbing form (P_r) by subsequent exposure to far-red light (5, 6, 36).

If this reversible process were a pure photoreaction, it should be independent of temperature (6) and also of O_2. In order to examine this deduction, seeds were placed in N_2, or at 3°, 25°, and 35° in air, from the start, irradiated with red or far-red light at exactly 1.5 hours after the beginning of soaking, and transferred to air at 25° 0.5 hour later. In this way irradiation was carried out while the seeds were being held either in N_2 or in air at a given temperature. The results are summarized in table I, where each reading is an average of 2 runs.

The germination of N_2-treated seeds is promoted by red light and inhibited by far-red, and the red/far-red reversible reaction takes place in N_2 just as effectively as in air throughout. Essentially the same is true at 35° or 3°. From these observations it can be concluded that the pigment conversion is a pure photoreaction independent of temperature and oxygen.

Table I. *Effects of Temperature and of Nitrogen Atmosphere on Reversibility of the Pigment System for Germination*

Seeds were irradiated with red and far-red light, after 1.5 hours of soaking under N_2 atmosphere or at a given temperature in air. Nitrogen atmosphere was applied at 25°, and other temperatures given in air; either treatment was given for 2 hours from the beginning of soaking. All seeds were then transferred to air at 25° in the dark and germination was determined after 48 hours.

Treatment with light	% germination				
	N_2	Air	3°	25°	35°
Red (5 min)	77	93	87	95	94
Far-red (5 min)	13	12	5	10	9
Red (5 min), then far-red (5 min)	18	17	2	12	15
Far-red (5 min), then red (5 min)	74	94	94	98	91
Dark control	32	32	31	40	24

This conclusion was confirmed by studying the dose-response curve. For this purpose, seeds held either in N_2 or at a given temperature were exposed to various dosages of red light. The results (fig 4 and 5) show that 1 minute of red light brings about maximum germination in all treatments. Lower final values obtained in the 3° treatments can be attributed to the inhibitory effect of this temperature as observed in figure 2a, and the slight inhibitions due to N_2 and 35° are due to the fact that the treatments extended 30 minutes beyond irradiation (see next section). When these points are taken into account, all the dose-response curves agree very closely. Hence the induction phase is a phase of pure photoreaction.

Postinduction Phase. A previous study at 25° indicated that a dark period of about 9 hours intervenes between photoinduction and actual germination (22, 27). This dark phase is presumably initiated by the physiologically active form of phytochrome, P_{fr}, (4–6, 21, 36), and is temperature sensitive (6). The experiments of figure 4 and table I suggest that the photoreaction is followed by an oxidative process, which is partly inhibited in nitrogen. The nature of this inhibition was, therefore, further studied.

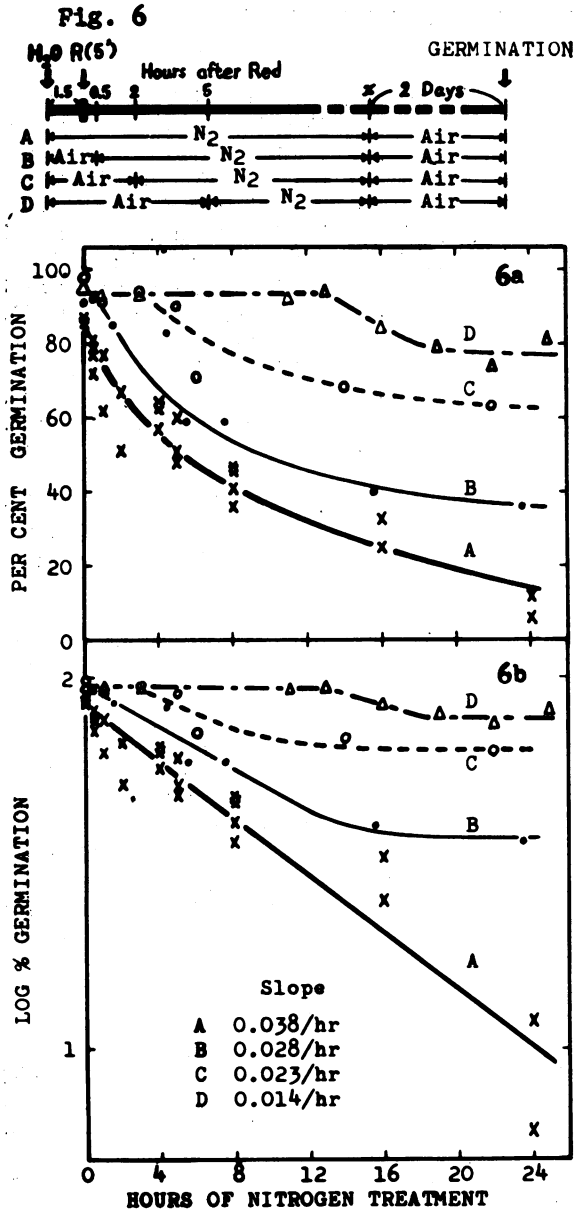
Nature of the First Postinduction Process. Seeds were kept at 25° throughout, and irradiated after exactly 1.5 hours of soaking, but placed in N_2 at 4 different times: A) at the beginning of soaking, B) 0.5 hour after red irradiation in air, C) 2 hours after red irradiation in air, and D) 5 hours after red irradiation in air. After various periods in N_2, the seeds were returned to air for germination, and scored after 48 hours. A diagram of the N_2 treatments and the data on a direct and a logarithmic plot are presented in figure 6. In treatment A, since N_2 had no significant effect during the preinduction period (fig 1), the duration in N_2 (abscissa) was measured from the red irradiation rather than from the start of treatment.

When seeds are treated with N_2 from the beginning of soaking (Curve A), inhibition of germination sets in immediately after red irradiation, and increases with time. This curve is comparable to the results which Wieser obtained with photosensitive tobacco seeds (38). The slight inhibition by N_2 previously observed (fig 4 and table I) can thus be ascribed to the 0.5 hour in N_2 after the red irradiation. When the onset of anaerobic treatment is

FIG. 3 (*upper right*). Effects of 4 different temperatures on water uptake.

FIG. 4 (*middle right*). Effect of N_2 atmosphere on dose-response curve. Seeds placed in nitrogen from the beginning of soaking to 0.5 hr after red irradiation. Red light (abscissa) given after 1.5 hrs of imbibition. Germination examined 2 days after transfer to air at 25°.

FIG. 5 (*lower right*). Effect of temperature on dose-response curve. Seeds treated at 3° and 35° from the beginning of soaking to 0.5 hr after red irradiation. Red light given after 1.5 hrs of imbibition. Germination examined 2 days after transfer to 25°.



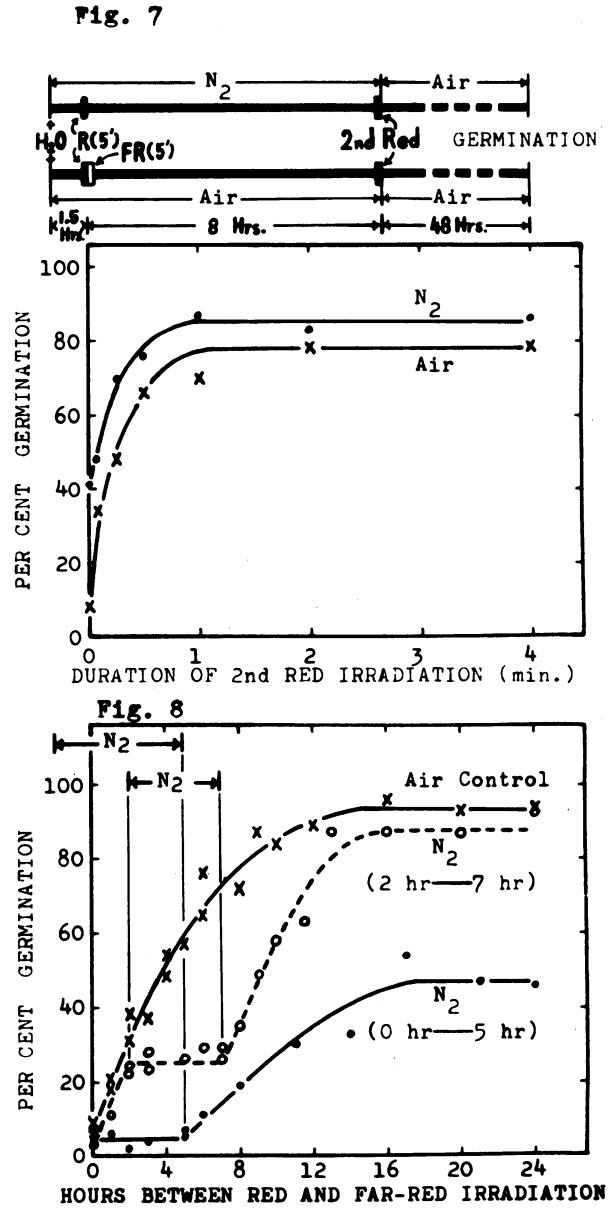


FIG. 6 (*left*). Effect on germination of the timing and duration of nitrogen treatment. Top diagram: experimental procedure. 6a: percent germination (ordinate) obtained with anaerobic treatments for various periods (abscissa). 6b: plot on logarithmic scale of the same data as in 6a. Abscissa for curve A refers to the hours in nitrogen after red irradiation. Temperature, 25°.

FIG. 7 (*upper right*). Dose-response curve for the reversal of N_2 inhibition by a second exposure to red light. Top diagram: experimental procedure. Temperature, 25°.

FIG. 8 (*lower right*). Effect of nitrogen atmosphere on the escape phenomenon. Seeds given N_2 atmosphere either from the beginning of soaking to 5th hour after red irradiation or from 2nd to 7th hour after red irradiation. Exposure to red light, 5 minutes at 0 time on abscissa; far-red light (5 min) given at various periods (abscissa) after red. Temperature, 25°.

delayed by 0.5 hour after red induction (Curve B), a lag period of about 0.5 hour precedes the inhibition, and the final degree of inhibition is less than in Curve A. Similarly, as the beginning of nitrogen treatment is increasingly delayed, the lag becomes longer and the inhibition less complete (Curves C and D).

During this series of experiments other effects of the N_2 atmosphere were also observed. Under our standard conditions (at 25° in air) the half-time

of germination is about 9 hours after red induction (22, 27). In treatment A, however, anaerobiosis delayed germination and the half-time increased from 9 hours to 16 hours (24 hr in N_2) in addition to the period in N_2. This effect was hardly noted with the seeds treated as B, C, and D. The germination of dark controls for treatments A, B, and C was essentially identical with that in figure 1, but no inhibition was observed with the dark controls for treatment D. It should be added that all the nongerminated seeds retained the ability to germinate, because they germinated 100 % in 3 days (in air) under diffuse light.

Since the 4 curves resemble a set of decay curves (fig 6b), it may be deduced that a substance responsible for the dark process of postinduction decays during the nitrogen treatment. Such a substance should be very closely associated with the phytochrome, since the inhibition took place immediately after the induction by red light (Curve A). Upon these assumptions the following 2 hypotheses are proposed: 1) the physiologically active form of the pigment, P_{fr}, decays under nitrogen, converting itself back to the physiologically inactive form, P_r; 2) a reaction which requires oxygen follows immediately after the formation of P_{fr} by red light. Those seeds which have completed this reaction would escape from the inhibitory effect of the nitrogen atmosphere, but those which have not would be inhibited. Observations of many workers that the rate of oxygen uptake by photosensitive seeds is promoted by photoinduction of germination (13, 19, 30, 33, 34, 35) would also support the second proposal.

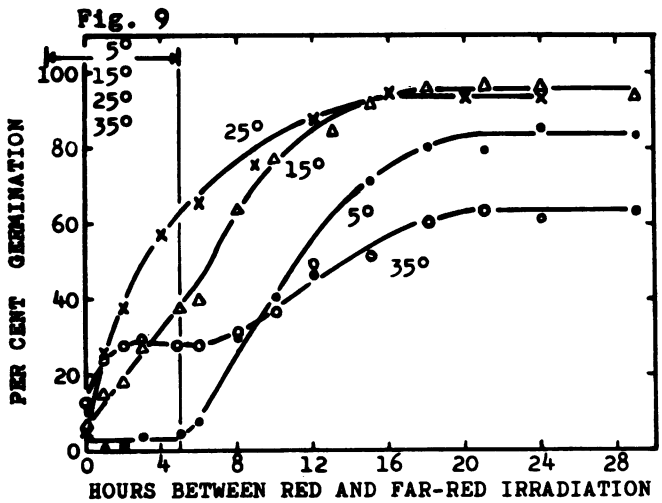
In order to prove the first hypothesis, seeds in N_2 were irradiated after 1.5 hours of soaking as before, but the anaerobic treatment was extended for 8 more hours thereafter. A second, varied red light exposure was then given while the seeds were still in N_2. Immediately thereafter, the seeds were transferred to air for germination. As air controls, seeds were irradiated with red light after 1.5 hours of soaking, but given far-red immediately afterwards. At the end of the 8th hour from the first red exposure, they were reirradiated with red light. The dose-response curves thus obtained in terms of the second red exposure are shown in figure 7 together with the diagram of treatments.

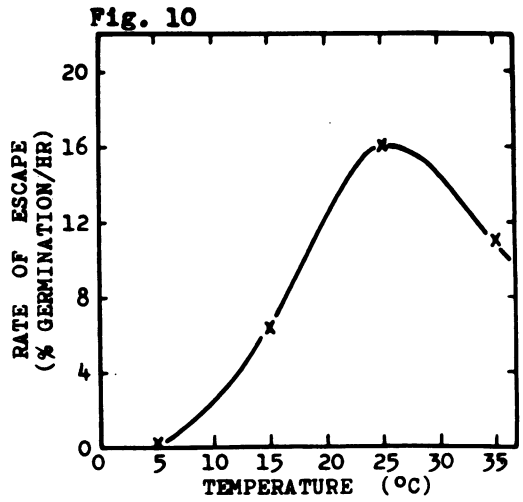
The results show that the saturating dose of red light is 1 minute of exposure in either N_2 or air, just as in figure 4. That the final values for air controls are lower than those for N_2 can be reasonably explained by the decreased sensitivity due to prolonged soaking (cf fig 1 and 2). If this is taken into account, the dose-response relationships agree remarkably well with each other. It follows that the P_r, which has been re-formed from P_{fr} during 8 hours in N_2, is reconverted to P_{fr} by the second red irradiation just as effectively as by the first.

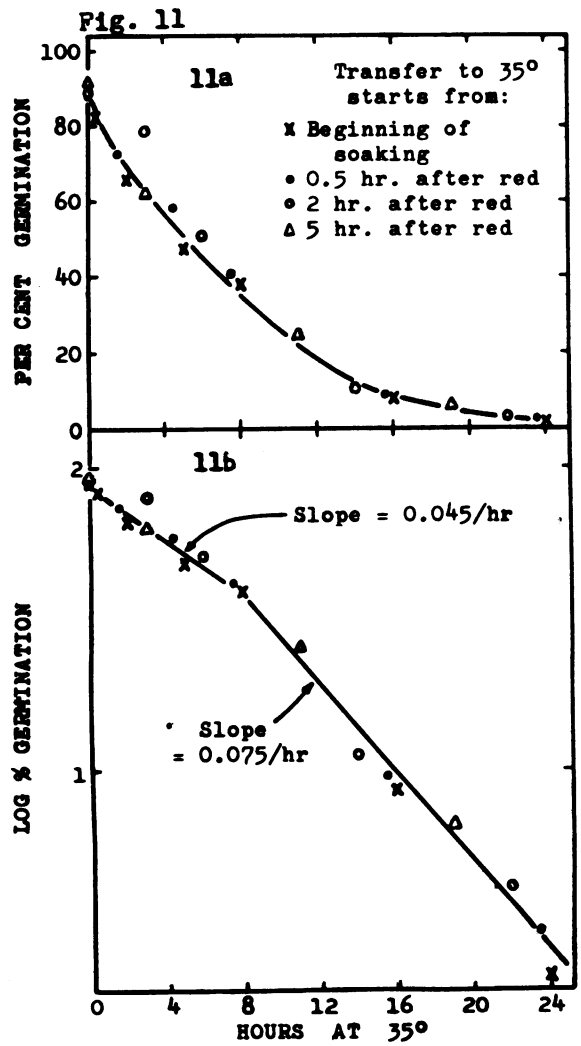
The seeds gradually escape from the inhibitory effect of far-red when it is given at progressively longer intervals after the red (6, 10, 24). The second hypothesis was, therefore, tested by using this phenomenon of escape. One group of seeds was placed in N_2 from the beginning of soaking, and returned to air at the 5th hour after the red irradiation (given after 1.5 hours of dark imbibition). Another group was exposed to red after 1.5 hours of soaking, then put in N_2 for 5 hours from the second to the seventh hour after red irradiation. Far-red light was given at various periods after the red. As controls, seeds were imbibed in air throughout the experimental period and irradiated similarly with red and far-red light. Figure 8 shows the results. First, 2 minor complications must be noted: (1) As the interval between red and far-red irradiation increases beyond 6 hours in air, some seeds start germinating (27); the data are not corrected for those seeds which had already germinated at the time of far-red irradiation. (2) The control curve in air is a little different from that obtained previously (24); the escape took place immediately after the red induction in the present experiments, whereas a lag of about 1 hour was observed previously. This discrepancy may be ascribed to the different types of seeds employed; high dark germinating type in the present experiments vs. low dark germinating type in reference (24).

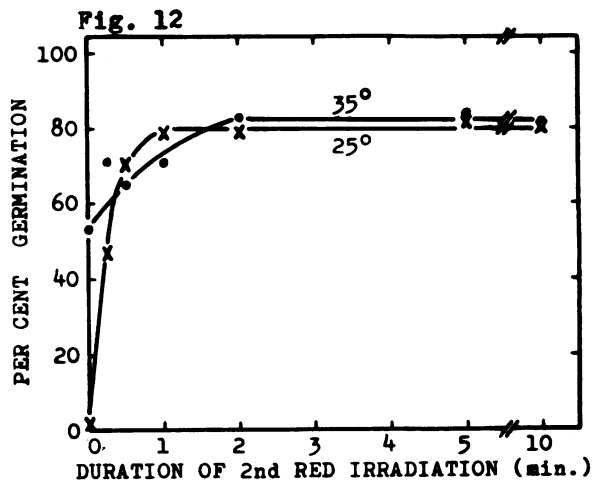
The results show that the seeds in air escape gradually from the inhibitory effect of far-red light; when far-red is not given until 12 hours after red, escape is complete. However, no escape takes place when the seeds are given far-red while still in N_2. The fact that N_2 treatments yielded a lower final germination than air controls is of course expected from the inhibitory effect of the anaerobic atmosphere (cf fig 6). The finding that N_2 inhibits the escape phenomenon strongly supports the idea that N_2 blocks an oxidative reaction which immediately follows the formation of P_{fr}. Furthermore, these results show a close association between the escape phenomenon and the oxidation reaction. It can, therefore, be concluded that the seeds in which the metabolism requisite for germination has completed this oxidation are no longer inhibited by far-red light or anaerobic conditions.

Since the escape phenomenon is a dark process, it should be dependent on temperature. Seeds were, therefore, allowed to imbibe at a specified temperature, irradiated with red light, and transferred to 25° at the 5th hour after exposure to red. Far-red light was given at various periods after red induction. Control seeds were imbibed and kept at 25° throughout. Figure 9 shows that the rate of escape is virtually zero at 5°, but increases progressively as the temperature is raised. When seeds are transferred to 25° from 5° or 15°, their rate of escape becomes comparable with that of 25° controls. However, 35° behaves differently; as the period of treatment increases, the escape is gradually prevented, then stops, or even slightly decreases. Furthermore, the inhibition does not recover fully even after the seeds are returned to 25°. The inhibitory action of









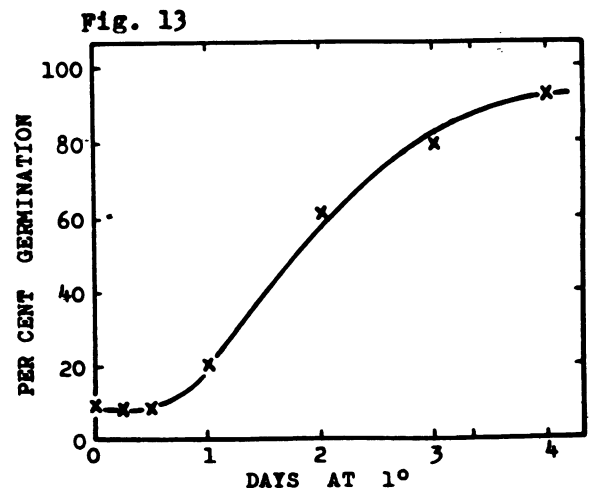


FIG. 9 (*upper left*). Effect of 4 different temperatures on the escape phenomenon. Temperature treatment, from the beginning of soaking to 5th hour after red irradiation. Red light given at 1.5 hours of soaking for 5 minutes (0 time on abscissa). Far-red irradiation, 5 minutes. All seeds transferred to 25° after temperature treatment.

FIG. 10 (*upper right*). Effect of temperature on the rate of escape.

35° is evidently different from that of the N_2 atmosphere (cf fig 8, also below).

The rates of escape at different temperatures, measured from the curves in figure 9, are plotted in figure 10. The optimum temperature for escape is about 25°, at which the rate is 16 % per hour. The Q_{10} values can be obtained from the figure as follows: $Q_{5-15°} > 10$, $Q_{15-25°} = 2.5$, and $Q_{25-35°} = 0.7$. From these values the activation energy (E_a) for the temperature range of 15° to 25° is 16,000 calories.

It can be concluded that the anaerobic treatment not only facilitates the dark conversion of P_{fr} to P_r, but also blocks the oxidative process which follows immediately after the formation of P_{fr}. Furthermore, this oxidative reaction controls the escape phenomenon, which is highly temperature sensitive.

Effects of High Temperature. Although 35° inhibits the overall germination of lettuce seeds (6, 22), it affects very little the first phase of radicle growth (22) or the first two phases of the germination processes (fig 2b, 5, and table I). These results indicate that the inhibitory effect of this temperature is exerted on a later stage of germination. In order to shed some light on the nature of the inhibition, therefore, a series of experiments was carried out essentially with the same procedure as that in figure 6. Figures 11a and 11b show the results.

Regardless of the starting time of the treatment, inhibition sets in as soon as the seeds are exposed to 35°, and progresses, apparently in 2 steps, with time at 35°. This behavior clearly differs from that in N_2 (fig 6). It can be concluded, therefore, that 35° exerts a general inhibition of processes of the postinduction phase, probably in later stages. The fact that the inhibition is hardly detectable if the seeds are treated at 35° only during the phases of preinduction and induction supports this conclusion further.

One possible explanation of so broad an effect, both on postinduction and on escape, would be that 35° acts by converting the P_{fr} back to P_r in the dark. This point was examined by reversing a partial inhibition due to 35° by a second exposure to red light, using a procedure similar to that for the N_2 experiments (cf fig 7). The dose-response curves obtained are shown in figure 12.

The control curve for 25° agrees well with those in figures 4 and 7; i.e., the saturating dose is 1 minute exposure to red light. The curve for 35°, on the other hand, indicates that at least 2 minutes' irradiation are required for maximum induction of germination. Thus the effect of 35° again differs from that of N_2 (cf fig 7). Nevertheless, the main fact shown is that the second exposure to red light fully reverses the inhibition caused by 35°; hence 35° must have caused the dark reversion of P_{fr} to P_r. However, the difference in shape of the dose-response curves indicates that this temperature causes other inhibitory effects as well. In view of the reports that the isolated pigment system is highly heat-labile (2, 8) partial denaturation of the phytochrome system at 35° might be one of those effects, though this alone may not explain all the observations.

Effects of Low Temperature. It was earlier shown that dark-imbibed seeds germinate to the full extent at a constant temperature of 3° (22). These findings have been confirmed and extended by allowing seeds to imbibe in darkness at 1° ± 1° from the beginning, and then transferring to 25° for germination after various periods of time (0–4 days). No red light was given at any time and no sign of visible germination was noted during the cold treatment. The final germination was examined 2 days later as usual. The results (fig 13) show that induction of germination takes place gradually after the first day and reaches a maximum 4 days later. This compares with the dark germination shown in figure 2. Thus low temperature appears to *substitute for red light,* though very slowly.

This raises the question as to whether the low temperature effect is inhibited by far-red. Far-red irradiation was, therefore, given for 5 minutes to the seeds either before or after the treatment at 2°. When given before the cold treatment, the seeds were imbibed at 25° for 1.5 hours first. After the cold treatment, all were transferred to 25° for germination.

The results (table II) show that germination is inhibited to the same extent by far-red light whether this is given before or after the cold treatment. This inhibition is greater with the low temperature for 1 day than with that for 3 days, but there is clearly *no reversal* of the stimulation caused by the cold. Thus, just as with gibberellins (18, 24, 25), the action of low temperature is to stimulate germination at a point other than that controlled by phytochrome.

Discussion

The Behavior of Phytochrome in Lettuce Seeds. The photoconversion of the pigment has been demonstrated by table I and figures 4 and 5 to be a pure photoreaction independent of temperature and O_2,

FIG. 11 (*middle and lower left*). Effect on germination of the timing and duration of treatment at 35°. Treatments started at 4 different periods of soaking (inset) and given for the periods on abscissa. For seeds given 35° from the beginning of imbibition, the abscissa refers to the period after red irradiation (5 min). 11a: data as obtained. 11b: plot on logarithmic scale.

FIG. 12 (*middle right*). Dose-response curve for the reversal of 35° inhibition. Seeds treated at 35° from the beginning of imbibition to 6th hour after first red exposure (5 min at 1.5 hr of soaking). The second red irradiation (abscissa) given immediately before transfer to 25°. Germination of 25° control seeds inhibited by far-red light (5 min) immediately after first red, restimulated by second red (abscissa) at 6th hour after the first.

FIG. 13 (*lower right*). Effect of cold temperature (1°) on dark germination.

Table II. *Effects of Far-Red Light on Germination of Grand Rapids Lettuce Seeds Treated with Low Temperature (2°)*

Irradiation with 5 minutes far-red light was given at 25° either immediately before or immediately after the cold treatment. Control seeds imbibed and were germinated at 25° throughout, and irradiated with red or far-red light at 1.5 hours after the beginning of soaking.

Treatment before final transfer to 25° for germination	% germination	% inhibition*
1 day at 2°, then far-red	6	80
Dark control	30	..
1.5 hours at 25°, then far-red, then 1 day at 2°	14	73
Dark control	51	..
3 days at 2°, then far-red	59	23
Dark control	77	..
1.5 hours at 25°, then far-red, then 3 days at 2°	65	29
Dark control	92	..
25° control, 5 min red	92	..
" , 5 min far-red	5	70
" , dark control	17	..

* Calculated for far-red inhibition with respect to corresponding dark control.

whereas the dark reversion of isolated phytochrome from P_{fr} to P_r is reported to be temperature-sensitive (10, 21). Our results indicate that the postinduction phase, when the pigment is in the P_{fr} form, is sensitive to both nitrogen and high temperature (fig 6, 8, 11), while the preinduction phase, in which the pigment is P_r, is not sensitive to either treatment (fig 1, 2a, b). These observations, together with the fact that dark reversion occurs only from P_{fr} to P_r (fig 7, 8; refs. 3, 7, 9, 21), imply that the P_{fr}, though physiologically active, is less stable than P_r.

Dark reversion of P_{fr} to P_r occurs in vivo in N_2 (fig 6, 7), and it apparently follows a first order kinetics (fig 6b). However, as the start of N_2 treatment is delayed, the start of inhibition by N_2 is also delayed, and the rate of dark reversion lowered. This suggests that the P_{fr} in the seed is protected from reversion once it has become involved in the succeeding reaction. This idea is further supported by the fact that the photosensitive seeds, once photoinduced to germinate and then dried in darkness, retain high ability to germinate upon rewetting in the dark (cf 11).

Role of P_{fr} in the Germination Processes. Figures 6, 8, and 9 show that a highly temperature-sensitive, oxidative reaction follows immediately after the formation of P_{fr} by red light. This reaction is, furthermore, shown to control the escape mechanism. Apparently it is irreversible in vivo, and seeds which have finished the reaction are no longer inhibited by far-red. Thus the oxidative reaction is probably one of the major steps in photocontrolled germination. Its optimum temperature is about 25°, and the minimum 5° (fig 11). Since at 25° the $t_{half\text{-}max}$ for escape is about 3 hours (measured from control curve in fig 9), this reaction seems to take up no more than one-third of the time of the postinduction phase. At 15° the $t_{half\text{-}max}$ values are 18 hours for germination (22) and 7 hours for escape (estimated from fig 9).

A further kinetic analysis of this reaction can be made. If the fraction of P_{fr} (F) which has been used by the oxidative reaction leads to germination, the following sequence of processes can be formulated:

$$\underset{(1-F)}{P_{fr}} \xrightarrow{O_2} \underset{F}{\text{Product}} \longrightarrow \longrightarrow \underset{aF}{\text{Germination}}$$

where a is a proportionality factor, a constant. Since the concentration of O_2 will not limit the reaction under normal, aerobic conditions, the reaction rate is considered to be proportional to the concentration of P_{fr} available for the reaction. Thus, $daF/dt = k(1 - F)$. Upon integration we obtain $\ln(1 - F) = -k_t/a + C$. At $t = 0$, $F = 0$ and hence $C = 0$. Hence, $\ln(1 - F) = -k_t/a$ or $\log(1 - F) = -K_t$, where $K = 1/2.303 \cdot k/a$. Since the escape curve in air (fig 8) can be regarded as showing the fraction of P_{fr} which has been used in the oxidative reaction prior to the removal of the pigment from the reaction by far-red irradiation, the value (100 − % germination) can be interpreted as $(1 - F) \cdot 100$ of the above equation. In figure 14, the data of the escape curve in air (fig 8) are replotted. The experimental variations increase as time lengthens. Nonetheless, a linear relationship between $\log(1 - F) \cdot 100$ and t is clearly shown, with $K = 0.073$/hr. The escape reaction is therefore of first order with respect to P_{fr};

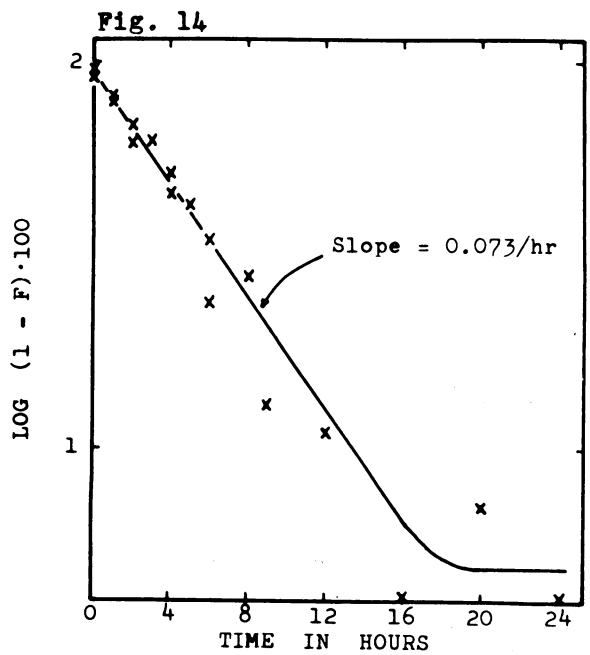


FIG. 14. Kinetic analysis of escape reaction. Same data as those of air control in figure 8 are plotted in terms of log (100 − % germination) vs. hours between red and far-red irradiation.

in other words, the P_{fr} may be the limiting factor for this reaction.

Comparison of the K value with the decay constant for the dark reversion (see inset for curve A of fig 6b) brings out another point. Should P_{fr} undergo dark reversion in air at the same rate as in N_2, though half the rate of the oxidative reaction, 100 % germination could never be obtained with air-soaked seeds by a single, short irradiation. This happens in *Chenopodium botrys* seeds, in which at high temperatures the dark reversion is so rapid that several light exposures are required for optimum germination (10). The type of dark reversion of P_{fr} to P_r shown in figure 6 is, therefore, somehow prevented in vivo under aerobic conditions.

Using a direct spectrophotometric method to measure phytochrome in dark grown maize shoots, Butler et al. (7) recently reported that reversion of P_{fr} to P_r as well as a decrease in total amount of photoreversible phytochrome took place in the dark following a single brief exposure to red. Neither phenomenon was, however, detected when the plants were placed at low temperature or in N_2. In many respects their findings agree with ours, especially if the loss of total phytochrome content were due to participation of P_{fr} in the succeeding reaction. However, their observation that no dark reversion took place under N_2 disagrees with ours, since we could observe it only in N_2. This difference might be explained by different types of reactions in which P_{fr} participates, e.g., in an oxidative reaction in the case of lettuce seeds and perhaps in a nonoxidative process in dark-grown shoots (cf 34).

Our frequent observation that the photosensitivity of lettuce seeds gradually decreases after 1.5 hours of dark soaking at 25° (fig 1, 2) poses interesting questions as to the nature of the germination processes: 1) does the concentration of the phytochrome itself decrease, 2) does the concentration of substance(s) which react with the P_{fr} in the oxidation reaction become limiting, or 3) do other substances and reactions involved in a later stage of the postinduction phase cause the decrease of sensitivity? The answer to the first question can be obtained from the daily observation that seeds which have not germinated during experimental treatments, in air or in N_2, are capable of 100 % germination in 3 days if they are subsequently transferred to a laboratory bench under diffused light; controls kept in the dark remain ungerminated. If the concentration of phytochrome were to decrease as the pre-soaking period extended, 100 % germination would not be obtained on later exposure to diffused light. The first possibility thus appears highly unlikely. Using *Lepidium* seeds, Toole et al. (37) showed that a temperature alternation immediately before or after red irradiation favored maximum induction of germination by red light. If the oxidative reaction is common to all photosensitive seeds, this finding is highly suggestive of the participation of other reactant(s) here. Furthermore, when low temperatures were given only during the preillumination period, there was no decline in the sensitivity curves (fig 2a, b), suggesting that the escape of the substance(s) in question is somehow inhibited by low temperature. When induced seeds were exposed to high temperature, the dose-response relationship with a second red light was different from that with the first, whereas in 25° controls these remained identical (fig 5, 12). This indicates that the substance(s) which cause the decrease of sensitivity are more closely connected with the P_{fr} than with the postinduction reactions. The second alternative is thus very probable. It should be mentioned that a single-peaked sensitivity curve has been reported with other photosensitive seeds (1, 28, 29, 30, 38).

Possible Mechanism and Site of Germination in the Lettuce Seed. From our data and those of the Beltsville group (5, 6), the germination processes can be presented diagrammatically as in figure 15. During

Fig. 15

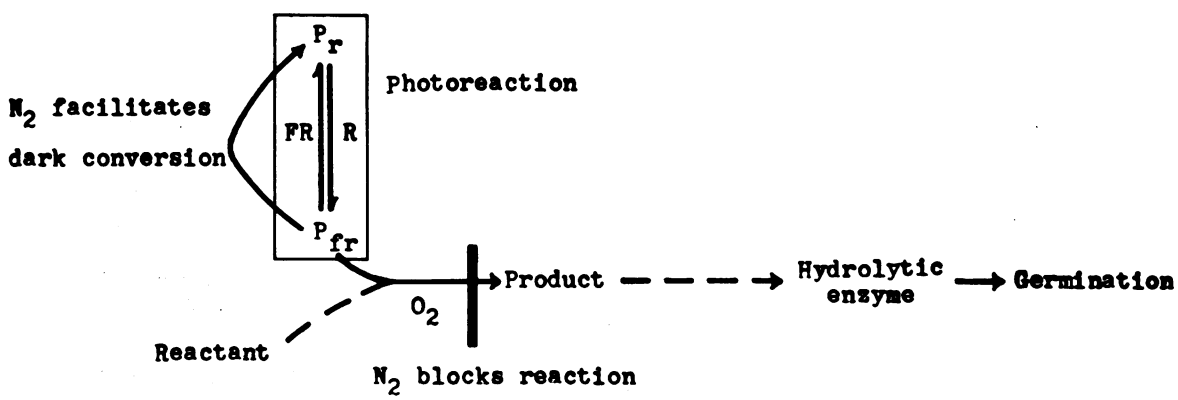


FIG 15. Schematic presentation of possible germination processes. P_r: red absorbing form of phytochrome. P_{fr}: far-red absorbing form of phytochrome. R: red light. FR: far-red light. Dotted lines: processes not critically examined.

imbibition the internal condition of the seed is gradually brought to a state where the phytochrome reaction can occur maximally. This state is attained when the water content of the seed has increased by 40 %. After the photoconversion of P_r to P_{fr}, the latter participates in a reaction in presence of O_2 and probably of another reactant. Nitrogen inhibits this reaction and thus facilitates the dark reversion of P_{fr} to P_r. The processes intervening between the oxidative reaction and the visible protrusion of radicles remain unknown. It has been concluded, however, that formation or activation of a hydrolytic enzyme explains most fruitfully the mechanism of radicle protrusion through the surrounding seed-coats (*27*).

It was previously observed that germination of the intact seed and the growth of the embryo both gave rise to the first sign of development at about the same time (*22, 27*). However, while germination was controlled by the reversible phytochrome system, radicle growth was not (*22, 27*). From cytological studies of the lettuce embryo, Haber and Luippold (16, 17) showed that the occurrence of mitoses and cell elongation could be regulated physically as well as chemically; e.g., mannitol alone or a combination of kinetin and high temperature (37°) promotes mitosis prior to actual germination, whereas a combination of thiourea and 37°, or that of gamma irradiation and kinetin, makes germination precede cell division. Their findings support our conclusion that the growth of the radicle and the germination of the seed are not identical phenomena. The above scheme is put forward to explain the germination processes per se.

Klein and Preiss (31) have shown that the photosensitivity of lettuce seeds does not change even when they are turned over in the interval between red and far-red irradiation. The participation of P_{fr} in an oxidative reaction and its presence mainly in the axis half of the seed (*23*) fits well with its location close to the extreme tip of the seed, where light can act from either side and O_2 is readily accessible.

Furthermore, the most reasonable explanation for the last phase of germination is to assume an enzymic breakdown of the mechanically restricting endosperm (*27*). In view of failure to demonstrate the enzyme in the axis, it is probably produced in only minute quantity in the vicinity of the action site. All these observations suggest strongly that all the processes of germination take place at the same site, namely close to the tip of the embryonic axis.

Summary

The physiological nature of the germination processes in Grand Rapids lettuce seeds was analyzed by applying a nitrogen atmosphere or a range of temperatures during the different phases of germination. Controls were germinated in air at 25°.

The preinduction phase, which takes about 1.5 hours in our standard procedure, is not influenced by placing the seeds in nitrogen, but is sensitive to temperature. Since there is a correlation between water content in the seed (roughly 40 % above air-dry weight) and maximum induction of germination by red light, and since water uptake by the seed is sensitive to temperature, the temperature effect on this phase was ascribed to its effect on water uptake.

The induction phase, in which the reversible reaction of the phytochrome functions maximally, is shown to be a pure photoreaction independent of temperature and oxygen.

The postinduction phase, which then takes about 9 hours, starts with a highly oxidative reaction which follows immediately after photoinduction. In seeds kept under anaerobic conditions this oxidation is inhibited and the escape from inhibitory far-red light stops, but the pigment becomes converted in the dark from the far-red absorbing form (P_{fr}) back to the red absorbing form (P_r). Under aerobic conditions both the oxidation and the escape phenomenon occur, but the dark reversion of the pigment is difficult to detect, and in all probability it occurs only to a minor extent. It is concluded that P_{fr} participates actively in the oxidation reaction and that this reaction directly controls the escape phenomenon. Escape is sensitive to temperature, showing an optimum at 25°.

Inhibition of germination caused by 35° is quite different from that due to nitrogen treatment, in that it occurs regardless of the time at which the seeds are transferred to 35° and is not reversed by a second red irradiation in the same manner as is the nitrogen inhibition. It is concluded, therefore, that 35° causes a general inhibition of the postinduction phase.

Low temperature (2°) can substitute for red light, causing full germination in the dark on subsequent transfer to 25°. However, this effect of low temperature is not reversed by far-red, so that, like gibberellin, it must act at a point other than that controlled by phytochrome.

Possible mechanisms and the site of the germination processes in lettuce seed are discussed and a scheme of the sequence of germination processes is presented.

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