# ON PHYTOCHROME IN LEMNA MINOR AND OTHER LEMNACEAE

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SUMMARY

The level of phytochrome in *Lemna minor*, grown in darkness interrupted by short during illuminations on a medium containing sucrose, is strongly affected by quality and timing of these illuminations. Red light induces a decrease in phytochrome. During a subsequent dark period, there is a recovery persisting for several days. This recovery was found to be independent of the initial degree of reduction, caused by the illumination. It is also insensitive to auxins and actinomycin D.

Phytochrome could also be demonstrated spectrophotometrically in *Lemna perpusilla*, *Lemna gibba* and *Spirodela intermedia*.

## 1. INTRODUCTION

Growth of *L. minor* on a sterile nutrient medium with 1% sucrose decreases to a low level when the cultures are transferred from light to darkness. After some days in darkness a short illumination with red light stimulates multiplication and frond expansion; this effect is abolished by subsequent illumination with far-red (HILLMAN 1957, ROMBACH 1965, 1966). This points to phytochrome as the photoreceptor for the growth-promoting effect of red light. Probably phytochrome is also involved in photoperiodicity of flowering in *L. perpusilla* (HILLMAN 1966). Effects of red and far-red on growth and flowering of *L. gibba* are described by KANDELER (1956, 1966) and by CLELAND & BRIGGS (1968). However, HILLMAN (1964) could not assess the presence of phytochrome by difference spectrophotometry in L. *perpusilla*.

Our success in finding phytochrome in *L. minor* (EVANS 1964, ROMBACH 1965) led us to examine also the following species, interesting from the point of view of photoperiodic reactions: *L. perpusilla* strain 6746, being a short day plant, and *L. gibba* strain G3, a long day plant. Further, *Spirodela intermedia* was examined, as suggested by Dr. J. W. McClure, Miami University, who found interactions of red and far-red on flavonoid synthesis in this plant (MCCLURE 1968).

## 2. METHODS

The strain of *L. minor* was obtained from Dr. W. Lindeman of this laboratory, *L. perpusilla* strain 6746, *L. gibba* G3, and *Spirodela intermedia* were supplied by Dr. J. W. McClure.

All strains were grown in sterile culture with mineral salts, 15 g sucrose,

0.26 g casein hydrolysate, 0.005 g tryptophane and 0.0008 g ($3.10^{-6}$M) kinetin per liter. Temperature was 22 °C. Kinetin is necessary for a sufficient growth rate of *L. minor* in cultures receiving only a few minutes illumination per day. Phytochrome is also found in cultures grown without kinetin.

Unless mentioned otherwise, plants were grown with a standard illumination program, consisting in 5 minutes red light of 5500 erg/cm²/sec once a day. Red light was obtained from fluorescent tubes combined with a filter consisting of 1 cm of an aqueous solution of 30 g/l $CuSO_4$ and 3 mm red Plexiglass Röhm und Haas 501. Far red was obtained from incandescent lamps in combination with a filter consisting of 5 cm water and 2,5 mm RG 9 glass from Schott-Mainz.

The growth rate of the cultures was constant for 3 weeks; during this period an inoculum of 4–6 fronds yielded 150–200 fronds.

*L. minor*, grown under these conditions, contained a small amount of chlorophyll, contributing to the relatively high apparent optical density (OD) of the samples. Changes in OD at 730 nm relative to OD at 800 nm in a layer of packed fronds of mostly 1 cm depth were measured with a dual wavelength spectrophotometer (DE LINT & SPRUIT 1963). An example of a measurement is given in *fig. 1*. Phytochrome content, as measured in this way, does not represent a quantity that is directly related to concentration of the pigment in the material. This is because the effective optical path-lengths in the highly scattering samples are unknown. For this reason, comparisons between phytochrome contents, measured in different materials, such as different parts of the same plant, or similar parts that had been grown under different conditions, should only be made with great caution. It is not always certain, therefore, that observed changes in the apparent phytochrome content are due to real changes in the amount of phytochrome. Because our material is, morphologically, rather homogeneous, and because exact methods to estimate the effects of changes in light-scatter and cellular distribution of pigment were not available, we have disregarded their possible effects in the present discussion.

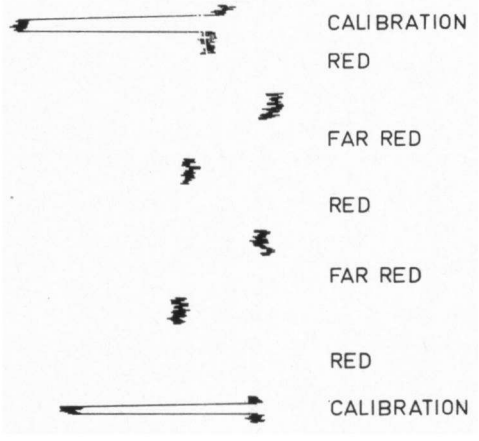


Fig. 1.
Example of a phytochrome estimation in *L. minor*. Optical density changes at 730 nm relatively to 800 nm in response to alternate 1 min. exposure to red (653 nm) or far red (737 nm); shift to the left indicates increased absorption at 730 nm. The calibration marks at the beginning and end represent an optical density increment of about $4 \times 10^{-3}$ at 800 nm. Depth of sample 10 mm.

Tightness of the content of the sample cell to some degree influenced the results of the measurement: 40% more material occupying the same volume gave a 30% higher Δ(ΔOD) value. Care was taken to fill the sample cells always with equal amounts of fronds (± 1,7 gram fresh weight); when carried out in this way measurements were reasonably reproducible.

## 3. RESULTS

### 3.1. Location of phytochrome

In etiolated seedlings the phytochrome concentration appears to be highest in the meristematic parts (BRIGGS & SIEGELMAN 1965). Meristematic tissue in adult fronds of *Lemna* is located in reproductive pockets at both sides of the proximal end of the frond. The proximal parts of a certain amount of *L. minor* fronds were cut off and the phytochrome in them and in the distal parts determined separately. Most of the phytochrome was found in the proximal parts as is shown in *table 1*. On the basis of dry weight, however, the phytochrome content does not appear to differ significantly in parts with and without meristems.

Table 1. Distribution of phytochrome in *L. minor*; Δ(ΔOD) values measured in sample cells of 6 mm depth.

| | Δ(ΔOD) × $10^4$ | Δ(ΔOD) × $10^4$ per gram fresh weight | Δ(ΔOD) × $10^4$ per 100 mg dry weight |
|---|---|---|---|
| Part with meristems | 15,1 | 12,7 | 10,8 |
| | 14,9 | 12,4 | 10,6 |
| Part without meristems | 8,2 | 10,0 | 12,0 |
| | 6,5 | 8,0 | 9,5 |
| Entire fronds | 13,4 | 14,7 | 13,1 |

### 3.2. Time course of phytochrome content in the dark interval between two illuminations

In two experiments with *L. minor* plants, treated with 10 minutes red light every 24 hours, phytochrome was measured at regular intervals during the dark period. Statistically there was no difference between the results of these experiments, and the averages of the two sets of values are plotted in *fig. 2*. This figure shows a decrease in total phytochrome content during four hours after illumination. Thereafter the phytochrome content again rises slowly to about the original value.

In *L. minor* decrease is relatively slow and incomplete after illumination compared with maize coleoptiles (DE LINT & SPRUIT 1963), the lowest concentration being still 50% of the original level. The phytochrome level is not much further reduced by a second illumination, given 3,5 hours after the first one *(fig. 3)*. The same graph shows that also a radiation with red light during 6 hours did not reduce the phytochrome level more than a radiation of 10 minutes.

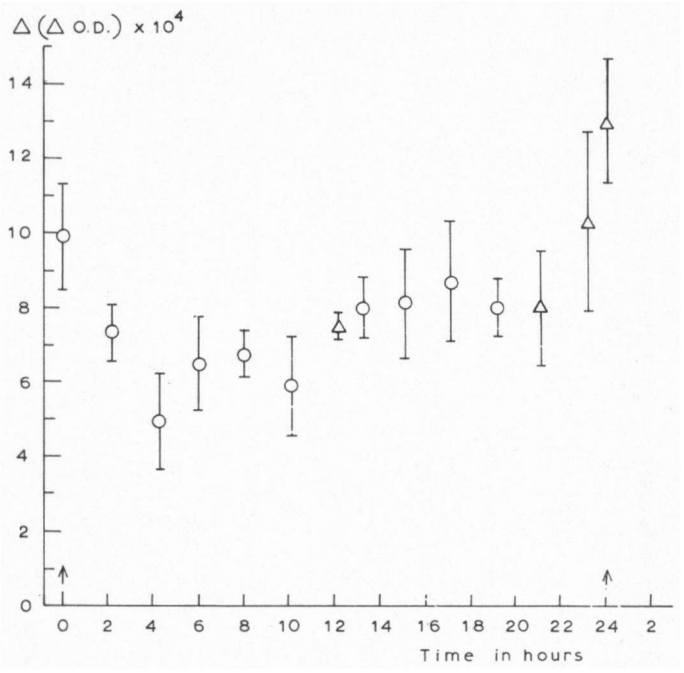


Fig. 2. Time course of total phytochrome content during a dark interval in *L. major* grown with 10 minutes red light once per day.
Standard deviations $s = \sqrt{\Sigma(x - \bar{x})^2/(n-1)}$ are indicated with bars; n = number of observations.
o: n = 4; Δ: n = 2; ↑ time of illumination.

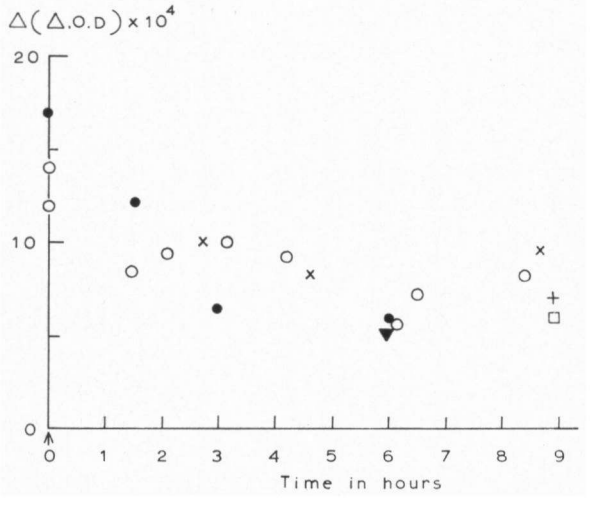


Fig. 3. Time course of the phytochrome content after ten minutes of red irradiation at zero time. Experiment a: O no additions; + same, but a second illumination after 3,5 hours; × $10^{-4}$ M IAA added at zero time; □ same, but second illumination after 3,5 hours. Experiment b: ● control; ▼ 6 hours continuous red light λ 660 nm, starting after illumination at zero time.

CLARKSON & HILLMAN (1967) reported that apparent phytochrome synthesis in *Pisum* was inhibited by actinomycin D, naphtalene acetic acid (NAA) or indole acetic acid (IAA). If these substances would have the same effects on phytochrome synthesis in *L. minor*, without interfering with phytochrome destruction, a steeper decrease to a lower level might result from their application, no restoration to the original level after 24 hours of darkness would occur, but a more clear picture of phytochrome destruction would be obtained.

In a first experiment, $10^{-4}$M IAA was added at the moment of illumination (fronds of *L. minor* grown at this concentration are very stunted). Some cultures were illuminated for a second time to bring about a further decrease in phytochrome level. At different moments after illumination, phytochrome levels were measured. *Fig. 3* shows the results; there is no influence of IAA on the phytochrome level, the decrease after illumination being the same as without IAA. The influence on the restoration level of phytochrome, 24 hours after an illumination, is studied with different concentrations of IAA, 1-NAA, and actinomycin D. The results are listed in *table 2*. For cultures treated with actinomycin D and with 1-NAA phytochrome determinations were also made 3–4 hours after one or two illuminations, to make sure that the phytochrome decrease after an illumination was not affected by these substances.

*Table 2* shows that IAA in none of the concentrations tested has an influence on the restoration level of phytochrome.

With 1-NAA the outcome is different: $10^{-4}$ M without a preceding illumination causes a disappearance of almost all phytochrome within 16 hours, so also the $P_{660}$ form of phytochrome proved to be unstable. However, when the plants were examined it was found that most of them were killed. The plants could stand $10^{-5}$ M NAA somewhat longer, but after an incubation time of 48 hours plants were dying at this concentration also. Before that time there was no effect on the phytochrome level.

Actinomycin D was applied in three concentrations, but in no case an effect on decrease or restoration of the phytochrome level after an illumination was found.

### 3.3. Phytochrome level of *L. minor* as affected by illumination quality and illumination schedule

In connection with experiments concerning $P_{730}$ formation and growth of *L. minor* (ROMBACH 1965) the question had to be answered whether the phytochrome level in plants grown under different conditions of illumination was the same. CLARKSON & HILLMAN (1967) demonstrated that in *Pisum* destruction and synthesis of phytochrome are both influenced by the amount of $P_{730}$ generated by light. If the same holds also for *L. minor*, great differences can be expected with different light treatments.

Different degrees of conversion of $P_{660}$ to $P_{730}$ were obtained by using either different amounts of red or of far-red after a saturating red illumination. The percentage of phytochrome conversion was estimated from the effect of light treatment on the multiplication rate (ROMBACH 1965: 50% phytochrome con-

Table 2. Effect of indole acetic acid (IAA), 1-napthalene acetic acid (NAA), and actinomycin D on the phytochrome level after a red illumination in *L. minor*. IAA was dissolved in ethanol, controls obtained the same amount of ethanol.

| Substance and concentration | hours of incubation with substance | Dark interval from illumination to measurement in hours | | Δ(ΔOD) × $10^4$ |
|---|---|---|---|---|
| | | from first illumination | from second illumination | |
| Ethanol 0,08% | 24 | 24 | | 13 |
| | 32 | 32 | | 18 |
| IAA $10^{-6}$ M | 22 | 22 | | 11 |
| | 31 | 31 | | 16 |
| IAA $10^{-5}$ M | 23 | 23 | | 11 |
| | 32 | 32 | | 17 |
| IAA $10^{-4}$ M | 23 | 23 | | 11 |
| | 32 | 32 | | 15 |
| control | | 48 | | 25 |
| | | 4 | | 10 |
| | | 7 | 3 | 9 |
| | | 24 | | 15 |
| $10^{-5}$ M NAA | 16 | 48 | | 25 |
| | 24 | 3½ | | 23 |
| | 23 | 7 | 3 | 9 |
| | 48 | 24 | | 11 |
| $10^{-4}$ M NAA | 16 | 48 | | 4 |
| | 4 | 4 | | 17 |
| Actinomycin D | 16 | 48 | | 19 |
| 5 mg/L | 19½ | 3½ | | 14 |
| | 23 | 7 | 3 | 7 |
| | 48 | 24 | | 18 |
| 10 mg/L | 23 | 24 | | 28 |
| | 6 | 3½ | | 15 |
| | 24 | 11 | | 17 |
| 50 mg/L | 23 | 24 | | 28 |
| | 6 | 3½ | | 17 |
| | 24 | 11 | | 16 |
| control | | 24 | | 25 |
| | | 3½ | | 20 |
| | | 11 | | 19 |

version gives a growth stimulation of 84% and is reached with $9.10^5$ quanta/$cm^2$ red λ658 or with $15.10^4$ ergs/$cm^2$ red light of the quality used in these experiments). The light treatment was repeated once in 24 hours, during a period of 7 days. Phytochrome was measured 24 hours after the last illumination.

The resulting phytochrome values, presented in *fig. 4*, show that cultures exposed every day to a saturating amount of red light contained half as much phytochrome as plants kept in continuous darkness for 7 days.

The phytochrome level is also affected by frequency of illumination. This is shown in *table 3*. In this experiment, cultures were grown for 20 days under the indicated conditions.

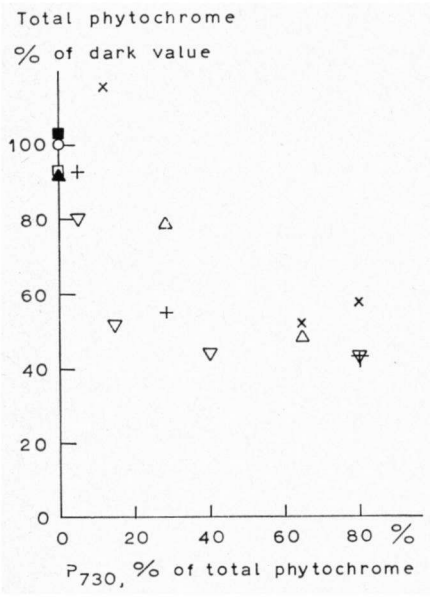


Fig. 4.
Influence on the phytochrome level in *L. minor* of different degrees of phytochrome conversions brought about by illuminations repeated once in 24 hours during 7 days. Phytochrome conversion was brought about by a short irradiation (not exceeding 10 minutes) with different amouts of red or far red light. The degree of phytochrome conversion (abscissa) is calculated from effect of light treatment on growth rate. Phytochrome is measured 24 hours after the last light treatment, values are percentages of controls in continuous darkness for 7 days. Experiment a: + red; □ red followed by far-red; ■ far-red. Experiment b: ▽ red. Experiment c: × red λ658 nm; △ red λ658 nm followed by far-red λ740 nm; ▲ far-red λ740 nm.

Table 3. Influence of frequency of illumination (5 minutes red light of 5500 ergs/cm² sec) on phytochrome level measured at the time of illumination in *Lemna minor*.

| Number of illuminations per 24 hour | $\Delta(\Delta OD) \times 10^4$ |
|---|---|
| 1 | 21 |
| 2 | 11 |
| 4 | 4 |
| 8 | 3 |

*Table 3* shows that phytochrome level, measured at the end of a dark period is lower the shorter the dark period between illuminations is. This is according to expectation: *figures 2* and *3* show that an illumination is followed by a decrease in phytochrome content whereas restoration of the phytochrome level is slow.

If the dark period is extended beyond 24 hours, the phytochrome level rises further. This is shown by *fig. 5*, giving results, expressed as percentages of controls measured after 24 hours darkness, of experiments in which phytochrome was measured after different times of prolonged darkness. The cultures were grown with the standard illumination program (5 minutes red light every day) before the prolonged dark period started. Crosses in *fig. 5* are derived from phytochrome values in *fig. 2*. The figure shows that the rate of phytochrome increase during the first interval of 24 hours after illumination is much greater than later on. This may be caused by self inhibition of phytochrome synthesis. Other data show, however, that 24 hours after illumination growth

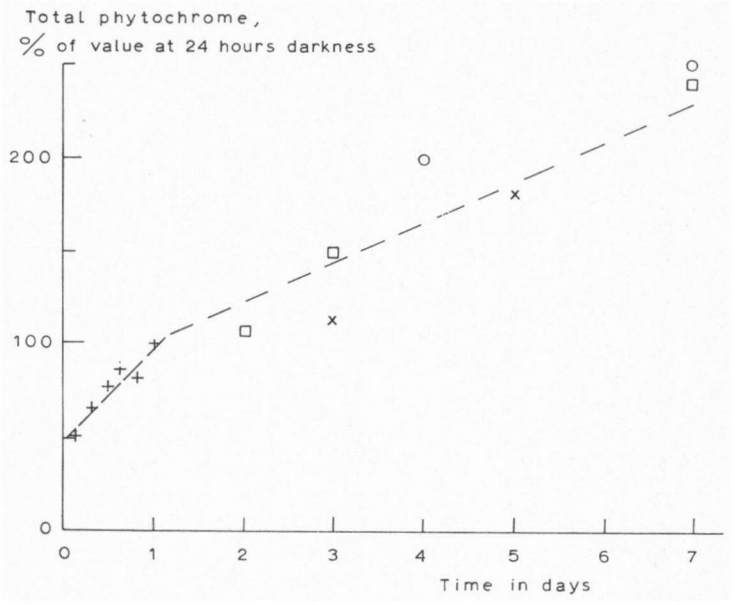


Fig. 5. Influence of a dark period on the phytochrome level of *L. minor*. Values from different experiments have different marks. Cultures were grown initially in a 24 hours illumination cycle; after the last red illumination they were kept in darkness for the indicated periods. Thereafter phytochrome was estimated.

rate also decreases, so that metabolic activity in general appears to decrease after that time.

### 3.4. Phytochrome in other *Lemnaceae*

*Lemna perpusilla*, *Lemna gibba*, and *Spirodela intermedia* were grown with and without kinetin under the standard illumination program. Growth rate with kinetin was slightly higher, but the phytochrome level was not influenced by kinetin. *L. perpusilla* showed abundant flowering after three weeks of growth under these conditions, 5 minutes light per day evidently acting as short day condition.

Phytochrome values are given in *table 4*. *L. minor* and *L. perpusilla* show about the same phytochrome level, followed by *L. gibba*. *Spirodela intermedia* had the lowest level.

We do not know yet whether these differences are species-bound. It appears likely that, within each species, strains can be distinguished differing in level of phytochrome under the same cultural conditions.

Moreover, the multiplication rate of *L. perpusilla* is very high under the experimental conditions, but the fronds are much smaller than those of *L. minor* or *L. gibba*, so a sample cell with *L. perpusilla* contains much more plants than a sample cell filled with one of the other species. This makes it difficult to compare phytochrome values of *L. perpusilla* with those of *L. gibba*: if the

Table 4. Phytochrome levels in three *Lemna* species and in *Spirodela intermedia*. Measurements 24 hours after last illumination. Together with the $\Delta(\Delta OD)$ values the standard deviation s is given, according to $s = \sqrt{\Sigma(x - \bar{x})^2/(n-1)}$, in which n = number of observations.

| | | |
|---|---|---|
| *L. perpusilla* 6746 | $22 \pm 4{,}3$ (n = 6) | $29 \pm 9{,}5$ (n = 4) |
| *L. gibba* G3 | $16 \pm 4{,}7$ (n = 4) | $15 \pm 1{,}5$ (n = 4) |
| *L. minor* | | $21 \pm 2{,}6$ (n = 7) |
| *Spirodela intermedia* | | $9 \pm 2{,}6$ (n = 5) |

phytochrome content per frond is the same in both species, a sample cell with *L. perpusilla* will show a much higher phytochrome value.

## 4. DISCUSSION

The results of the above measurements lead to a very simple conclusion, *viz.*, that in *L. minor* phytochrome decreases after an illumination, and increases in darkness. It is not very fruitful to speculate about rates of disappearance and reappearance, because there are no data available on dark reversion with *L. minor*, and the amounts of phytochrome are too small to allow much precision. It seems, however, that initial decrease and subsequent increase are both rather slow, and after 6 hours of illumination with pure red light still a considerable amount of phytochrome is present. This is in accordance with expectation from physiological experiments: 16 hours after a red illumination phytochrome induced growth could still be reduced by irradiation with far-red (ROMBACH 1966).

Apparent phytochrome synthesis in *L. minor* does not seem to depend on $P_{730}$ formation or on decrease of the phytochrome level below a certain low value, this being at variance with the situation found in *Pisum* by CLARKSON & HILLMAN (1967). It must be considered, however, that the phytochrome level in *L. minor* is already very low compared with the level found in darkgrown *Pisum* seedlings. Also at variance with the data of Hillman is the absence of an influence of auxins on apparent synthesis of phytochrome in *L. minor*. However, their effect on growth of *L. minor* is also much smaller than on stem elongation of *Pisum*.

Actinomycin D has no influence either. This is remarkable, because 10 mg/l actinomycin D inhibits the stimulatory action of phytochrome $P_{730}$ on frond expansion completely. This indicates that for the restoration of the phytochrome level after an illumination no synthesis of messenger RNA is necessary, and that it is independent of growth.

It is encouraging that phytochrome can also be demonstrated in *L. perpusilla* and *L. gibba*. An investigation of the apparent behaviour of phytochrome in connection with photoperiodical responses in these species is in progress.

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