EFFECTS OF LIGHT AND PHYTOCHROME IN HETEROTROPHIC GROWTH OF *LEMNA MINOR* L.

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(with an additional summary in Dutch)

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD
VAN DOCTOR IN DE LANDBOUWWETENSCHAPPEN,
OP GEZAG VAN DE RECTOR MAGNIFICUS,
DR. IR. J. P. H. VAN DER WANT,
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OP VRIJDAG 13 FEBRUARI 1976
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H. VEENMAN & ZONEN B.V. - WAGENINGEN - 1976

STELLINGEN

I

Een veronderstelling krijgt pas wetenschappelijke betekenis wanneer deze een aangrijpingspunt biedt voor een poging tot weerlegging.

K. R. POPPER, Conjectures and refutations, the growth of scientific knowledge, Routledge and Kegan Paul, London, 1969.
W. S. HILLMAN, Ann. Rev. Plant Physiol. **18**, p. 311 (1967) (phytochrome paradoxes.)

II

De fotostimulatie van de vermenigvuldiging van Klein Kroos is tijdens de lichtperioden bij een veel lagere concentratie van het phytochroom-Pfr verzadigd dan tijdens de donkerperioden.

Dit proefschrift.

III

Phytochroom-Pfr en intermediairen van de fotosynthese kunnen beide betrokken zijn bij door ver-rood gestimuleerde fotomorfogenese processen; in welke verhouding zij dat zijn is onbekend.

H. MOHR, Lectures on photomorphogenesis, Springer Verlag, Berlin Heidelberg New York, 1972, p. 46.
Dit proefschrift.

IV

In tegenstelling tot het bij calluskulturen van tabak door BERGMANN en BERGMANN gevondene, is bij heterotroof gekweekt Klein Kroos de thiaminesynthese niet afhankelijk van de fotosynthese maar van de Pfr vorm van phytochroom.

L. BERGMANN und A. L. BERGMANN, Planta **79**, 84–91 (1968).
Dit proefschrift.

V

De veronderstelling dat de afwezigheid van donker-reversie van phytochroom-Pfr, die bij enkele grassen is gevonden, een eigenschap van éénzaadlobbigen in het algemeen zou zijn, ontleent zijn wetenschappelijke betekenis aan zijn weerlegbaarheid.

R. E. KENDRICK and W. S. HILLMAN, Am. J. Bot. **58**, 424–428 (1971).

VI

Bij het evalueren van fotoperiodiciteitsproeven met planten die gegroeid zijn onder omstandigheden die voor de synthese van koolhydraten en eiwitten beperkend zijn, moet rekening gehouden worden met de mogelijkheid dat het mechanisme van bloeiremming teveel is verzwakt voor het tot uiting komen van het mechanisme van bloei-inductie.

D. DE ZEEUW, Proc. Kon. Ned. Akad. Wetensch. C **56**, 418–422 (1953).
M. K. JOUSTRA, Meded. Landbouwhogeschool Wageningen **69-13**, 1–10 (1969).
S. KLAUTKE und W. RAU, Planta **112**, 25–34 (1973); Ber. Deutsch. Bot. Ges. **86**, 571–576 (1973).
W. HAUPT, in: The induction of flowering, L. T. EVANS ed. McMillan of Australia, Melbourne 1969, p. 398.

VII

De waarneming dat bij *Pharbitis nil* CHOIS. en *Chenopodium rubrum* L. de bloei-inductie afhankelijk is van twee door phytochroom gecontroleerde processen, waarvan de een bloeibevorderend is en gedurende de gehele donkerperiode werkzaam is, terwijl de ander de bloei tegengaat en alleen korte tijd na een stoorbelichting optreedt, duidt er op dat het hierbij betrokken phytochroom zich in verschillende compartimenten bevindt.

A. TAKIMOTO and K. C. HAMNER, Plant Physiol. **40**, 859–864 (1965).
B. C. CUMMING, S. B. HENDRICKS and H. A. BORTHWICK: Can. J. Bot. **43**, 825–853 (1965).

VIII

De conclusie van BEZEMER-SYBRANDY et al., dat de door van EYK gesignaleerde kinetine - foliumzuur interactie een gevolg is van de door foliumzuur versnelde afbraak van kinetine, is niet gerechtvaardigd.

J. VAN EYK, dissertatie Leiden 1963.
S. M. BEZEMER-SYBRANDY, J. G. TASSERON-DE JONG and H. VELDSTRA, Biochim. Biophys. Acta **161**, 568–571 (1968).

IX

De waarneming dat bij proeven over de veroudering van plantendelen het effect van cytokininen op de afbraak van chlorophyllen en eiwitten afhankelijk van de ontwikkelingsfase en van correlatieve beïnvloeding versnellend of vertragend kan zijn, wijst er op dat de werking van cytokininen in dit geval

gezocht moet worden in stimulatie van het genetisch reguleringssysteem, met inbegrip van de translatie.

D. PENNER and F. M. ASHTON, Plant Physiol. **42**, 791–796 (1967).
J. DE BOER and J. FEIERABEND, Z. Pflanzenphysiol. **71**, 261–270 (1974).
K. V. THIMANN, R. R. TETLEY and T. V. THANH, Plant Physiol. **54**, 859–862 (1974).

X

Bij de studie van veranderingen in enzymactiviteiten onder invloed van belichtingsschema's en fytohormonen kunnen de veranderingen in groeisnelheid en groeipatroon kardinale informatie geven.

J. DE BOER and J. FEIERABEND, Z. Pflanzenphysiol. **71**, 261–270 (1974).

XI

De microflora van de fyllosfeer geeft het blad een bescherming tegen pathogene fungi die door bespuitingen met fungiciden belangrijk wordt verminderd.

N. J. FOKKEMA, J. A. J. VAN DE LAAR, A. L. NELIS-BLOMBERG and B. SCHIPPERS, Neth. J. Pl. Path. **81**, 176–186 (1975).
C. H. DICKINSON, Pestic. Sci. **4**, 563–574 (1973).

XII

Door kultuurmaatregelen (waaronder inoculatie met micro-organismen) kan de microflora van de fyllosfeer zodanig beïnvloed worden dat de schade door infectie met pathogenen belangrijk afneemt.

D. D. BHATT and E. K. VAUGHAN, Pl. Dis. Reptr. **46**, 342–345 (1962).
J. VAN DEN HEUVEL, Meded. Phytopath. Lab. Willie Commelin Scholten **84** (1970).

XIII

Ervaring met de geïntegreerde insectenbestrijding doet verwachten dat in de toekomst biologische gewasbeschermingsmethoden een overheersende positie zullen innemen.

J. DE WILDE, Ambio **4**, 105–111 (1975).
P. GRUYS, Proc. 8th British Insecticide and Fungicide Conference, Vol. 3, 1975.

J. ROMBACH, Wageningen, 13 februari 1976.

VOORWOORD

Graag wil ik hen bedanken die mij in mijn opleiding gestimuleerd en gericht hebben. Daartoe behoren in de eerste plaats mijn ouders. Zij hebben door hun grote vertrouwen en steun de opleiding mogelijk gemaakt tot een beroep, dat in belangrijke mate aan mijn geluk heeft bijgedragen. De jaren van mijn studie, die ik bij de familie VAN DER LEK heb doorgebracht zijn vol dierbare herinneringen; dat alles in de natuur van elkaar afhankelijk is en dat ook de mensen tot deze natuur behoren is mij door de gesprekken met Dr. H. A. A. VAN DER LEK steeds meer bewust geworden.

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CONTENTS

1. INTRODUCTION

1.1. THE PHOTOMORPHOGENETIC EFFECT OF LIGHT ON THE GROWTH OF ETIOLATED PLANTS

According to SACHS (1863), MC DOUGAL (1903), and WASSINK et al. (1957), Charles BONNET (1754) was one of the first naturalists to investigate experimentally 'l'ettiolement' of plants. He observed that seedlings of peas and beans and a shoot of vine, grown in dark chambers, developed long flexible stems with only very small leaves of white colour, whereas seedlings grown under transparent glass bells behaved the same as the controls growing in the open air. At the end of his account he raised the question: 'l'obscurité produiroit-elle donc seule l'ettiolement; le grand jour suffiroit-il seul à le prévenir?' and further: 'tout ceci mérite un examen plus approfondi. Je me borne actuellement à mettre les physiciens sur les voies'. The expression 'ettiolement' was, according to BONNET (1754), used by gardeners for the symptoms brought about on plants by prolonged darkness: pale colour and excessive elongation of stems.

One of the first followers of BONNET was MEESE (1775 a, b, 1776) who worked in the Netherlands at the former university of Franeker until his death at the age of twenty-one. Through the mediation of BONNET and the diligence of VAN SWINDEN, professor at Franeker, his work was published posthumously. It is of historical interest because of the large number of plants used, his measurements of growth rate and the presentation of the experimental data by tabulating the absolute and relative values. One of his conclusions was that etiolation is promoted by high temperature, by moisture and by darkness; he observed that after transfer to darkness plants often dropped their green leaves but retained the very small dark grown leaves for a long time.

The literature from 1686 to 1902 has been reviewed by MC DOUGAL (1903). More recent reviews are by BURKHOLDER (1936), ÅBERG (1943), GORHAM (1950), WASSINK and STOLWIJK (1956), WILLIAMS (1956), HUMPHRIES and WHEELER (1963).

Our knowledge of etiolation phenomena in different species of plants still is due mainly to the work of MC DOUGAL (1903). MC DOUGAL described nearly 100 species of plants growing in the dark room at the expense of storage organs and he compared the dark grown forms with those of light grown controls. Darkness was not completely continuous as once a day the room was illuminated for a few minutes by a candle or by a four candle power electric hand lamp. It is therefore likely that MC DOUGAL gives information on photomorphogenetic sensitivity rather than on growth in absolute darkness. From MC DOUGAL'S observations it is however clear that in most dark grown plants differentiation of the tissues is retarded or incomplete. If angles or ribs were present on the stems of light grown plants, in the etiolated ones they were often absent, the vascular

strands were less developed and the cells of the cortex were mostly larger with more intercellular spaces and thinner cell walls. Especially the lower stem internodes of plants grown in the dark were often longer than those of plants grown in the light. Mesophyll tissue of leaves in particular was reduced, differentiation in palissade and sponge parenchyma was poor, intercellular cavities were absent. The petioles on the other hand often responded in the same way as the stems.

MC DOUGAL described a wide variety of modifications of these rules. A few examples may suffice to illustrate the various phenomena encountered. In the group of ferns in most cases stipes and rachis of dark grown plants were both elongated, the distances between the pinnae were increased, and the pinnae themselves did not unroll or expand. In some instances, mainly the stipe was elongated (*Polystichum acrostichoides* (MICHX.) SCHOTT), in others mainly the rachis (*Pteris longifolia* L.), whereas in *Woodwardia radicans* SM. the fronds emerged in much larger numbers but were not longer than in the light grown controls. Dark grown fronds of all Pteridophytes investigated contained some chlorophyll. In Monocotyledons stems generally were strongly elongated. In plants with parallel venation leaves were either of normal length or longer; *Hemerocallis* sp. was the only species in this group that in darkness was found to have smaller leaves than normal.

In leaves of Monocots with open or reticulate venation wide variation was observed in the degree of development of the laminae. The laminae of *Arisaema dracontium* (L.) SCHOTT and *A. triphyllum* (L.) TORR. remained very small. The laminae of *Caladium esculentum* VENT. (= *Colacasia esculenta* (L.) SCHOTT) and *Calla palustris* L. were almost as large as in the light grown controls. These last two plants were very sensitive to the small amount of light used occasionally for illumination of the dark room, forming small amounts of chlorophyll in the leaves.

In Dicotyledons the laminae of the leaves were almost always very small in darkness. Exceptions were *Brassica campestris* L., with narrow leaf blades of 9 cm length, *Taraxacum* sp., with very narrow leaf blades and elongated petioles, and species of *Rheum* and *Rumex*. *Beta vulgaris* L., according to SACHS (1863), also belongs to these exceptions. In *Rheum* and *Rumex* and to a lesser degree also in *Beta*, the venes were growing faster than the mesophyllous parts of the leaves; in some cases the mesophyll between the venes was torn apart.

Besides the incidental weak illumination of the dark room, the light conditions under which the buds were formed also influenced the degree of etiolation. With *Aesculus hippocastanum* L., MC DOUGAL found that illumination of the seedling until the plumule 'was almost disengaged from between the cotyledons' had an after effect not only on the first leaf but on all subsequent leaves (4 pairs) developed within a month. FUNKE (1944) repeated this experiment and found that 3 days of light were sufficient to produce this effect. SACHS (1863) supposed that differences between species of plants in leaf growth in darkness were connected with the growth pattern of these plants. Growth would be unaffected by darkness if the leaves during the greater part of their growth were covered by

bud scales or leaf sheaths. For species with leaf growth mostly in the open, it would be inhibited during continuous darkness.

1.2. On chemical factors substituting for light

Sachs (1887) considered etiolation as a disease caused by lack of specific substances required for their proper nutrition. There were several arguments for this. Prantl (1873) had measured the diurnal periodicity of leaf growth on plants of several species periodically placed under a black cover during the night and observed that growth during the dark period was not less than that during the light period. Moreover, partial etiolation experiments, with plants growing with the upper part of their stems in a dark chamber and the basal parts in daylight, showed that the leaves growing in darkness developed almost to the same size as those in light, although very little chlorophyll was formed. From this kind of experiment, of which that with *Cucurbita pepo* L. (Sachs, 1865) is the best known, it was concluded that substances required for leaf growth are produced in the photosynthesizing leaves and transported to those in darkness. With *Tropaeolum majus* L., however, Sachs (1865) found that the leaves of the darkened part of the shoot remained very small. Several investigators tried in vain to reproduce Sachs' results with *Cucurbita pepo* L.. For instance, Teodoresco (1899) found that in all climbing plants he used (*Humulus lupulus* L., *Phaseolus multiflorus* Lam. and *Cucurbita pepo* L.), leaves grown in darkness on partly etiolated shoots remained as small as those on plants grown entirely in darkness, whereas the stems showed almost normal differentiation of the vascular tissues. With plants with self-supporting stems, however, Teodoresco found effects similar to those found by Sachs. The dark grown leaves of partially etiolated *Atriplex hortensis* L. and *Faba vulgaris* Mnch. (= *Vicia faba* L.) were intermediate between the leaves of the dark grown plants and those of the light grown ones. Dark grown internodes of *Helianthus tuberosus* L. developed very long petioles and very small leaf blades, whereas completely dark grown plants hardly developed at all. Funke (1944), on the other hand, confirmed the findings of Sachs. Jühren and Went (1949) observed in *Cucurbita pepo* L. fed with sucrose a small positive growth response of leaves further upwards on the stem if one leaf was illuminated.

It is very difficult to obtain absolute darkness in cabinets used for partial etiolation. Sachs (1865) was aware of this but considered it to be of no great importance because: 'Zur Feststellung dieser Abhängigkeit bedarf es keiner absoluten Finsternis, eben so wenig wie es zur Feststellung des Gesetzes zwischen Dampfspannung und Temperatur einer Beobachtung beim absoluten Nullpunkt der Temperatur bedarf.' The majority of investigators in this field held the same opinion. Mc Dougal (1903), however, was among the first to be aware of the very high sensitivity to light of some of his objects and, more recently, it has been shown that even the incidental use of a green safe-light may bring about reactions in dark grown plants (Huisinga, 1964; Wilkins, 1965;

BRIGGS and CHON, 1966; BLAAUW et al., 1968; RAVEN and SPRUIT, 1973).

The partial etiolation experiments led to the question as to whether light was acting by way of photosynthesis or otherwise. Several investigators have compared plant growth with and without CO_2. MC DOUGAL (1896), comparing the results of his own experiments with those of previous authors, concluded that most plants expand their leaves normally in the absence of CO_2, provided there is a sufficient supply of food from storage organs. An exception to this rule was *Arisaema triphyllum* L., expansion of the leaves of this plant being inhibited by the absence of CO_2 almost to the same degree as by darkness, even though the food reserves were not exhausted. Similar experiments were made by TEODORESCO (1899) with seedlings. Although carbohydrate supply probably was the limiting factor for their growth, and darkness probably was not maintained very strictly, withholding of CO_2 never reduced the size of the leaves as much as darkness.

The best answer as to whether leaf expansion was due to substances provided by photosynthesis probably was given by BATALIN (1871), who illuminated bean seedlings with diffuse daylight for one or two hours once every 3 days. The primary leaves of these plants developed almost as well as in daylight. Since no greening took place, the light effect could not be due to photosynthesis.

On the other hand, it is obvious that light acts on a biochemical reaction, and SACHS, with his theory that specific substances, synthesized by the illuminated parts of the plants, are required for the expansion of leaves, put the problem on a molecular basis. This idea was pursued further by WENT (1938) in his search for specific substances, which he called rhizocaline, caulocaline and phyllocaline, and were supposed to control the growth of the different plant organs.

1.2.1. *Experiments with intact plants*

WENT (1938) made observations on the interdependence of the development of roots, the increase in length of stems and the area increase of leaves of etiolated seedlings of *Pisum sativum* L.; the rate of increase in length of 1 cm pea seedlings on a 2% sucrose solution was reduced to half by taking away either the roots or the cotyledons; when both roots and cotyledons were removed, the growth rate was only 10% of that of the controls.

Another effort to analize the factors affecting stem growth and leaf growth was made by JÜHREN and WENT (1949). *Cucurbita pepo* L. plants raised under controlled light conditions were placed in a dark room and fed by injections of a sucrose solution into the hollow petioles. If the injections began immediately after transfer to darkness, the elongation of stems and petioles remained rapid during the next 3 weeks and was independent of the preculturing conditions. The leaf blades grew normally until they had attained a length of 10 mm, but failed to expand further. Several substances were added to the sucrose solution: amino acids, yeast extract, adenine, indolyl acetic acid, a diffusate from light grown *Cucumis* leaves, but no extract nor any substance increased the effect of sucrose by more than 20%.

1.2.2. *Experiments with detached leaf primordia*

In the study of nutritional and hormonal requirements for leaf development, the culture of detached leaf primordia and leaf discs is of great value, since influences from other parts of the plants are then excluded. DE ROPP (1945, 1946a, 1946b) measured increase in length, fresh weight and dry weight in stem tip cultures of rye embryos and in leaf discs and whole leaf primordia sectioned from the center of the cabbage head of *Brassica oleracea* L.. Increase in volume of isolated leaves and leaf discs from cabbage was entirely due to the enlargement of existing cells; the final size of the epidermal cells depended on their size at the moment of excision; the cells of the older primordia increased from 25 to 58 units while those from the youngest primordia increased from 4.5 to 12.2 units.

In *Helianthus annuus* L. and *Nicotiana tabacum* L. STEEVES et al. (1957) found a similar relation between the initial and ultimate sizes of explants. Addition of vitamins, casein hydrolysate or plant diffusates to the culture medium had no effect; only sucrose was required (DE ROPP, 1945). Light reduced the final length of an expanding primordium in rye but it promoted initial increase (DE ROPP, 1946a). In cabbage leaf cultures, light resulted in chlorophyll synthesis allowing sufficient photosynthesis to render leaf expansion independent of sucrose, but induced no further development (DE ROPP, 1947). Apparently light did not satisfy the requirements for development to the size the leaves would have reached if kept attached to the plants.

In stem tip cultures of rye (DE ROPP, 1946b) only the lower and largest leaf primordium grew out; further outgrowth was obtained only if roots generated on the stem tips. Isolated roots, cultivated together with the stem tips, did not have this effect, therefore the substance responsible for the effect was not excreted in the medium. The promoting effect of rooting was observed also in stem tip cultures of cabbage; rooting of isolated primordia had no effect (DE ROPP, 1947).

DE GREEF and CAUBERGS (1972), working with dark-grown bean seedlings, similarly observed that expansion of the primary leaves depended on the presence of other parts of the plants: expansion of excised leaves depended on sugar supply and was much increased by regeneration of roots on the petiole. For leaf expansion illumination was required, with intact plants not only the leaf but also the plumular hook had to be illuminated. It must be noticed that these observations differ from those of WENT (1938) on *Pisum*, leading to the statement that leaf expansion was independent of the presence of roots.

In the fern *Osmunda cinnamomea* L., STEEVES and SUSSEX (1957) found again that the ultimate size in culture of the isolated leaf primordium depended on the size at the time of excision. Cell division in the primordium, however, continued after excision. Nevertheless, the onset of expansion was accelerated by excision so that the period of meristematic growth was much shortened.

In light, the period of cell division of isolated primordia was further reduced. The final length of the frond in illuminated cultures was less than that in cultures kept in darkness. Illumination was required for unrolling and extension of

the rachis and pinnae, but it increased their final size only if applied after termination of the phase of meristematic growth. Evidence was obtained that in nature the period of meristematic growth was extended for many months by the presence of older primordia and expanding leaves and by the darkness inside the apical bud under the surface of the soil. In the case of ferns this might fully explain the much smaller final size of the isolated fronds in comparison with the attached fronds.

1.2.3. *The effect of cytokinins on leaf expansion*

The experiments of JÜHREN and WENT (1949) and STEEVES and SUSSEX (1957) have shown that darkness does not reduce meristematic growth of leaf primordia but mainly affects expansion growth. Leaves or parts of leaves that have finished their meristematic phase of growth therefore appear to furnish a good test system for substances promoting leaf growth in darkness.

A rapid bio-assay was developed by BONNER et al. (1939) and by MILLER (1951). Leaf discs cut from the primary leaves of etiolated beans were floated on media of different composition. With this test it was found that cobalt had a stimulating effect on leaf disc expansion, additive to that of light (MILLER, 1952). In a similar test, using stem sections, the effect of cobalt was opposite to that of light, however, increasing the length (BERTSCH, 1963).

Meanwhile, a very potent stimulator for cell division and bud initiation in tissue culture was isolated from yeast extract by MILLER and coworkers (1955). The substance was identified as 6-furfuryl-amino-purine. This substance promoted cytokinesis in tissue culture and was therefore named kinetin.

Kinetin, and its even more potent analog benzyl-amino-purine (BAP) proved also to promote expansion of leaf discs (SCOTT and LIVERMAN, 1956), but here promotion was mainly due to cell expansion (POWEL and GRIFFITH, 1960). In darkness, the effect of kinetin was much larger than in the light, so it appears that cytokinins could substitute for light to some degree. The same was found in the germination of lettuce seed (MILLER, 1956).

HILLMAN (1957) arrived at the same conclusion with *Lemna minor* grown on sucrose in axenic culture. Five minutes red light given every 3 or 4 days stimulated the multiplication rate; the same stimulation was obtained with kinetin in the dark.

The conclusion that cytokinins could substitute for light in several light dependent processes was, however, soon disputed. MILLER (1958) reexamined the lettuce seed germination, this time maintaining more strict darkroom conditions than in his earlier experiments. He observed that the sensitivity of the seeds to light was much increased by kinetin, so that they responded already to the safe-light of the dark room. Later, IKUMA and THIMANN (1963) found that the site of action of kinetin is in the cotyledons, the expansion of which was promoted by kinetin and was further increased by light. Light primarily acted on the ability of the radicle to penetrate the seed coat, probably by inducing the activity of hydrolytic enzymes. With *Lemna minor*, ROMBACH (1961) found that the increase of multiplication rate and frond size by kinetin was greater at a very

low light intensity than in continuous darkness. OOTA (1966) found a similar relation between kinetin and light effects in *Lemna gibba* strain G 3.

1.3. SOME REMARKS ON CYTOKININS

According to the definition given by LETHAM (1967) cytokinins are substances with kinetin-like activity. Natural cytokinins have been found in the bleeding sap of various plants and can be extracted from root tips, growing fruits, seeds and leaves (LETHAM, 1967; SKOOG and ARMSTRONG, 1970). It was found by MOTHES et al. (1959) that when kinetin is applied to the lateral half of a detached leaf, the other side shows rapid yellowing, proteins are degraded and the degradation products are transported towards the treated half which remains green. KULEAVA (1962), who was one of the first to consider cytokinins as functional hormones in plant growth, used this phenomenon as a test for cytokinins. She found that applied kinetin induced no rapid yellowing when the leaf was left in xylem connection with the root system. She concluded that cytokinins were supplied by the roots. If growth was inhibited by nitrogen deficiency or if the xylem connections between the roots and the shoots were disrupted, the cytokinin level of the shoot dropped so much that the leaves responded to exogenously applied kinetin. Observations of SHIBAOKA and THIMANN (1970) support this conclusion: they failed to find cytokinin activity in the extracts of roots of etiolated *Pisum sativum* seedlings, developing only rudimentary leaves, whereas plenty of cytokinins were found in extracts from plants grown in the light. Although the supposition that roots provide the cytokinins required for shoot growth is interesting, it must be kept in mind that not all plant species were found to be sensitive to the kinetin test (KULAEVA, 1962). In general, young leaves failed to respond; maybe the roots are not the only source of cytokinins.

Uptake and retention by *Lemna minor* cultures was studied by VAN EIJK (1963) for kinetin and by BEZEMER-SYBRANDY (1969) for BAP. Translocation from the root to the frond was very poor, but retention in plants transferred to a medium without the cytokinin was, in comparison with translocation and retention of adenine, very strong. Cytokinins are rapidly metabolized; in biotests cytokinin activity was found in the fractions without polynucleotides, but also in the alkaline hydrolysate of transfer RNA. In both cases the activity was found in cultures grown with added cytokinin as well as in those without it (BEZEMER-SYBRANDY and VELDSTRA, 1971).

No evidence was obtained for incorporation of BAP in ribonucleotides in *Lemna minor*. On the contrary, since BAP added to the homogenate was strongly adsorbed to the polynucleotide fraction, any BAP in the hydrolysate of the polynucleotide fraction of plants grown on a BAP-containing medium can be suspected to be a contamination. On the other hand, the cytokinin 6-(3-methyl-2-butyrylamino)-9β-D-ribofuranosylpurine is a functional group in some tRNA species in plants and animals. It is doubtful, however, whether applied cyto-

kinins are involved in the formation of these tRNA species (HALL 1973).

VAN EIJK (1963) demonstrated that in *Lemna minor* 12 hours after kinetin addition the incorporation into RNA of several compounds, but especially formate, was stimulated. Incorporation of formate in protopectins and in DNA was decreased.

In long-term experiments, with *Lemna* cultures in light, BAP stimulates both the synthesis and the degradation of RNA (TREWAVAS, 1970) and increases the rate of protein synthesis (TREWAVAS, 1972). In cultures with a decreasing rate of growth resulting from transfer from a complete medium to water, BAP decreases the rate of degradation of proteins (TREWAVAS, 1972). These reports show that by addition of cytokinins some metabolic processes are accelerated and others slowed down.

No information has been obtained on the primary site of action of cytokinins, the first effects not being observed until 6 hours after kinetin addition. Moreover, the evaluation of the experimental data are complicated by the low rate of transport of kinetin in the plants. One very rapid effect of kinetin is reported, however: LIVNE and GRAZIANI (1972) observed an increase in the rate of rehydration of epidermis-free tobacco leaf discs within 2 minutes after kinetin application, which could mean that water permeability of the plasmalemma is directly influenced.

1.4. THE EFFECT OF LIGHT

1.4.1. *The effect of brief illuminations on leaf expansion and the involvement of phytochrome*

As mentioned in section 1.2.3, a brief period of illumination induces expansion of leaf discs cut from the primary leaves of etiolated seedlings. This effect was abolished when far red light was given within a short time after the illumination. The action spectra of these light effects have been determined by PARKER, HENDRICKS, BORTHWICK and WENT (1949) and by DOWNS (1955). The photoreceptor pigment involved was isolated by BUTLER et al. (1959) and called phytochrome.

By difference spectrophotometry of living plant tissue several forms of phytochrome have been detected, the two more stable forms are known as Pr and Pfr, with absorption maxima respectively at 660 and 730 nm. The action spectra of photoconversion of Pr into Pfr and of Pfr into Pr (BUTLER et al., 1964) correspond with the action spectra for leaf expansion.

In the plant, Pr is very stable but Pfr disappears under aerobic conditions at room temperature with a half life that differs for each plant species but is usually less than 3 hours.

There are, however, indications that the Pfr involved in some physiological reactions has a much longer life time. For this reason it is suggested that there is a difference in stability (perhaps owing to binding to cellular structures) between the bulk fractions of phytochrome as measured spectrophotometrically and a

more stable physiologically active fraction (BUTLER and LANE, 1965; HILLMAN, 1965). The effect on *Lemna minor* of short illuminations with a narrow spectral band of red light, and its reversion by far red are described by ROMBACH (1965). The presence of phytochrome in *Lemna* was demonstrated by ROMBACH and SPRUIT (1968) with dual wavelength spectrophotometry. On a dry weight basis, the phytochrome content was the same in the non-growing parts of the plants as in the parts containing the meristems. The phytochrome content was reduced if the frequency of the illumination was raised or if conversion to Pfr was more complete. Determinations of Pfr after illumination (ROMBACH and SPRUIT, unpublished) show that the half life of Pfr was about 2.5 hours at 23 °C, but Pfr was shown to be physiologically active up to 15 hours after illumination (ROMBACH, 1966).

1.4.2. *The effect of extended periods of illumination*

Often a further increase in light response is observed if exposure time and light intensity are increased above the energy level required for maintaining the photostationary state of phytochrome. This has been found by MEYER (1959) and by ENGELSMA and MEYER (1965b), studying the inhibition of the growth of hypocotyls and the promotion of expansion of cotyledons in *Cucumis sativus* L. by light. EVANS et al. (1965) have shown that in two garden varieties of *Petunia* and in *Lactuca sativa* L. a brief illumination with red light inhibited hypocotyl growth only when preceded by a longer period of radiation, the maximum effectivity of which was in the blue. With very young lettuce seedlings, another maximum of action was found at 720 nm, an observation made previously by MOHR and WEHRUNG (1960). There was a difference, however, between the action of blue and of far red pretreatments since during illumination growth of the hypocotyls was strongly reduced by blue light but promoted by far red, whereas during the subsequent dark periods growth of plants pretreated with far red or with blue light was the same.

The effects of long periods of illumination with blue and far red light are often similar (WASSINK and STOLWIJK, 1956) but in many cases differences were also found (GRILL and VINCE, 1965, 1966; ENGELSMA, 1967 a, b). Far red probably works via the phytochrome system (HARTMANN, 1966; ENGELSMA, 1967b), perhaps by mobilizing storage products in the cotyledons (HÄCKER, 1967), but there are also indications that parts of the photosynthetic reaction chain, especially cyclic photophosphorylation by system I, are involved (KANDELER, 1969; SCHNEIDER and STIMSON, 1971). This might apply also to etiolated plants since OELZE-KAROW and BUTLER (1971) found that dark-grown bean seedlings slowly accumulated some chlorophyll during prolonged illumination with far red (700–1000 nm). After 12 hours irradiation, the leaves developed the capacity for DCMU-insensitive photophosphorylation, whereas the capacity for oxygen evolution began to appear only after 24 hours of far red irradiation (DE GREEF et al., 1971). When the illumination period is increased, synthetic processes connected with photosynthesis and other light dependent processes can be expected to increase in importance also in *Lemna minor*. A period with high photosyn-

thetic activity has an after effect on the growth rate during the first days of a subsequent dark period (ROMBACH, 1961, 1971). We may, therefore, expect to find multiple relations between the actions of phytochrome, cytokinins and photosynthesis in the effect of light on the growth of plants.

Four kinds of effect of phytochrome on metabolism have been observed or deduced from the results of experiments.

1. A rapid effect on the permeability of bio-membranes beginning within a minute after the start of illumination.
2. An increase in the amount of extractable growth substances.
3. Regulation of enzyme synthesis.
4. Effects on messenger RNA production and on ribosomal activity.

A rapid effect of phytochrome Pfr on permeability of bio-membranes is deduced from the action of red and far red light on the leaf movements of some Leguminosae (FONDEVILLE et al., 1966; SATTER and GALSTON, 1971) and from rapid changes of electropotential at the surface of root tips (JAFFE, 1968) and *Avena* coleoptiles (NEWMAN and BRIGGS, 1972). The rapid far red reversible changes in the ATP-level observed by WHITE and PIKE (1974) in etiolated pea plumules after a brief illumination may be related to transport through membranes.

The studies with polarized light by BOCK and HAUPT (1961) and HAUPT (1970) on chloroplast movements in *Mougeotia* indicated localization of phytochrome in the plasmalemma.

An increase of extractable gibberellic acid in etiolated wheat leaf segments was observed by BEEVERS et al. (1970). A maximum yield of extractable gibberellic acid was found 15 minutes after the beginning of a 5 minute irradiation. These experiments are of interest because addition of GA induced an unrolling of the etiolated leaf segments similar to that after treatment by light (POULSEN and BEEVERS, 1970).

Rapid effects of phytochrome on regulation of enzyme synthesis in dark-grown seedlings of *Sinapis alba* were reported by OELZE-KAROW et al. (1970) for the temporary repression of lipoxygenase synthesis and by RISSLAND and MOHR (1967) for the resumption of phenylalanine ammonia lyase (PAL) when a second illumination followed after a period of darkness.

For many phytochrome controlled enzymatic reactions it takes some time before the first increase in activity after illumination is detectable. The induction of PAL synthesis by the first illumination of an etiolated plant, for instance, has a lag phase of about 1 hour (ENGELSMA, 1967a, b; BELLINI and HILLMAN, 1971; SCHOPFER and MOHR, 1972). In cotyledons of *Sinapis alba* this first induction of PAL is inhibited by actinomycin D (RISSLAND and MOHR, 1967), which indicates that messenger RNA production is required. In this case phytochrome control may be at the site of mRNA production (according to MOHR, 1966, a case of gene activation). If this proves correct, it probably is not the only site where phytochrome regulates PAL synthesis since stimulation of PAL syn-

thesis by a second illumination is only partly inhibited by actinomycin D (RISSLAND and MOHR, 1967).

A far red reversible effect of red light on ribosome activity was found by TRAVIS et al. (1974). They measured amino acid incorporation in monoribosomes from dark-grown maize leaves isolated at various times after 5 minutes of red illumination. Maximum stimulation was found after 2 hours, but a small increase was already detectable after 30 minutes. This ribosome activation was independent of mRNA availability in the in vitro reaction medium.

In mitochondria isolated from etiolated peas, illumination increased the ability to reduce exogenous NADP. This effect, reversible by far red, was found by MANABE and FURUYA (1974). In 1975 they reported that a small part of the phytochrome was bound to mitochondria and microsomes, and that this part increased after illumination of the intact plumules of etiolated pea seedlings in proportion to the Pfr content.

The first illumination of plants grown in complete darkness causes changes in physiological sensitivity which made RAVEN and SPRUIT (1973) suppose that after this initial illumination a change in the distribution of phytochrome in the cells takes place. SPRUIT (1972) suggested that the apparent phytochrome decay observed with in vivo spectrophotometry after the first illumination might be an optical artifact due to a change in distribution.

RUBINSTEIN et al. (1969) observed that in homogenates of etiolated tissue the phytochrome could be separated in a fraction pelletable by centrifugation, and a supernatant fraction. MARMÉ et al. (1973) and MARMÉ et al. (1974) found that after a brief illumination part of Pfr was associated with pelletable membrane fractions free from mitochondria and other particles. BOISARD et al. (1974) reported Pfr decay during the first hour after in vivo illumination in *Cucurbita pepo* to be due to decay of the Pfr bound to the membrane fraction. The ability of this fraction to bind Pfr in response to a second illumination was not restored within 2 hours; 24 hours later, however, the binding capacity was as before. Therefore decay of total Pfr, decay of bound Pfr and decay of the binding capacity of the membrane fraction were similar during the first 2 hours after a brief illumination. The decay of the binding capacity of the membrane was prevented by far red following the red illumination. It was concluded that after decay of the bound Pfr during the first hours after an illumination the binding sites on the membrane were occupied by non photoreversible phytochrome.

QUAIL et al. (1973) reported that in hooks of *Cucurbita pepo* seedlings the red-induced binding of Pfr was partly reversible by far red, but not in coleoptiles of *Zea mais*. In *Zea*, in vivo illumination induced an immediate increase of binding of Pr as well as of Pfr. QUAIL and SCHÄFER (1974) observed that in *Zea* the binding, when induced by brief illuminations, depended on the Pfr level. With longer illuminations and a low photostationary state of Pfr, the binding was a function of both light intensity and duration of illumination. They proposed a model in which the occupation of the membrane by phytochrome was a function of the rate of 'cycling' between Pr and Pfr.

The membrane model of CHANGEUX and THIÉRY (1968) has served as a guide

in the studies mentioned above. A discussion of this model in relation to phytochrome has been given by WASSINK (1974).

The experiments discussed above point to a migration of phytochrome after illumination, reinforced by an effect of Pfr on the membranes. Nevertheless, it is not likely that these binding reactions are the same as those postulated by SPRUIT (1972), since in that case the pelletable fraction of phytochrome should be expected to increase in the dark after an illumination at the same rate as the apparent dark decay of phytochrome measured in the spectrophotometer.

It is likely that the primary action of phytochrome is different for different reactions induced by Pfr, and that membrane processes may be involved. There are indications in literature that cytokinins influence permeability (the work of LIVNE and GRAZIANI is mentioned in section 1.3). This makes both the resemblances and the differences between the effects of cytokinins and Pfr significant.

1.5. THE MATERIAL

1.5.1. Lemna minor *as an experimental plant*

Lemnaceae have been used as experimental plants by many investigators in the fields of plant physiology and biochemistry, as appears from the review by HILLMAN (1961). The small size of the plants and the ease of aseptic cultivation on well defined media are advantages in experimental studies. Their small size, however, is also a disadvantage: a culture of duckweeds consists of plant tissue that is never homogeneous; roots, old fronds, young fronds and meristems are difficult to separate. According to ARBER (1919), the morphology of *Lemna* is related to that of *Pistia*. A frond of *L. minor* bears a reproductive pocket on each side (fig. 1.1. **c** and **e**) from which new fronds grow out in succession. The younger of these 'daughter' fronds always appears above the older. The 'daughter' fronds remain connected with the 'mother' frond by a thin connective stalk which is thrown off by both mother and daughter fronds after some time. The adult frond tapers towards the side of the connective stalk; ARBER (1919) regards the part of the frond between this stalk and the implantation of the root as a stem; this part bears the reproductive pockets.

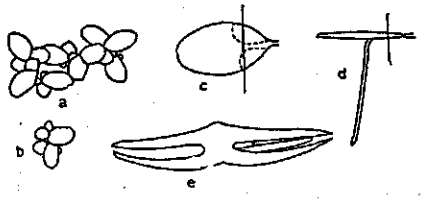


FIG. 1.1. *Lemna minor* grown on a medium with 1% sugar; **a** and **b** grown at 0.5 μW cm^{-2} continuous light, **a** with 3×10^{-6} M kinetin, top view; **c**, **d**, and **e**: fronds grown at 1000 μW cm^{-2}, **c**: top view, **d**: side view, **e**: cross section in the plane indicated in **c** and **d**, the right side reproductive pocket contains two frond primordia, the lower one is the oldest. Enlargements: **a** and **b**: 1×; **c** and **d**: 2×; **e**: 8×.

The root is initiated in the region between the reproductive pockets; in analogy with *Pistia* it is considered to be an adventitious root, issuing from the node. The reproductive pockets are covered on the underside of the frond with a flap; this flap has grown together with the margin of the frond in such a way that the reproductive pocket remains closed until the first daughter frond breaks through. Since these daughter fronds are not contaminated and make no contact with the disinfectants which generally kill the mother plant, this is very helpfull in sterilization.

The other part of the frond, from the root implantation to the distal end, is considered to be a petiolar phyllodium. This description implies that the proximal part of a *Lemna* plant should behave in physiological respect like a stem, and the distal part like a leaf stalk. In practice, however, the entire frond responds to light like a leaf blade.

1.5.2. *Nutritional requirements of* Lemna minor *for growth in darkness*

GORHAM (1950) found that a combination of sucrose, casein hydrolysate and yeast extract enabled the plants to multiply in darkness for an indefinite time, be it at a low multiplication rate. Without casein hydrolysate and yeast extract almost no increase in number of fronds took place. GORHAM concluded from the beneficial effect of casein hydrolysate that in darkness *L. minor* was unable to use nitrate or an amonium salt as a source of nitrogen. This was contradicted by BORNKAMM (1970) who demonstrated nitrate assimilation in the absence of casein hydrolysate in long term dark cultures. In these experiments addition of yeast extract proved indispensable, however.

GORHAM (1950) tried to replace casein hydrolysate by single amino acids. From 25 amino acids tested aspargine, DL-phenylalanine, β-alanine, DL-isoleucine, leucine, DL-methionine, DL-treonine, L-proline and DL-amino-butyric acid, each had about 50% of the effect of casein hydrolysate while the others were ineffective or even toxic at low concentrations.

In the absence of yeast extract the cultures died after 2 months in darkness. GORHAM (1950) tried to replace the yeast extract by the following vitamins: ascorbic acid, thiamine, riboflavine, pyridoxine, para-amino-benzoic acid, calcium-panthothenate, niacin, inositol, choline, biotin and folic acid. A mixture of these vitamins was inferior to yeast extract. Coconut milk and a mixture of adenine sulfate, guanine hydrochloride, xanthine and uracil likewise had no additional effect.

HILLMAN (1957) added the following substances to this list: kinetin, benzyl-amino-purine and various other purine analogues, adenosine, cobalt, arginine, 4-chloro-phenoxy-iso-butyric acid, cysteine, gibberellic acid, indole-acetic acid and uridine. The most active of these components was benzyl-amino-purine at a concentration of 10^{-6} M. Kinetin had a similar effect, at a three times higher concentration. The four other purine analogues were either much less effective or not effective at all; 10^{-4} M adenosine had 20% of the kinetin effect; 10^{-4} M cobalt nitrate gave 40% of the kinetin effect; the other substances were ineffective.

The stimulation of growth by kinetin and benzyl-amino-purine was considerable; during a period of 2 days following the cytokinin addition, the multiplication rate was 6 times higher than before. TASSERON-DE JONG and VELDSTRA (1971) observed that this stimulation was maintained for only 7 days, after 3 weeks the cultures had died. ROMBACH (1974) found that after addition of thiamine, stimulation by kinetin to 150% was maintained over dark periods longer than 80 days. Although it has not been possible to simulate completely the effect of light by chemical substances added to the medium, there are some promising aspects.

With *L. minor*, GORHAM (1950) observed that during the first two days of darkness there was an 'inoculum effect', consisting of a relatively high multiplication rate. During the subsequent 5 days in darkness this inoculum effect was followed by a lag phase. The inoculum effect was increased when the sugar content during the light period was higher than that during the dark period or if photosynthesis was increased by increasing the light intensity or the CO_2-content of the air. Probably, the inoculum effect was due not to the fresh medium but to the light used during inoculation. ROMBACH (1961, 1971) found that the period of increased growth was lengthened from one day to seven days by the presence of kinetin and that the effect was increased by feeding casein hydrolysate, by lowering the temperature during the preceding light period, or by increasing the light intensity. This suggests that during the preceding light period products of photosynthesis accumulate which stimulate the multiplication rate in a subsequent period of darkness.

Tissue cultures grown in light are often very different in appearence from those grown in darkness, but the rate of dry weight increase is mostly not influenced by light. An intersting exception is the growth of tobacco callus cultures requiring kinetin. BERGMANN and BÄLZ (1966) found that on an optimal medium with kinetin and thiamine, increase in darkness was only slightly below that in light. DIGBY and SKOOG (1966) and LINSMAIER-BEDNAR and SKOOG (1967) found that on a medium without kinetin and thiamine tobacco callusses did not increase in darkness nor at low light intensity. The cultures stayed alive and the endogenous thiamine concentration stayed at a relatively high level. If kinetin was supplied growth was considerable but this was accompanied by a drastic decrease in the endogenous concentration of thiamine. After three weeks the cultures turned brown and died. During this period the endogenous thiamine had evidently been consumed without being replenished by biosynthesis. With a 30 times higher (5×10^{-6} M) kinetin concentration growth continued, thiamine biosynthesis being stimulated (DRAVNIEKS et al., 1969). At high light intensities, preferably in blue light, the cultures synthesized chlorophyll and became independent of exogenous thiamine and cytokinin (LINSMAIER-BEDNAR and SKOOG, 1967; BERGMANN and BERGMANN, 1968).

This case history of tobacco tissue culture suggest that also for *Lemna minor* it may be possible to find the missing factors for growth in darkness.

1.6. Scope of the investigation

The investigations discussed above demonstrate that light allows *Lemna minor* to grow on media without thiamine and kinetin. Since addition of these substances to cultures grown in the dark is not sufficient to simulate the effect of light, the latter evidently contributes still one or more factors for growth. Several experimental approaches to this aspect of the action of light are possible.

In the first place, the influence of illumination on metabolism could be analyzed. At present this approach is being followed by several groups. Examples are: Mohr and coworkers at Freiburg i. Br. (Mohr, 1972), and Key and coworkers at Athens, Georgia (Travis et al., 1974).

In the second place chemical factors essential for growth, generated through the action of light, could be identified by studying the effect of the addition of specific chemicals to dark-grown cultures. Examples of this type of approach are given in the previous section.

Thirdly, action spectra, dose-response relations and other characteristics of the effect of light, such as photoreversibility, could be studied, in order to obtain information on the pigments involved.

To explore the potentialities of the third approach we made an experimental survey of the action of phytochrome in the photostimulus reaction in *Lemna minor* grown on a nutrient medium with sugar and casein hydrolysate. Since kinetin had the effect to increase the responses on low amounts of light and to prevent clumping of the fronds so that mutual shading was less, the response to light could be evaluated better in cultures grown with added cytokinin than in those without it. Most of the experiments were therefore made in two parallel series, one with kinetin and the other without it. Presupposing that kinetin is a substitute for endogenous cytokinin, we assumed that this might also enable us to obtain indications about the light requirement of endogenous cytokinin production.

2. METHODS

2.1. MATERIAL

A strain of *Lemna minor*, originally introduced in this laboratory by Dr. W. LINDEMAN (1952), was used. At the start of our investigations the plants were disinfected by successive dipping in 50% ethanol, immersion in 0.1% $HgCl_2$ for 40 seconds, and rinsing with sterile water. Young fronds with the reproductive pockets still closed, gave the best results.

2.2. CULTURE CONDITIONS

2.2.1. *Culture medium*

In most experiments a standard medium, modified from GORHAM (1950), was used:

$Ca(NO_3)_2 \cdot 4H_2O$	0.5 g
$MgSO_4 \cdot 7H_2O$	0.25 g
KH_2PO_4	0.136 g
H_3BO_3	2.86 mg
$MnCl_2 \cdot 4H_2O$	1.81 mg
$ZnSO_4 \cdot 7H_2O$	0.22 mg
$(NH_4)\ Mo_7O_{24} \cdot 4H_2O$	0.18 mg
$CuSO_4 \cdot 5H_2O$	0.07 mg
$Co(NO_3)_2 \cdot 6H_2O$	0.08 mg
NH_4VO_3	0.01 mg
Fe^{3+} EDTA	4.26 mg

distilled water to make up to 1 liter.

In experiments under continuous illumination the concentration of calcium nitrate was doubled. Furthermore, the medium contained 15 g saccharose, 0.26 g casein hydrolysate vitamin free (DIFCO Detroit USA) and 0.005 g tryptophane per liter. The nitrogen content of casein hydrolysate is 10%. Accordingly, 30% of all nitrogen was supplied by this nutrient.

Kinetin (NBC, Cleveland, Ohio), 3×10^{-6} M (0.8 mg/l), was added to the medium when indicated.

2.2.2. *Sterilization of the medium*

Glass tubes, 15 cm long, 2.6 cm internal diameter, with 25 ml of medium containing macro-elements, micro-elements and saccharose, were autoclaved for 15 minutes at 120°C. At the end of autoclaving pH was 4.5. During this treatment saccharose inverted to reducing sugars for about 10%. Iron with peat extract was autoclaved together with the minerals and saccharose, but the EDTA iron complex had to be autoclaved separately, otherwise a precipitate was formed.

Casein hydrolysate with the added tryptophane was also autoclaved separately, as was kinetin. These substances were added to the basal medium after sterilization.

In some experiments less stable substances, to be added to the culture medium, were sterilized by filtration through a glass filter G 5 (SCHOTT Mainz), or through a cellulose nitrate membrane filter SM 11307 pore size 0.2 μ of SARTORIUS, Göttingen.

2.3. TECHNIQUES OF MEASUREMENT

2.3.1. *Multiplication rate*

As long as there were no senescent fronds in the cultures, frond number increased exponentially. The rate of increase in frond number, dn/dt, is then proportional to the number of fronds n_t at time t.

$$dn/dt = G_f n_t \tag{1}$$

equation 1 gives

$$\ln(n_1/n_o) = G_f\,(t_1\text{-}t_o) \tag{2}$$

G_f is the rate constant for frond multiplication, it is also the increase in frond number per frond per unit of time, or the relative rate of increase in frond number. Conversion to Briggian logarithms and introducing the multiplication rate MR ($MR = 434\ G_f$), gives

$$MR = 1000\ 1/t\ \log(n_1/n_o) \tag{3}$$

$t = t_1 - t_o$ (days)

2.3.2. *Rate of cell division*

The colchicine method, described by EVANS, NEARY and TONKINSON (1957) was used for determination of the rate of cell division. The description is in chapter 7.

2.3.3. *Frond area and increase in length of fronds*

Photographs of the cultures by shadow print technique were taken on high speed panchromatic film (ILFORD HP4) against a green safe-light. The amount of light received by the plants was below 75 μW sec cm^{-2} per exposure. The spectral composition of the light is given in section 2.4.4. In experiments with *Pisum* plumula, exposition to an amount of energy of 9×10^3 μW sec cm^{-2} resulted in conversion of 5% of Pr to Pfr, so we estimate that in *Lemna* the order of magnitude of Pfr formed per photograph was less than 0.1% of the total phytochrome content. From enlarged prints, the area of the fronds was measured with a planimeter, the length with a ruler.

2.3.4. *Carbohydrate determinations*

The total amount of reducing sugars in the medium was determined with the colorimetric method of NELSON (1944). Saccharose was inverted by boiling for

15 minutes with 0.5 N HCl. The amount of saccharose was then obtained by subtracting the value of reducing sugars before inversion from that determined after inversion. For determination of the sugars in the plants, the cultures were extracted with 80% ethanol. Glucosides were removed from the extract by clarification of the ethanol extract with charcoal. In one experiment glucose and fructose were determined separately after paper chromatography (VERHOEKS, 1965).

Since the inversion procedure influenced the colorimeter readings, a separate calibration curve had to be made for the saccharose determinations.

Starch was extracted with the method of PUCHER et al. (1948). The precipitate obtained was hydrolyzed and determined as reducing sugar. With pure starch about 85% was recovered with this method.

2.3.5. *Photosynthesis and respiration*

In some experiments, manometric measurements of gas exchange were carried out with the WARBURG technique. The WARBURG vessels used in these experiments had a circular 'gallery' alongside the inner wall, provided with 1 ml of PARDEE's CO_2 buffer (4 M diethanolamine + 0.1% thiourea) (KREBS, 1951) and a slip of filter paper to increase its gas exchange with the air. At the start of the experiment, the buffer was equilibrated with 0.5% CO_2. The vessels contained 60–80 mg fresh weight of *Lemna* fronds. Different light intensities were obtained by filters attached to the bottom of the vessels. As light source 40 W fluorescent lamps colour 33 were used.

It was assumed that the differences in manometric pressure were due to changes in oxygen pressure only and that the assimilatory and respiratory quotients were 1.0.

2.3.6. *Estimation of chlorophylls and carotenoids*

Fresh plants were extracted with pure acetone at 5 °C under our green safelight by grinding with sea sand and a little calcium carbonate (BRUINSMA, 1963; RAVEN, 1973). Extinctions of the extracts (80% acetone) were determined at the wavelengths 573 nm, 628 nm, 646 nm, 652 nm and 664 nm in a ZEISS model PMQ II spectrometer equipped with a grating monochromator M 20. Chlorophyll concentrations in μg/ml were calculated, using the absorption coefficients of MACKINNEY (BRUINSMA, 1963; RAVEN, 1973).

The absorption spectrum of an acetone extract of *Lemna* plants, grown in darkness interrupted every 3 hours by a red illumination of 2 minutes 500 μW cm^{-2} (fig. 2.1), suggests that absorption in the blue is mainly due to carotenoids.

An approximate carotenoid content was derived from the extinction value at 473 nm, the contribution of the chlorophylls at this wavelength being less than 3%. We have assumed an extinction coefficient of 220 liter per gram per centimeter for these carotenoids.

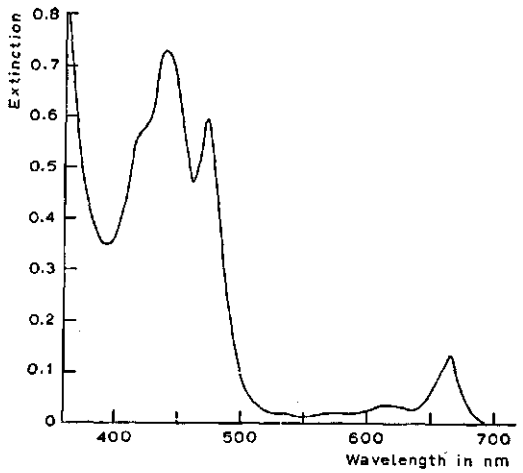


FIG. 2.1. Absorption spectrum of a pigment extract from *Lemna minor*. 1 gram (fresh weight) was extracted with a total volume of 125 ml 80% acetone. Light path 1 cm. Plants grown for 14 days in a regime of 2 minutes red light, 500 μW cm^{-2}, every 3 hours.

2.3.7. *Fresh weight, dry weight*

Immediately after removal from the culture medium, the surface of the plants was dried with filter paper. The plants were transferred to stoppered weighing bottles and weighed without delay. For dry weight determinations the plants were heated to 100 °C for 15 minutes and then dried to constant weight at 75 °C for an additional 24 hours.

2.3.8. *Calculation of standard deviations*

When considered useful in comparing results, the standard deviation of the mean is given:

$$\bar{\sigma} = \sqrt{(x-\bar{x})^2/n(n-1)} \tag{4}$$

x = single observation, $\bar{x}$ = mean value of the observations, n = number of observations. In some cases the standard deviation of the single observation is considered to be of intererest:

$$\sigma = \sqrt{(x-\bar{x})^2/(n-1)} \tag{5}$$

2.4. EQUIPMENT FOR ILLUMINATION

2.4.1. *Cabinets for short periods of illumination*

For experiments with short periods of light, 4 cabinets with 3 compartments each were used (fig. 2.2).

Above each cabinet 3 lamp houses were constructed (fig. 2.2b); the central lamp house was used for far red, the two others in most cases for red light. Light intensity in each compartment was adjusted with adjustable lattice screens, con-

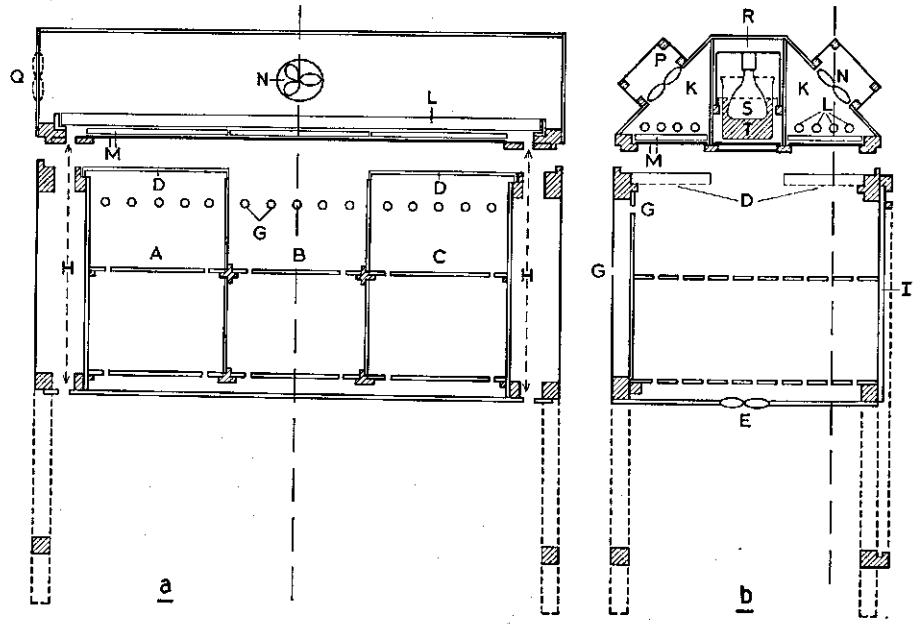


FIG. 2.2. Cabinet for growing plants with illuminations of short duration.
a: Front view; A, B and C compartments for different intensities of light, light intensity is regulated with lattice screens D. Ventilation by ventilator E (in b) and air outlets G. Removable boards at half height of the cabinets, with two rows of openings for ventilation, give the possibility to place objects nearer to the lamps. N: Ventilator of the lamp house of fluorescent lamps, Q: ventilator of the far red lamp house (see R in b) H: air inlet for the lamp houses. The lamp house, with filters of coloured glasses and copper sulfate solutions M, is placed upon the cabinets.
b: side view; I: sliding doors giving entrance to the cabinets; K: lamphouses for fluorescent lamps L, M: light filters; N: ventilator with ventilator shaft P; R: far red lamphouse with incandescent lamps S with internal reflector, the bulbs cooled by water filter T.

sisting of 2 glass plates, one on top of the other, on each of which evenly spaced black paper strips were glued. By moving the upper glass plate the light transmission could be varied. Red light was obtained from 120 W white fluorescent lamps, colour 33 (PHILIPS), combined with a 3 mm layer of red Plexiglas nr 501 (RÖHM und HAAS) and a 10 mm layer of an aqueous solution of 30 g/l $CuSO_4 \cdot 5H_2O$.

Blue light was from 120 W white fluorescent lamps, colour 55 (PHILIPS), combined with a 3 mm layer of Plexiglas nr 0248.

Far red was obtained from incandescent lamps combined with 4 cm water and 2.5 mm of the RG 9 glass of SCHOTT; the ratio of the energy at 660 nm to that at 730 nm was about 10^{-4} for this light source. The transmission curves of the filters are given in figure 2.3. The curve for the transmittance of water was derived from data of JONES and GUY (1913).

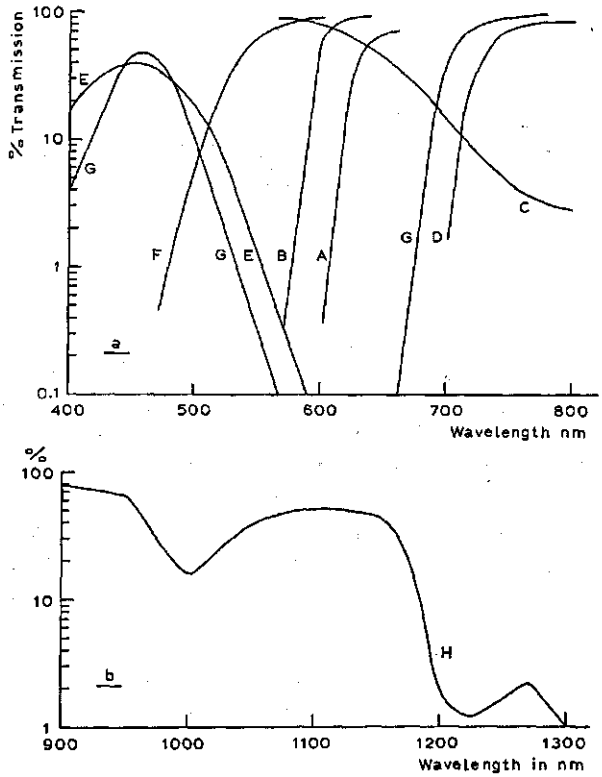


FIG. 2.3. Transmission curves of the filters
a: A = commercial red glass 2.5 mm;
B = Plexiglas nr 501, 3 mm;
C = copper sulfate solution 30 g/l, 10 mm;
D = coloured glass SCHOTT, Mainz, RG 9, 2.5 mm;
E = Plexiglas nr 0248, 3 mm;
F = Cinemoid sheet, colour 47, 0.1 mm;
G = Plexiglas nr 627, 3 mm, in the far red transmission is close to that of 1 mm RG 8 (SCHOTT, Mainz).
b: H = water, 4 cm, 25°C.

2.4.2 *Equipment for illumination with narrow wavelength bands*

The parallel light beam from a 500 W slide projector first passes a layer of 1 cm water and subsequently an interference filter (BALZERS, Liechtenstein, Filtraflex B-40). The water filter reduces the radiation, transmitted by the filters at longer wavelengths, to 10% of the total transmitted energy. After passing the filter system, the beam was reflected downwards by a mirror and was then diffused by frosted glass. Different light intensities could be obtained by placing the objects at different distances from the frosted glass, as well as by wire screen filters.

2.4.3. *Spectral energy distribution of the lamp-filter combinations and their effectivity in phytochrome conversion*

The spectral energy distribution of the different lamp-filter combinations is given in table 2.2. The values are obtained by multiplying the emission spectra of the lamps, presented in table 2.1, with the transmission values of the filters, given in figure 2.2, and with a factor depending on the wavelength band, converting watts into einsteins. The emission spectra of the fluorescent lamps were derived from the data supplied by the manufacturer (see GAASTRA, 1969). Since study of phytochrome involvement was one of the aims of the experiments described in the following chapters, and direct measurements of the content of the active form Pfr is possible only under special conditions, we tried to estimate photoconversion from the spectral energy distribution of the light sources and the published absorption spectra of phytochrome. The spectral fluxes in table 2.2 were multiplied by the action spectra published by BUTLER, HENDRICKS and SIEGELMAN (1964) for phytochrome conversion in vitro, and by PRATT and BRIGGS (1966) for phytochrome conversion in vivo.

For each lamp-filter combination the integral of the values thus obtained was divided by the integral of the fluxes given in table 2.2. The resulting quotients

TABLE 2.1. Spectral energy distributions of the lamps. Values are fractions of the total energy between 400 and 700 nm emitted in the indicated wavelength bands multiplied by 1000.

Wavelength band nm	TL 33	TL 55	Wavelength band nm	Incandescent Lamp 150 W. T = 2800 °K.
300–320	5.2	4.0	600–620	93
320–340	0.7	0.5	620–640	103
340–360	1.7	2.1	640–660	113
360–380	17.2	18.1	660–680	122
380–400	13.8	15.9	680–700	130
400–420	48.9	54.7	700–720	138
420–440	102.6	105.6	720–740	145
440–460	45.4	60.5	740–760	152
460–480	51.0	71.1	760–780	158
480–500	50.3	74.9	780–800	162
500–520	49.6	73.8	800–820	166
520–540	57.8	68.0	820–900	700
540–560	119.8	97.2	900–950	448
560–580	138.0	62.1	950–1000	446
580–600	128.1	61.6	1000–1050	450
600–620	97.1	61.1	1050–1100	440
620–640	58.5	62.6	1100–1150	420
640–660	32.4	64.8	1150–1200	396
660–680	17.9	49.4	1200–1300	680
680–700	11.0	32.4		
700–720	6.9	23.9		
720–740	4.1	15.9		

TABLE 2.2. Relative spectral energy distribution of the light obtained from the different lamp-filter combinations. Values are the transmitted fractions of the values in table 2.1, converted to light quanta. Lamp-filter combination BC = TL 33 + filter B + filter C, 658 = incandescent lamp 500 W + interference filter λ 658 nm, E = TL 55 + filter E, Safelight = TL 55 + filter E + filter F, Far red I = incandescent lamp 150 W + filter G + B + 4 cm water, Far-red II = incandescent lamp 150 W + RG 9 + 4 cm water, Far-red III = incandescent lamp + interference filter λ 740 nm.

Wavelength band	Lamp-filter combinations				Wavelength band	Lamp-filter comb.		
	BC	658	E	Safe-light		Far red		
						I	II	III
360–380			0.5		660–680	7.2		
380–400			4.2		680–700	138.2	3.2	
400–420			38.0		700–720	465	75	5
420–440			125.0		720–740	666	336	105
440–460			89.0	0.07	740–760	750	583	190
460–480			95.0	0.38	760–780	815	724	10
480–500			74.0	1.8	780–800	896	795	
500–520			39.0	4.7	800–820	994	886	
520–540			14.8	5.2	820–900	4170	3560	
540–560			3.9	2.4	900–950	3460	1780	
560–580	2.0		1.0	0.9	950–1000	1070	586	
580–600	71.1		0.37	0.33	1000–1050	907	376	
600–620	273.5		0.13	0.12	1050–1100	1632	341	
620–640	164.5	8	0.04	0.04	1100–1150	1940	206	
640–660	70.6	105			1150–1200	537	33	
660–680	28.4	57			1200–1300	70	22	
680–700	10.5	6						
700–720	4.1							
720–740	1.6							

TABLE 2.3. The efficiency factors for phytochrome conversion calculated from the different lamp filter combinations in table 2.2 and the action spectra published by BUTLER et al. (1964) for phytochrome photoconversion in vitro and by PRATT and BRIGGS (1966) for that in *Avena* coleoptiles. When the light intensity is measured in einsteins $cm^{-2}sec^{-1}$, the Q values are 10^6 times higher. $Q_{(\lambda)(Pr)}$ = efficiency factor for conversion from Pr to Pfr, $Q_{(\lambda)(Pfr)}$ = efficiency factor for conversion from Pfr to Pr. (λ) = indication for the wavelength composition of the light.

		Lamp-filter combinations						
		BC	658	E	Safe-light	Far red		
						I	II	III
$Q_{(\lambda)(Pr)}$	BUTLER	19.2	31.1	1.1	1.2	0.08	0.008	0.017
	PRATT	38.6	91.4	1.3	5.4	0.29	0.019	0.011
$Q_{(\lambda)(Pfr)}$	BUTLER	5.2	8.1	1.7	0.3	1.6	1.4	11.6
	PRATT	12.0	28.4	1.7	1.7	5.5	3.7	29.4
Pfr/P_{total}	BUTLER	79%	79.4%	40%	77%	4.8%	0.53%	0.15%
	PRATT	77%	76.4%	52%	76%	5%	0.49%	–

$Q_{(\lambda)(Pr)}$ and $Q_{(\lambda)(Pfr)}$ are the efficiency coefficients of phytochrome photoconversion in light of the spectral composition (λ):

$$dPfr/dt = E\, Q_{(\lambda)(Pr)}[Pr] \tag{6}$$

$$\text{and } dPr/dt = E\, Q_{(\lambda)(Pfr)}[Pfr] \tag{7}$$

Herein E is light energy, [Pr] and [Pfr] are the fractions of total phytochrome present as Pr and Pfr respectively and t is the time.

Since there is an overlap of the spectra of Pr and Pfr, the photoreactions Pr→Pfr and Pfr→Pr take place simultaneously. Therefore the net rate of change of the Pfr fraction is:

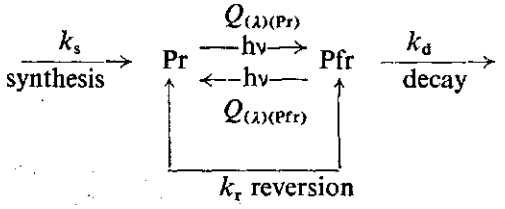
$$dPfr/dt = E\, Q_{(\lambda)(Pr)}\, Pr - E\, Q_{(\lambda)(Pfr)}\, Pfr \tag{8}$$

$$E\, t = Pfr/Q_{(\lambda)(Pr)}\, \ln\{(Pfr_\infty - Pfr_o) - \ln\,(Pfr_\infty - [Pfr])\} \tag{9}$$

$$Pfr_\infty = Q_{(\lambda)(Pr)}/Q_{(\lambda)(Pr)} + Q_{(\lambda)(Pfr)} \tag{10}$$

In this equation Pfr_∞ is the photostationary fraction in Pfr; Pfr_o that at the beginning of the illumination; [Pfr] and [Pr] are the fractions at time t.

The stationary fraction of Pfr during illumination does not depend upon the light reactions only but also upon the rate k_s of de novo Pr synthesis, the rate k_r of dark reversion and the rate k_d of Pfr decay:



Since, at light intensities above 10 μW cm^{-2}, these reactions are relatively slow compared with the photoconversion, they have been neglected in the calculation of the photostationary states in table 2.3.

The accuracy of the data in table 2.3 is open to criticism; the lamp-emission data are only approximate; furthermore, the spectral data of BUTLER et al. are for phytochrome in solution. Obviously, the optical and physical conditions in such extracts differ from those in vivo. This is demonstrated by the Q values in table 2.3: there is agreement between the values in the blue, but in the red the in vivo values of PRATT and BRIGGS are much higher than the in vitro values. Considering that there is considerably more absorption by screening pigments in vivo than in vitro, one would have expected the opposite. We are unable to offer an explanation for this discrepancy.

In view of this discrepancy, we prefer direct Pfr measurements above values calculated with equations 9 and 10. Direct spectrophotometric Pfr measure-

ments were impossible in most cases because the phytochrome concentrations were too low. If, however, the cultures were kept for 7 days in continuous darkness, the phytochrome content increased to a sufficient level (ROMBACH and SPRUIT, 1968). With *Lemna minor* cultures treated in this way, photoconversion as a function of light dose could be measured spectrophotometrically. The results are presented in fig. 2.4. As a check, phytochrome photoconversion in plumules of etiolated seedlings of *Pisum sativum* c.v. Alaska was also measured. As the phytochrome content of this material is much higher, the measurements are more reliable than those in *Lemna minor*.

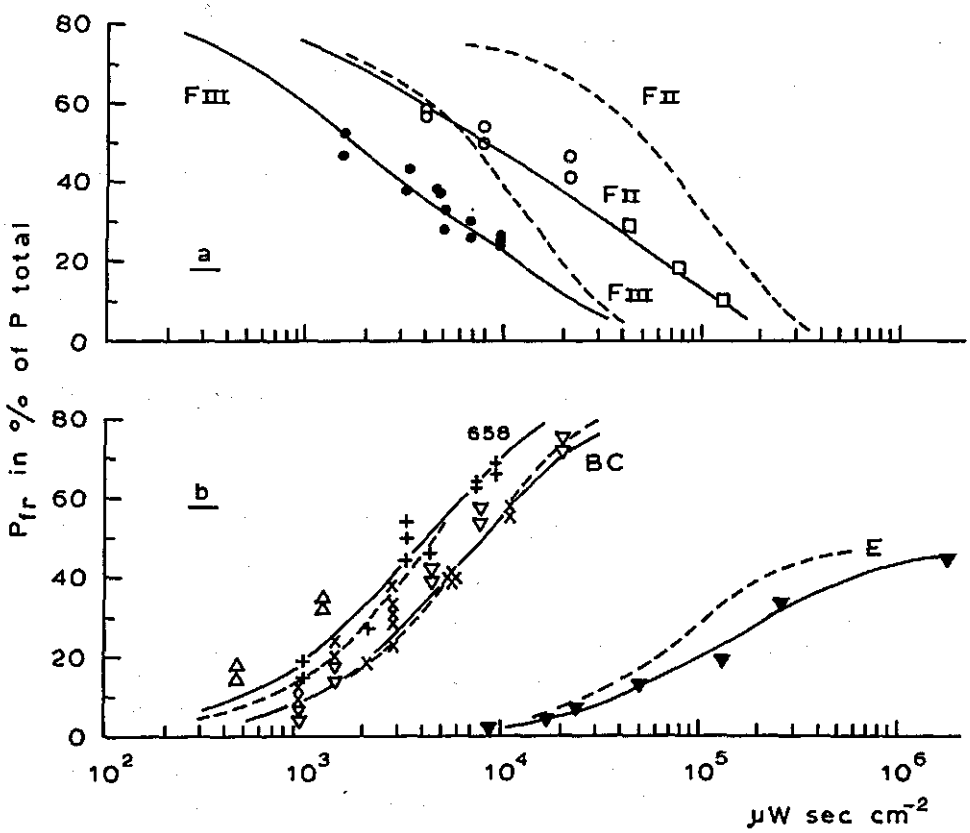


FIG. 2.4. Photoconversion of phytochrome as a function of the dose of light in *Lemna minor* illuminated in the culture tubes and in plumules of 8 days old *Pisum sativum* seedlings illuminated in 2 mm sample cells. Broken lines: photoconversion of phytochrome calculated from data of BUTLER et al. (1964). The full lines are drawn through the marks.
a: Photoconversion of Pfr to Pr; (○), (□) and FII = far red II; (●) and FIII = far red III (interference filter 740 nm); (○) and (●) = *Lemna minor* (□) = *Pisum sativum*.
b: Photoconversion of Pr to Pfr; (×), (▽) and BC = red light from lamp filter combination BC; (+), (△) and 658 = lamp filter combination 658 (interference filter 658 nm); (▼) = blue light, lamp filter combination E; (×) and (+) = *Lemna minor*; (▽), (△) and (▼) = *Pisum sativum*.

The broken lines in fig. 2.4 refer to the photoconversion calculated with equation 9 from the Q values according to BUTLER et al. (1964). Comparison with the measured values shows that there is good agreement in the red part of the spectrum, but in the blue, and especially in the far red the slope and the position of the measured curves is different. Since a considerable fraction of the incident light is scattered back, the intensity in the first layer of the tissue exposed to the light will be lower than that measured directly. The light intensity inside the tissue decreases with the depth of the layer by absorption, this counts especially in the blue by the presence of carotenoids. The decrease in apparent photochemical activity caused by these effects is counteracted by the increase in path length inside the tissue (BUTLER, 1972; HARTMANN and UNSER, 1972). In the case of far red this may more than compensate for the backscattered light; in the red an accidental balancing of these factors may result in the good correspondence between the measured and the calculated phototransformations. Additional measurements, however, are required to settle this.

No differences were found between the measured and calculated photostationary states of phytochrome.

2.4.4. '*Safe-light*'

Green 'safe-light' was obtained from 20 W fluorescent lamps colour 55 (PHILIPS) combined with a far red cut off filter of Plexiglas 0248 of RÖHM und HAAS (transmission curve E in fig. 2.3) and a yellow Cinemoid filter nr. 47 of Stran Electric Comp. (F in fig. 2.3). The spectral energy distribution of this light i given in table 2.2.

2.5. LIGHT MEASUREMENTS

Radiation intensity was measured with a cosine corrected selenium barrier layer cell connected to the AL4 galvanometer of KIPP en Zonen, Delft. The photocell was calibrated for every lamp-filter combination against a MOLL large surface thermopile (E 11 KIPP en Zonen, Delft). Intensities lower than 100 $\mu W\ cm^{-2}$ could not be measured directly with the galvanometer-thermopile combination. Lower intensities, required for calibration of the photo cell, were obtained by varying the distance from a point shaped light source or by interposition of wire-screen filters of known transmission. Readings of the galvanometer-selenium cell combination were linear with light intensity up to 40 μA.

3. THE EFFECT OF VARIATION OF SOME CULTURE CONDITIONS

Experimental conditions in general were chosen to ensure the maximal response to the light regime; this implies that other limitations should be removed as far as possible. Effects of variation of some environmental conditions are reported below.

In most cases the aspect of growth studied was the multiplication rate, since preliminary experiments had shown that this was easy to measure and the response to light was very marked. Multiplication rate, however, is the result of several growth processes, as there are cell division, expansion growth, correlation phenomena and processes of carbon assimilation. This induced us to give some attention to these aspects too.

3.1. THE INFLUENCE OF TEMPERATURE ON THE MULTIPLICATION RATE

Cultures were grown in continuous light at 12.4 μW cm^{-2} and at 1800 μW cm^{-2}. At 12.4 μW cm^{-2} photosynthesis was very low and multiplication rate was largely dependent on sucrose assimilation from the medium. At 1800 μW cm^{-2} however, photosynthesis supplied most of the energy required for growth (section 3.6).

At the higher light intensity the optimum temperature for multiplication rate was 28 °C. The multiplication rate at the lower light intensity was almost independent of temperature in the interval from 17° to 30 °C (fig. 3.1). This shows that at low light intensities photochemical processes are rate limiting for frond multiplication, while at higher intensities non-photochemical processes are rate limiting. Cultures with kinetin added responded in the same way to temperature as those without.

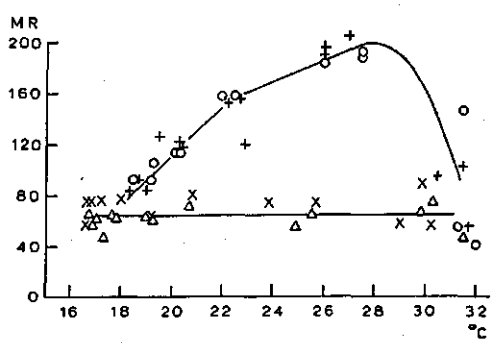


FIG. 3.1. Multiplication rate (*MR*) as a function of temperature at two light intensities of continuous white fluorescent light (PHILIPS TL 40, colour 33). Medium with 1.5% sugar; (×) and (△) = 12,5 μW cm^{-2} light; (+) and (○) = 1800 μW cm^{-2} light; (○) and (△) = no kinetin added; (×) and (+) = 3 × 10^{-6} M kinetin.

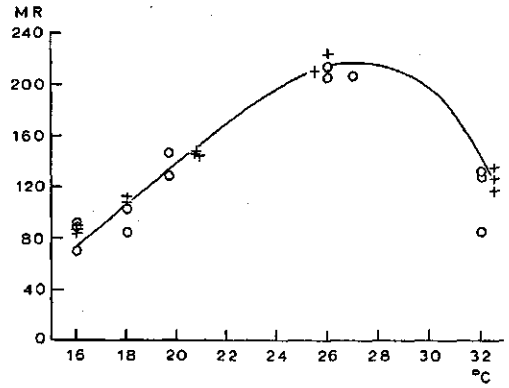


FIG. 3.2. Multiplication rate (*MR*) as a function of temperature under a regime of 18 hours light and 6 hours darkness. Fluorescent light (colour 33) 2200 μW cm^{-2}. Medium with 1.5% sugar. (○) = no kinetin; (+) = 3×10^{-6}M kinetin.

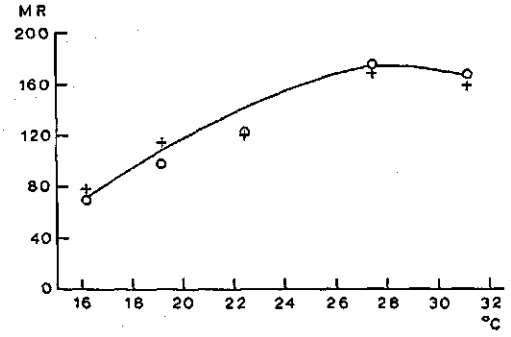


FIG. 3.3. Multiplication rate (*MR*) in continuous light under a periodic change of temperature from 16°C for 8 hours to the temperature indicated on the abscissa for 16 hours. Fluorescent light (colour 33) 1800 μW cm^{-2}. Medium with 1.5% sugar. (○) = no kinetin; (+) = 3×10^{-6}M kinetin.

In continuous light, growth was inhibited at temperatures above 30°C and the fronds showed necrotic spots. When a dark period of 6 hours per day was inserted (the light intensity was increased) the growth reduction above 30°C was less pronounced (fig. 3.2). The damaging effect of 30°C in continuous light could also be reduced by lowering the temperature to 16°C during 8 hours per day (fig. 3.3). Similar results were obtained by HILLMAN (1956) with respect to chlorosis of tomatoes grown in continuous light.

Finally a temperature of 22°C was chosen for most experiments, at higher temperatures the cultures being less healthy. Moreover, as fig. 3.1 shows, at 22°C frond multiplication below $MR = 150$ is rate limited by light and not by temperature.

3.2. THE INFLUENCE OF KINETIN CONCENTRATION

Since kinetin was a component of the nutrient solution in most experiments, the effect of its concentration was tested at different levels of light intensity. In fig. 3.4 the effect on multiplication rate and in fig. 3.5 that on the final size of the fronds is given. At all light intensities tested, the optimum concentration was between 15 and 45 $\times$ 10^{-7} M, in agreement with the results of HILLMAN (1957). At 135 $\times$ 10^{-7} M growth inhibition was observed, in accordance with the findings of VAN EYK (1963). The final size of the fronds proved somewhat more sensitive to kinetin than the multiplication rate, this applies both to the stimulatory effect of low and to the inhibitory effect of high concentrations.

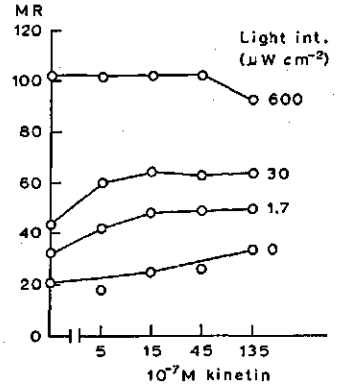


FIG. 3.4. Multiplication rate (*MR*) at different kinetin concentrations (abscissa) and different light intensities. Fluorescent light (colour 34). Medium with 1% sugar.

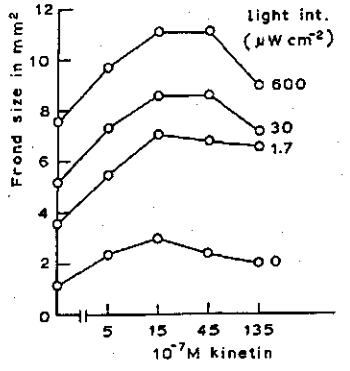


FIG. 3.5. Final size of fronds grown at different kinetin concentrations (abscissa) and different light intensities. Same experiment as in fig. 3.4.

3.3. The influence of folic acid and its interaction with light and kinetin

Van Eyk (1963) observed a favourable effect of folic acid on frond multiplication and found that it reversed the inhibitory effect of high kinetin concentrations. Bezemer-Sybrandy et al. (1968) demonstrated photochemical destruction of kinetin, especially in the presence of folic acid. She attributed the results of Van Eyk to this reaction. Benzyl-amino-purine was much more stable under these conditions. Accordingly, we made some experiments to see in how far this may have interfered with our work. Some results are shown in table 3.1. Folic acid stimulated growth in continuous light both with and without kinetin in the medium, confirming the observation of Van Eyk (1963). It had no influence on the multiplication rate when brief illuminations in cycles of 5 minutes light and 36 hours darkness were given.

Table 3.1. Effect of folic acid and kinetin on the multiplication rate of *Lemna minor* on standard medium in continuous light 770 $\mu W\ cm^{-2}$ from fluorescent lamps colour 55, or in darkness interrupted once every 36 hours with 5 minutes red light 200 $\mu W\ cm^{-2}$.

Concentrations in μM		Multiplication rate			
Kinetin	Folic acid	Continuous light		5 min. light/36h	
		Exp 1	Exp 2	Exp 1	Exp 2
0	0	–	160	–	30
0	1.5	–	190	–	30
0	3.0	190	190	30	30
3	0	160	170	44	45
3	1.5	180	180	45	45
3	3.0	190	190	47	45

The effect of light on media containing both folic acid and kinetin was tested by measuring the multiplication rate on media exposed previously to different amounts of light from fluorescent lamps, colour 55.

Kinetin absorbs radiation only in the ultraviolet and infrared parts of the spectrum. Folic acid has an absorption maximum at λ 368 nm. Fluorescent light, colour 55, has only 0.015% of its radiation in the spectral region from 380 to 420 nm, and light absorption by kinetin is only small as compared with that of folic acid. Red light is absorbed by none of these substances and is, therefore, suitable in a kinetin test. In table 3.2, multiplication rates are given of cultures grown in darkness interrupted by 200 $\mu W\ cm^{-2}$ red light for 5 minutes every 24 hours. The growth stimulation by this treatment is much increased by kinetin (chapter 5). Table 3.2 shows that the multiplication rate was decreased when a medium containing kinetin had been previously exposed for 8 days or more to 5000 $\mu W\ cm^{-2}$ fluorescent light colour 55. With kinetin and folic acid both present, growth inhibition was visible in media pre-irradiated for

TABLE 3.2. Effect of radiation on destruction of kinetin. Culture tubes without plants with standard medium or this with 3×10^{-6} M kinetin or folic acid or both were illuminated at 30°C for different periods with 5000 $\mu W\ cm^{-2}$ light from fluorescent lamps, colour 55. Hereafter the tubes were inoculated with *Lemna minor*; the cultures were illuminated once a day with 200 $\mu W\ cm^{-2}$ red light during 5 minutes. Multiplication rates (at 25°C) were determined after 21 days.

Time of preparative irradiation in days	Multiplication rate			
	Standard medium	Folic acid	Kinetin	Kinetin + Folic acid
0	50	–	60	–
6	–	–	60	53
8	45	47	60	47
15	45	25	48	25
21	–	–	33	19

only 6 days. Fronds were smaller and some dead fronds were observed.

These data show that at high light intensities growth inhibiting substances are formed in media with kinetin. In experiments to be described in chapters 3 and 4, the light intensity was always much lower than 5000 $\mu W\ cm^{-2}$. With 2000 $\mu W\ cm^{-2}$ fluorescent light, colour 33, the medium became covered with fronds within 10 days. The medium was then shielded against further irradiation. No indications were found for kinetin destruction by light in these experiments.

3.4. THE INFLUENCE OF CASEIN HYDROLYSATE, CYSTEINE, YEAST EXTRACT AND THIAMINE ON GROWTH RATE AND LONGEVITY IN DARKNESS

The literature discussed in section 1.5.2 induced us to test the requirement for casein hydrolysate and some vitamins.

Table 3.3 shows the effects of different substances on the increase in frond number and longevity in continuous darkness. The plants were harvested after different periods of growth. In the absence of yeast extract or thiamine dead fronds appear in the cultures with benzyl-amino-purine (BAP) as well as in those without. On the basal medium with added BAP dead plants were often found after 20 days already, confirming the observation of TASSERON-DE JONG and VELDSTRA (1972). It is not likely that this is due to formation of toxic substances, as in most experiments the surviving fronds were vigorous. Addition of casein hydrolysate delayed the appearance of dead fronds in the presence of BAP (table 3.3). Cysteine, which according to MARTIN (1964) promoted frond multiplication in darkness of *Spirodela polyrrhiza*, was without effect on *Lemna minor*. No mortality was observed in the presence of yeast extract or thiamine. A 100 fold increase in frond number ($N = 2$) was obtained with BAP, sucrose and the

TABLE 3.3. The effect of various substances on frond production and longevity. Frond production (N) expressed as increase in the logarithm of the number of fronds; mortality (M) expressed in percent dead fronds at time of harvest. Cas. = 130 mg/l casein hydrolysate, BAP = benzyl-amino-purine. Basal medium = 1.5% sucrose + minerals + EDTA-iron complex.

Substances added to the medium	Culture period					
	20 Days		40 Days		80 Days	
	N	M	N	M	N	M
Without BAP						
Basal medium	0.52	0	1.10	0	1.46	10
Cas.	0.44	0	1.07	0	1.65	5
cysteine 4 mg/l + Cas.	0.42	0	1.05	0	0.85	5
yeast extract 100 mg/l + Cas.	0.37	0	1.01	0	1.73	0
thiamine 5×10^{-6}M	0.47	0	–	–	1.78	0
With 10^{-6}M BAP	20 days		40 days		60 days	
Basal medium	0.81	0	1.14	38	1.58	47
Cas.	0.73	0	1.30	21	1.58	37
Cysteine 4 mg/l + Cas	0.81	0	1.46	12	1.63	47
Yeast extract 130 mg/l + Cas	0.72	0	1.40	0	1.74	5
Thiamine	0.86	0	1.55	0	2.00	0
2-methyl-4-aminopyrimidyl-5-methanesulfonic acid* 10^{-5}M	0.81	0	1.15	4	1.80	30
4-methyl-5(β hydroxyethyl)-thiazole* 10^{-5}M	0.85	0	1.35	0	1.95	0

* The pyridine and thiazole moieties of thiamine were obtained by cleavage of thiamine with bisulfide (WILLIAMS et al. 1935). The reaction products were purified and identified by W. CH. MELGER and G. P. LELYVELD, Dept. of Organic Chemistry, Agricultural University Wageningen, using silica gel thin layer chromatography and infrared spectrophotometry.

TABLE 3.4. The effect of different illumination cycles on longevity of cultures grown on a medium with minerals, EDTA-iron complex, 1.5% sugar and 1×10^{-6} M benzylamino-purine with or without 5×10^{-6} M thiamine. The duration of a cycle was 48 hours. 2 min R = 2 minutes 600 μW cm^{-2} red BC light, 5 min F = 5 minutes 5000 μW cm^{-2} far red II, 48 hr D = remainder of cycle in darkness. Frond production (N) and mortality (M) are expressed as in table 3.3. Total chlorophyll content is given in μg/gram fresh weight.

Irradiation schedule	28 Days		47 Days		Chlorophyl content
	N	M	N	M	μg/g
Basal medium					
2 min R – 48 hr D	1.38	0	1.82	0	–
2 min R – 5 min F – 48 hr D	0.94	0	1.22	27	–
5 min F – 48 hr D	1.00	0	1.43	17	–
Dark control	0.99	11	1.38	18	–
Basal medium with thiamine					
2 min R – 48 hr D	1.39	0	2.00	0	0.38
2 min R – 5 min F – 48 hr D	0.96	0	1.46	0	0.42
5 min F – 48 hr D	1.05	0	1.73	0	0.05
Dark control	1.10	0	1.89	0	0.01

EDTA iron complex as the only organic additions to the medium. Thiamine could be substituted by its thiazole moiety, the pyrimidine moiety was inactive.

Further experiments, presented in table 3.4, show that one illumination of 2 minutes red light, given once every 48 hours, was sufficient to make the increase in frond number independent of thiamine supply. The effect of red light was reverted by far red, pointing to phytochrome control of endogenous thiamine synthesis. It is not likely that this control was via the development of the chloroplasts; in the red – far red treated cultures chlorophyll content was not lower than in those treated with red light alone.

3.5. The influence of different concentrations of some sugars

At low light intensities growth of *Lemna minor* depends on assimilation of sugar or other carbon sources. From the survey by Hillman (1961) it appears that sucrose, glucose and fructose are superior to glycerol, tartrate, succinate and acetate in sustaining heterotrophic growth of Lemnaceae. Data of Tasseron-de Jong (1968) on *Lemna minor* show a preferential uptake of glucose from a mixture of glucose, fructose and sucrose in concentrations of 0.1%, whereas at a concentration of 1%, fructose and glucose were taken up at the same rate. Sucrose was converted to glucose and fructose, but no invertase activity was found in the medium. Mannitol was not metabolized.

We confirmed these data; fructose and glucose produced no better growth than sucrose, mannitol was not utilized, sorbose was toxic. Sucrose was inverted for 5–10% during autoclaving the medium, and further inversion took place during growth of the cultures. During the incubation of 250 fronds (500

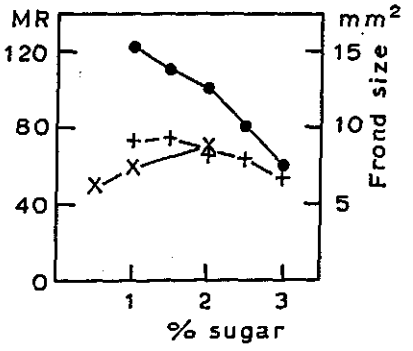


Fig. 3.6. Multiplication rate (*MR*) and final size of fronds at different initial sugar concentrations in the medium (abscissa) and two intensities fluorescent light (colour 34). Medium with 3×10^{-6} M kinetin; (×) = multiplication rate at 18 μW cm^{-2}; (+) = multiplication rate at 25 μW cm^{-2}; (•) = final size of fronds at 25 μW cm^{-2}.

mg fresh weight) for 7.5 hours on 10 ml medium with 1.5% sucrose at 22°C under aseptic conditions the amount of reducing sugars increased from 5 to 22 mg, in 24 hours to 28 mg and in 48 hours to 44 mg; thus, after the first 7.5 hours, the rate of sugar inversion decreased. This experiment showed that in a medium where initially sucrose had been the only sugar, glucose and fructose soon appeared. Media supplied with sucrose will be indicated therefore as media with sugars

The effect of different concentrations of sugar is shown in fig. 3.6. Since at 2.5 $\mu W\ cm^{-2}$ there was almost no photosynthesis, growth depended on sugar uptake from the medium. While multiplication rate had its maximum at a concentration of 1.5%, the final frond size was smaller the higher the sugar concentration.

3.6. PHOTOSYNTHESIS

A number of measurements of photosynthesis were made with the WARBURG technique, using PARDEE's buffer for maintaining a constant pressure of CO_2. The results, presented in fig. 3.7, show that photosynthesis, when related to fresh weight and corrected for respiration, was equal for cultures grown with or without kinetin. Kinetin did not affect the respiration rate either: with kinetin, as well as in its absence, 24 mm^3 O_2 per hour per 100 mg fresh weight was respired in the presence of sugar, half that value without sugar in the medium. Cultures grown at 10 and at 2 $\mu W\ cm^{-2}$ had a reduced rate of photosynthesis. Their chlorophyll content was also deminished; when photosynthesis was related to chlorophyll content, much of the difference disappeared.

Photosynthesis measurements at CO_2 pressures similar to those during the actual growth experiments and at higher light intensities were possible with the diaferometer technique described by PIETERS (1971). Figure 3.8 shows that at 0.03% CO_2 light saturation of photosynthesis was reached at 2000 $\mu W\ cm^{-2}$. Photosynthesis per unit area of cultures grown without kinetin exceeds that of kinetin cultures by a factor 1.2. This does not contradict the WARBURG measurements reported above because weight per cm^2 frond area of cultures without kinetin was also 1.2 times higher than that of the kinetin cultures.

3.7. THE RATE OF SUGAR UPTAKE

The standard deviation of the sugar determinations sets a lower limit of a few hours to the time period over which uptake of sugar from a 1% solution could be measured. In long term experiments the uptake per frond per day of growing cultures can be calculated if the number of fronds present at any time during the observation period is known.

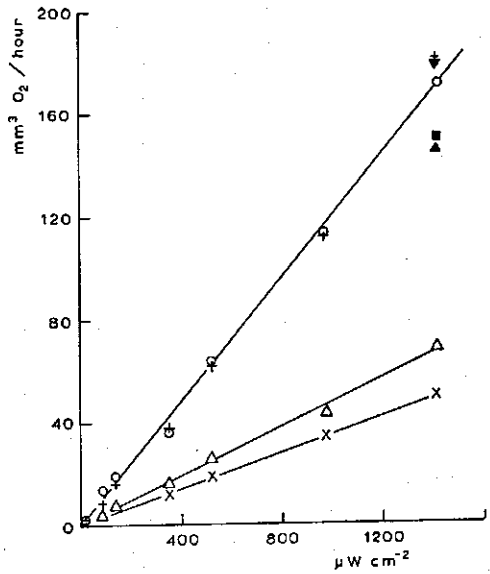


FIG. 3.7. Oxygen evolution per 100 mg fresh weight, corrected for respiration (24 mm^3), at different intensities of white fluorescent light (colour 33) of *Lemna* cultures grown at the intensities indicated below. The medium contained 1% sugar. Measurements with the WARBURG technique. CO_2-content of the air was 0.5%, temperature 22°C.
(○) = cultures grown at 1400 $\mu W\ cm^{-2}$ without kinetin;
(+) and (▼) = cultures grown at 1400 $\mu W\ cm^{-2}$ with kinetin;
(△) and (▲) = cultures grown at 10 $\mu W\ cm^{-2}$ with kinetin; (×) and (■) = cultures grown at 2 $\mu W\ cm^{-2}$ with kinetin.
(▼) and (▲) and (■) = O_2-production per relative unit of chlorophyll.

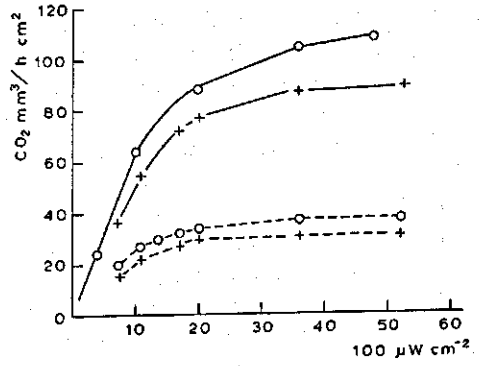


FIG. 3.8. CO_2-consumption per cm^2 frond area in incandescent light. Intensities pertain to the wavelength range between λ 400 and λ 700 nm. The cultures were grown at 400 $\mu W\ cm^{-2}$ fluorescent light (colour 34), the medium contained 1% sugar. The values are corrected for respiration. (——) = 5% CO_2; (– – –) = 0.03% CO_2; (○) = no kinetin; (+) = 3×10^{-6} M kinetin. Temperature 22°C.

If the rate of sugar uptake per frond per day is W and y_t is the number of fronds in the culture, then the rate of sugar uptake by the culture is $y_t \times W$, and the sugar uptake U over a period of t days is equal to

$$U = \int_{t_o}^{t_t} W y_o \, e^{G_f t} \, dt \tag{11}$$

$$U = W y_o / G_f \, (e^{G_f t} - 1) \tag{12}$$

In this equation G_f is the rate constant of growth (see section 2.3).

The decrease of the sugar content of the medium was measured in two experiments at three light intensity levels, the results are given in table 3.5. Sugar uptake was in the range of 0.03 to 0.06 mg per frond per day. No stimulation of the uptake by light was found.

The amount taken up per frond per day is greater than the increase of dry weight per frond per day. The difference is due to respiration and to the difference in chemical composition of the sugar and that of the resulting dry matter.

In media without sugar an increase in frond number was only observed at 96 and at 960 $\mu W\ cm^{-2}$. At 96 $\mu W\ cm^{-2}$ the number of fronds doubled in 31 days, thus photosynthesis at that intensity scarcely outweighed respiration.

3.8. Dry matter production and growth limitation on media with and without kinetin

From the preceding section it appeared that carbohydrate uptake per frond was not influenced by growth rate or light intensity, but, when temperature was not varied, only by sugar concentration. This could mean that the rate of production of dry matter per frond or per frond area is constant under conditions of heterotrophic growth, and that this sets an upper limit to the growth stimulation by light when contribution by photosynthesis to carbohydrate supply is negligible. The rate of dry matter production of cultures under different light intensities was calculated from the multiplication rate and the data on frond dry weight. Since light intensity affects frond size, the data were related to leaf area.

Table 3.6 shows that below 100 $\mu W\ cm^{-2}$ the rate of dry weight increase per unit area is rather constant. In the cultures with kinetin the rate is lower than in those without, notwithstanding a higher multiplication rate. This is due to the lower dry weight per cm^2 frond area. This is demonstrated in fig. 3.9.

Figure 3.10 shows the rates of dry matter production at different light intensities in cultures with and without sugar. The figure shows that the additional dry weight production due to sugar is remarkably independent of light intensity. Since frond production was limited by morphogenetic effects controlled by light and kinetin, this incremental dry matter production does not result in

TABLE 3.5. Number of fronds at different sampling times, dry weight production and sugar uptake in cultures grown in continuous fluorescent light colour 34 at 25°C in aseptic cultures with nitrate as nitrogen source. Accuracy of sugar determinations 2%; light intensity E in $\mu W\ cm^{-2}$; n = number of parallel cultures per condition.

1	2			3	4	5	6	7	8
Conditions	Average number of fronds after t days			Number of fronds integrated over incubation time	Sugar uptake in mg	Sugar uptake in mg per frond per day	Total dry weight production in mg	P/I in mg	Dry weight per frond in mg
	$t = 0$	$t = 11$	$t = 30$	I^*	U	U/I	P		
1% Sucrose									
$E = 960$ $n = 2$	2.5	29	922	5040	212	0.041	131	0.026	0.142
$E = 96$ $n = 2$	2.0	12.5	186	1335	70	0.052	–	–	–
$E = 10$ $n = 3$	2.7	6.3	26	332	13	0.039	4.5	0.014	0.173
0.5% Glucose									
$E = 960$ $n = 3$	3.0	42	880	5300	125**	0.024	92	0.017	0.105
$E = 96$ $n = 3$	2.3	23.0	256	1927	51	0.026	–	–	–
$E = 10$ $n = 3$	2.3	7.0	31	370	10	0.026	5.5	0.015	0.177
Darkness $n = 3$	2.7		9.3	168	5.7	0.034	2.0	0.012	0.214
Without sugar									
$E = 960$ $n = 5$	3.6	18.0	175	1470	–	–	8.3	0.006	0.047
$E = 96$ $n = 5$	3.0	5.0	6.0	–	–	–	–	–	–

* $I = (y_o/G_f)(e^{G_f t} - 1)$ (see equation 12)

** Sugar exhausted at conclusion of experiment

TABLE 3.6. Dry matter production in cultures grown at different light intensities of fluorescent light colour 34, without or with 3×10^{-6} M kinetin, on a medium containing 1% sucrose. Multiplication rate G_t is on base of the natural logarithm (section 2.3.2).

Light intensity $\mu W\ cm^{-2}$	Multiplication rate G_t	Dry weight mg/cm^2	Dry weight increase mg/cm^2 day
Without kinetin			
Darkness	0.04	8.2	0.32
1	0.06	5.0	0.30
3	0.08	4.0	0.32
8	0.10	3.0	0.30
30	0.16	2.1	0.34
110	0.20	1.9	0.38
300	0.29	1.9	0.55
1100	0.38	2.5	0.95
2400	0.39	3.0	1.17
With kinetin			
Darkness	0.06	3.1	0.19
1	0.12	1.9	0.23
3	0.14	1.6	0.22
8	0.16	1.5	0.24
30	0.17	1.2	0.20
110	0.22	1.2	0.27
300	0.28	1.5	0.42
1100	0.39	2.0	0.78
2400	0.40	2.5	1.00

TABLE 3.7. Multiplication rate and dry weight production of cultures on a medium with 3×10^{-6} M kinetin and 1.5% or 3% sugars. The cultures were illuminated with red BC light, 600 $\mu W\ cm^{-2}$ during 5 minutes, one, two, four or eight times per 24 hours. Far red (following red) was given for 5 minutes at an intensity of 5000 $\mu W\ cm^{-2}$.

Irradiation schedule	1.5% Sugar			3% Sugar		
	MR	Dry weight mg/frond	Dry weight production mg/frond per day	*MR*	Dry weight mg/frond	Dry weight production mg/frond per day
Red						
every 3 hours	91	0.14	0.029	83	0.19	0.036
every 6 hours	81	0.15	0.028	73	0.23	0.039
every 12 hours	76	0.18	0.032	59	0.27	0.037
every 24 hours	63	0.20	0.029	53	0.29	0.035
Red followed by far red						
every 3 hours	73	0.18	0.030	56	0.29	0.037
every 6 hours	62	0.19	0.027	49	0.32	0.036
every 12 hours	60	0.24	0.033	50	0.34	0.039
every 24 hours	44	0.28	0.029	32	0.43	0.032

a corresponding increase in rate of frond production at the different light intensities (see fig. 4.1, which gives the multiplication rates in this experiment).

Table 3.7 shows the results of a similar calculation for cultures growing on two different sugar concentrations and a cyclic illumination program that allowed no carbohydrate production by photosynthesis. Whereas the multiplication rate depended upon the light regime as well as upon the sugar concentration, dry weight production was influenced by the sugar concentration only.

The data presented above indicate that sugar uptake per unit frond area is constant at constant sugar concentration and temperature, provided that the

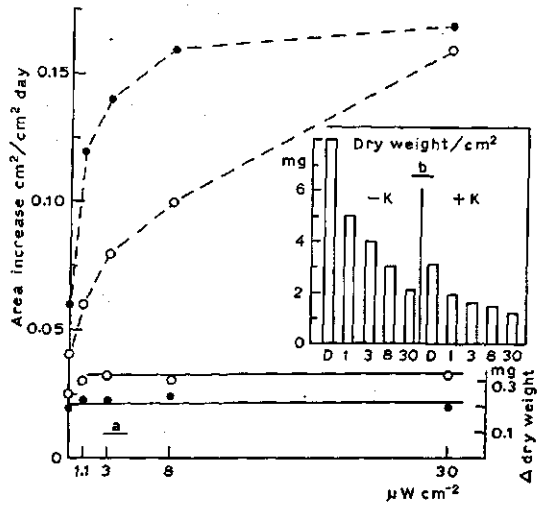


FIG. 3.9. Rate of increase of area and dry weight as a function of light intensity. Data from table 3.6; (– – –) = area increase; (——) = dry weight increase mg/cm² day; (○) = no kinetin; (●) = 3×10^{-6} M kinetin. *Insert: Dry weight as a function of light intensity in the presence* (+K) and absence (−K) of kinetin.

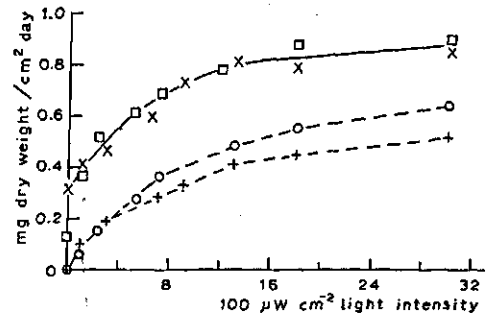


FIG. 3.10. Rate of dry weight production, in mg/cm² frond area per day, at different intensities. Fluorescent light (colour 33). Temperature 22 °C. All cultures are with 0.03% casein hydrolysate. (– – –) = without sugar; (——) = with 1.5% sugar; (○) and (□) = without kinetin; (+) and (×) = 3×10^{-6}M kinetin.

structure of the fronds is not altered by differences in kinetin supply. Any increase in multiplication rate is therefore at the expense of the dry weight per unit frond area. In order to know the maximum rate of area increase possible at a given dry weight production, one has to know the minimum dry weight per unit area under a given condition. Since a definite difference in osmotic pressure is required to keep the cells turgescent, an increase in concentration of solute in the medium can be expected to induce a corresponding change in osmotic value of the cell sap. This often means an increase in materials dissolved in the vacuole which results in an increase in dry weight percentage and dry weight per unit area. Moreover, increase in sugar concentration increases also the density of the structure of the fronds and so has an effect opposite to that of kinetin. The minimum dry weight per unit area depends therefore not only on the morphogenetic effects of light and kinetin on the structure of the fronds but also on the osmotic value of the nutrient medium. This means that our data give no information on the maximum rate of area increase which is possible with the sugar supply from the medium.

4. GROWTH OF *LEMNA MINOR* IN CONTINUOUS LIGHT

During growth of *Lemna minor* at low light intensities an interaction was observed between light and kinetin. Therefore most of the experiments to be described in this chapter were carried out simultaneously with two media, one containing 3×10^{-6} M kinetin, the other without kinetin.

4.1. THE INFLUENCE OF LIGHT INTENSITY ON THE MULTIPLICATION RATE

In figure 4.1 the relation between multiplication rate and light intensity is shown for cultures growing with or without 1.5% sugar in the medium. In both cases the medium contained 0.03% casein hydrolysate. At light intensities above 100 μW cm^{-2} the multiplication rates were almost independent of kinetin. Addition of kinetin raised the mean area per frond by 20% at all light intensities except the highest (3000 μW cm^{-2}).

The curve for the multiplication rate in the media without sugars was found to be very similar to the photosynthesis curve at 0.03% CO_2 (fig. 3.8). Addition of sugar increased the multiplication rate, especially at low light intensities when photosynthesis limits growth. The multiplication rate in darkness was very low, even in the presence of sugar. The stimulation by 100 μW cm^{-2} light was more than can be accounted for by photosynthesis, as appears from a comparison with the growth rates of cultures grown with and without sugar at the same light intensity (fig. 4.1).

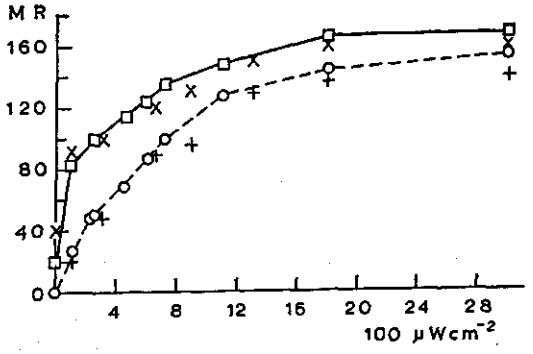


FIG. 4.1. Multiplication rate as a function of the intensity of fluorescent light colour 33. (– – –) = no sugar in the medium; (——) = 1.5% sugar; (×) and (+) = 3×10^{-6} M kinetin; (□) and (○) = no kinetin.

4.2. The influence of low intensity red and blue light on the multiplication rate

Most plant pigments absorb light predominantly in the red or in the blue region of the spectrum or in both. Therefore, both wavelength regions were tested.

For blue light, the lamp and filter combination E was used, red light was obtained from the BC combination (see section 2.4.3). The relative spectral energy distribution of the light is given in table 2.2. The results of the experiments are shown in figure 4.2. Red light is more effective than blue light. The ratio between the effectivities of red and blue decreases with increasing light intensity. This probably is a combined effect of phytochrome stimulation and photosynthesis, the latter becoming pronounced at light intensities in excess of about 100 $\mu W\ cm^{-2}$ (see section 4.8).

Kinetin increases the multiplication rate by an amount which is independent of light intensity and colour (fig. 4.2). This indicates that in this range of light intensities the effects of kinetin and light are additive. Kinetin increases also the dark value of the multiplication rate; here the increase is smaller than at low light intensities.

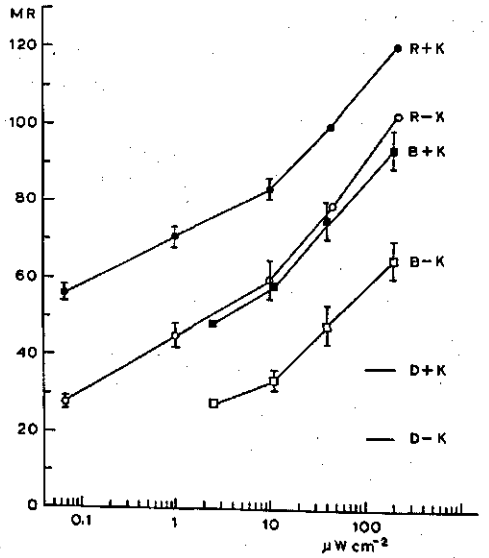


FIG. 4.2. Multiplication rate as a function of the intensity of red and blue light. Culture medium with 1.5% sugar. − K = no kinetin; + K = 3×10^{-6} M kinetin; R = red BC light; B = blue E light; D = dark control. (●) and (○) = red BC light; (■) and (□) = blue E light. Vertical bars indicate double standard deviation $\bar{\sigma}$ of the mean.

4.3. THE INFLUENCE OF DIFFERENT LIGHT INTENSITIES ON FROND GROWTH

By taking photographs of the cultures, frond elongation was followed from the moment a 'daughter' frond protruded from the reproductive pocket until it had reached its ultimate size. Only the part protruding from the reproductive pocket was measured, the inside part remaining the same during the period of examination. The increase in length in mm/day was linear with time during this period.

The rate of frond elongation at different light intensities is shown in fig. 4.3. The ratio of elongation rate to multiplication rate is given in brackets alongside the points in fig. 4.3. Except for the dark values, they do not differ much, showing that multiplication rate and frond elongation respond similarly to light intensity. In fact, when growth of 'mother' and 'daughter' fronds is followed, a fixed relation is found between the sizes of the expanding fronds. The first 'daughter' frond appears just before the 'mother' frond had attained its ultimate size.

The effect of light intensity on the final length of the fronds is shown in fig. 4.4. Light promoted the length of the fronds. Kinetin gave an additional increase in frond length, this increase was much the same in darkness and in light. The time required for frond expansion, from protruding from the reproductive pocket until the frond has reached its ultimate size, is obtained by dividing the values for final frond length (fig. 4.4) by those for growth rate (fig. 4.3). The

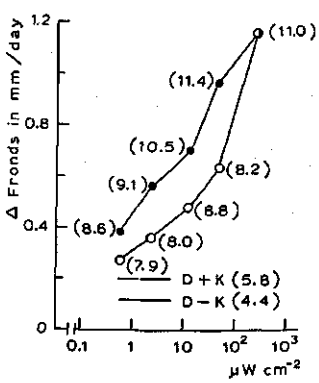


FIG. 4.3

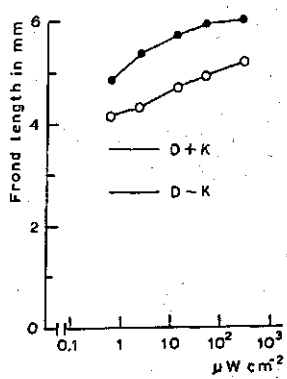


FIG. 4.4

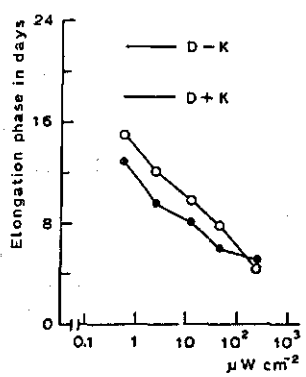


FIG. 4.5

FIG. 4.3. Increase in length of fronds as a function of the intensity of red light. Sugar concentration 1.5%; (○) = no kinetin; (●) = 3×10^{-6} M kinetin. In brackets: the rates of frond growth divided by the multiplication rates. D = dark control; − K = no kinetin; + K = kinetin added.

FIG. 4.4. Final length of fronds grown at different intensities of red light. (○) = no kinetin; (●) = 3×10^{-6} M kinetin; D = dark control; − K = no kinetin; + K = kinetin added.

FIG. 4.5. Duration of elongation phase in growth of fronds at different light intensities. (○) = no kinetin; (●) = 3×10^{-6} M kinetin; D + K = 18 days darkness, with kinetin; D − K = 22 days darkness, no kinetin. The values are obtained by dividing final length by daily increase in length.

results, given in fig. 4.5, show that the time of frond expansion was shortened by a few days by kinetin. The effect of the light is much greater. In darkness, a frond needed 3 to 4 weeks of expansion growth to reach its final length, with 300 μW cm^{-2} light only 4 to 6 days.

4.4. The structure of fronds grown at different light intensities with and without kinetin

The tissue of fronds grown in darkness consists of spherical cells of about 0.035 mm diameter; intercellular cavities were absent. In light grown fronds intercellular cavities developed, at the lower side of the fronds they were of irregular shape, at the upper side they were separated by ridges of tissue arranged in a honeycomb pattern. The cells of these ridges were about twice as long as wide. Kinetin increased the intercellular cavities, this gave rise to larger fronds without much increase in cell number. Diagrams of fronds grown at different light intensities, without and with kinetin, are presented in fig. 4.6. The shape of the fronds was hardly influenced by light or by kinetin.

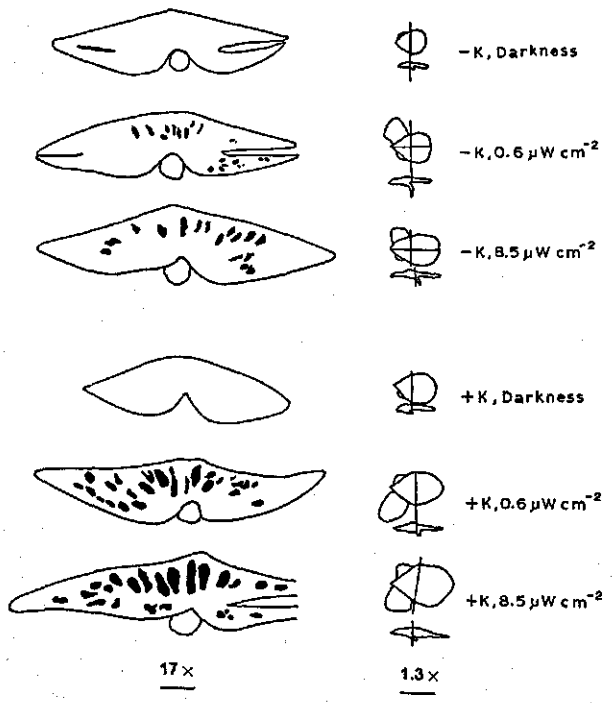


Fig. 4.6. Diagrams of fronds grown for 21 days under different conditions. Continuous illumination with red light at the intensities indicated. In black: intercellular cavities. − K = no kinetin, + K = kinetin added.

4.5. Fresh weight, dry weight and content of starch and sugars of cultures grown at different light intensities

In chapter 3 it was shown that great differences in multiplication rate were possible with the same rate of dry matter production, and that the dry weight percentage could give information whether or not growth was limited by carbohydrate supply. Figure 4.7 shows the dry weight per frond of cultures grown on media with or without sugars, fig. 4.8 the dry weight percentages. In cultures with sugar the dry weight per frond and the dry weight percentages are lowest when light intensity is high enough to stimulate the multiplication rate but too low for compensation of the respiration by photosynthesis. In cultures without sugar, dry weight per frond and dry weight percentage remain constant as long as multiplication rate is limited by photosynthesis. The exceptional dry weight of 8% of cultures grown without sugar at 100 μW cm^{-2} is due to the absence

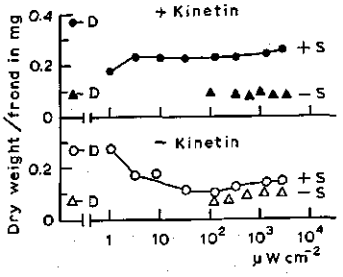


Fig. 4.7. Dry weight per frond in cultures grown in continuous white fluorescent light colour 34. Kinetin concentration: 3×10^{-6} M. − S = no sugar; + S = 1% sugar in the medium; D = dark control.

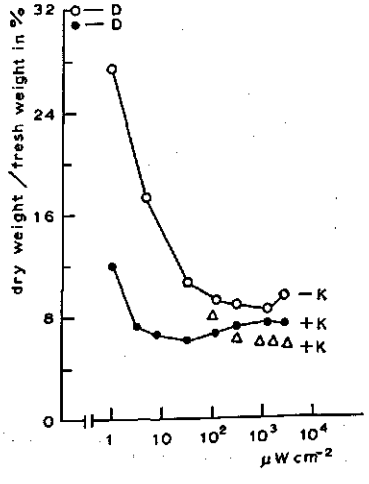


Fig. 4.8. Dry weight, in percent of fresh weight of cultures grown in continuous white fluorescent light colour 34. − K = no kinetin; + K = 3×10^{-6} M kinetin; (△) = no sugar in the medium; (○) and (●) = 1% sugar in the medium; D = dark control.

of small growing fronds. Differences in structure of the fronds were associated with differences in fresh weight and dry weight per unit area. For fresh weight this is shown in fig. 4.9. Cultures with kinetin had a higher proportion of air space in the fronds and, consequently, fresh weight per area was lower. In these experiments the area and the weight of whole cultures were measured. If only adult fronds were considered, the difference would have been even greater. Fig. 4.10 shows that at low light intensities the dry weight per unit area of cultures grown without kinetin was much higher than that for cultures grown with kinetin. This was due to the higher starch content of the former. Starch content of cultures grown at different light intensities is given in fig. 4.11. It was inversely related to multiplication rate, suggesting a competition between growth and starch formation for the sugar absorbed by the fronds. When the sugar content of the medium was increased, the starch content of the fronds was also higher.

For the determination of reducing and non-reducing sugars, cultures were extracted with 80% ethanol. The glucosides were removed from the extract by clarification of the ethanol extract with charcoal; in one experiment glucose and fructose were separated by paper chromatography (VERHOEKS, 1965). Table 4.1 shows that sugars were present in the fronds under all conditions. Evidently starch accumulation in darkness and at low light intensities was not coupled with depletion of sugars.

The ethanol-soluble part of the plant constituents is included in table 4.1. This part contains sugars, plant pigments, nitrogenous substances, organic acids and many other soluble low molecular weight compounds. At light intensities below 30 $\mu W\ cm^{-2}$ the ethanol-soluble part of dry matter was greater in cultures grown on a medium with kinetin than in those grown without kinetin, even when dry matter without starch was taken as reference value.

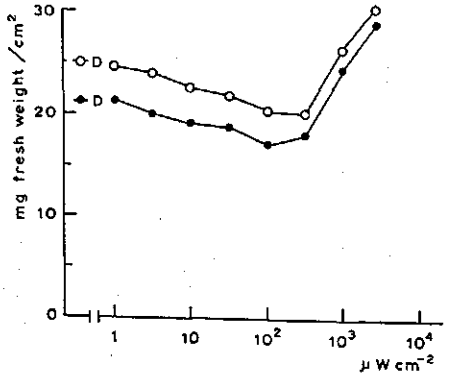


FIG. 4.9. Fresh weight per cm^2 frond area of cultures grown at different intensities of white light. Medium with 1% sugar. (○) = no kinetin; (●) = 3×10^{-6} M kinetin; D = dark control.

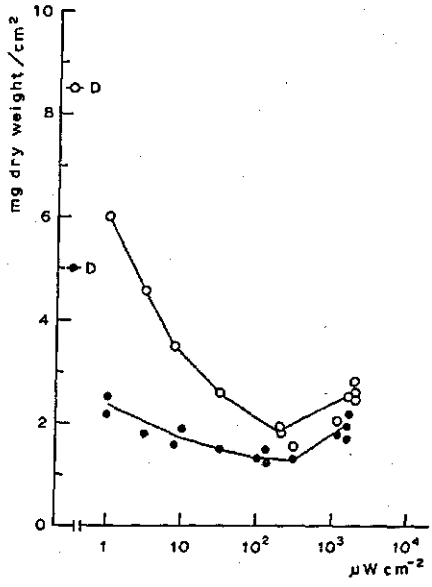


FIG. 4.10. Dry weight per cm² frond area of cultures grown at different intensities of white light. Medium with 1% sugar. (○) = no kinetin; (●) = 3×10^{-6} M kinetin; D = dark control.

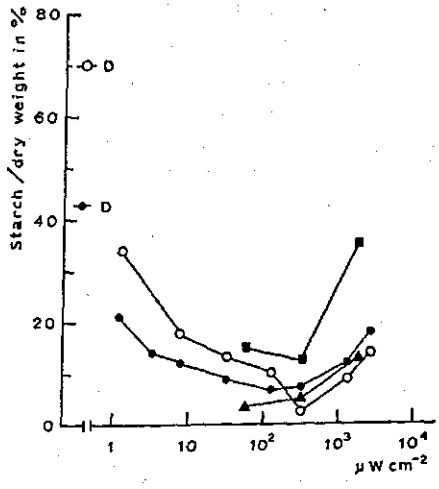


FIG. 4.11. Starch, in percent of dry weight, of cultures grown at different intensities of white fluorescent light colour 34. (○) = no kinetin; (▲), (●) and (■) = 3×10^{-6} M kinetin. Sugar concentration in the medium: (▲) = 0.5% sugar; (○) and (●) = 1% sugar; (■) = 2% sugar; D = dark control.

TABLE 4.1. Reducing and non-reducing sugars, starch and ethanol-soluble material in percentage of dry matter in cultures of *Lemna minor* grown without kinetin (−K) or with 3×10^{-6} M kinetin (+K) at different light intensities. 1% or 1.5% sugar in the medium.

Sugar content of medium	Light intensity in $\mu W\ cm^{-2}$	Sugars				Starch		Total ethanol soluble substance	
		Non-reducing		Reducing					
		−K	+K	−K	+K	−K	+K	−K	+K
	Darkness	–	2.5	–	1.7	64	44	–	15
	1	–	2.7	–	1.0	34	20	–	20
	3	–	3.0	–	0.8	28	13	–	22
	10	–	2.6	–	0.7	18	11	10	25
1.0%	30	1.0	–	0.0	–	12	8	18	26
	100	4.3	2.0	3.0	2.4	8	6	23	43
	300	1.6	5.8	3.0	0.7	3	5	45	43
	1000	4.6	4.0	1.3	0.5	4	10	39	32
	1600	5.0	4.0	1.2	0.3	15	19	33	31
	30	0.7	1.1	1.8	2.2				
	100	0.6	2.3	1.5	1.3				
1.5%	300	0.0	0.0	1.5	1.2				
	1200	1.0	1.8	2.2	1.6				
	1600	0.4	2.0	4.0*	3.1**				

* Glucose: 1.5, fructose 2.5, glucoside 0.7
** Glucose: 0.9, fructose 2.2, glucoside 0.6

4.6. NITROGEN CONTENT AT DIFFERENT LIGHT INTENSITIES

Nitrogen determinations were made by BONGERS (1956) with the micro-KJELDAHL method. If we accept that nitrogen in the plant is mainly in proteins, the average nitrogen content of the nitrogenous substances will be 16% and multiplication of the nitrogen percentage of the dry weight by 6.25 yields the percentage of nitrogenous substances. Table 4.2 column 4 shows that nitrogenous substances make up for 20% of the starch free dry weight under all conditions. This indicates that, in contrast to the starch, the content of nitrogenous

TABLE 4.2. Nitrogen content of cultures of *Lemna minor* grown on standard medium with 1% sugar at different intensities red light. Medium without kinetin is indicated by −K, medium with 3×10^{-6} M kinetin by +K.

1	2		3		4	
Light intensity $\mu W\ cm^{-2}$	Nitrogen (N) % of dry weight		Protein (N × 6.2)		Protein % of dry weight without starch	
	−K	+K	−K	+K	−K	+K
Darkness	1.12	1.98	6.9	12.3	19	22
0,8	2.04	2.14	12.6	13.3	19	17
6	2.49	3.16	15.5	19.7	20	22
8	2.67	3.08	16.5	19.0	20	20

substances was not influenced by the light treatment nor by the presence of kinetin.

4.7. Chlorophyll and carotenoid content of cultures grown at different light intensities

Figure 4.12 shows that in cultures grown at 1 μW cm^{-2}, chlorophyll content per cm^2 frond area was still low; between 1 and 10 μW cm^{-2} a steep rise was observed. Cultures grown with kinetin contained less chlorophyll per cm^2 frond area than those without. This must be ascribed to the difference in structure of the fronds (section 4.4). An estimation of the amount of carotenoids is given in fig. 4.13. The dark level of carotenoids was higher than that of chlorophyll, but the effect of light on concentration was similar.

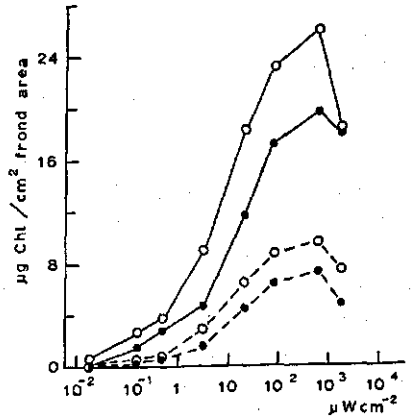


FIG. 4.12. Chlorophyll per cm^2 frond area of cultures grown at different intensities red light. (——) = chlorophyll a, (– – –) = chlorophyll b. Medium with 1.5% sugar. (○) = no kinetin; (●) = 3 × 10^{-6} M kinetin.

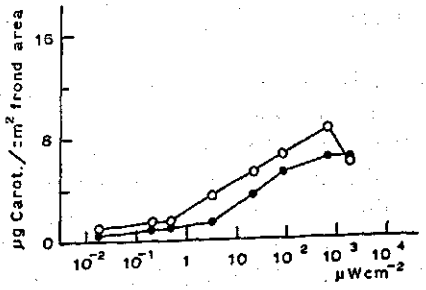


FIG. 4.13. 'Carotenoids' per cm^2 frond area of cultures grown at different intensities red light. (○) = no kinetin; (●) = 3 × 10^{-6} M kinetin. Same experiment as fig. 4.12. Dark level: without kinetin 0.4 μg cm^{-2}, with kinetin 1.0 μg cm^{-2}.

4.8. Discussion of chapter 3 and 4

In the relation between growth stimulation and light intensity, two ranges can be distinguished (fig. 4.2). In continuous darkness growth rate was very low. A considerable increase in multiplication rate is observed already at light intensities below 0.1 $\mu W\ cm^{-2}$. Red light was some 100 times more effective than blue, which points to phytochrome as the photoreceptor pigment in this response. Further evidence for this is given in section 6.2, where it is shown that far red admixed to red under specific conditions depresses the effect of red light.

At light intensities above 1 $\mu W\ cm^{-2}$ the chlorophyll content increases rapidly (fig. 4.12) and photosynthesis could begin to play a role. Since respiration is compensated by photosynthesis at 200 $\mu W\ cm^{-2}$ (fig. 3.7), below that intensity the contribution of photosynthesis to the energy supply will still be small. On the other hand in *Chlorella* photosynthetic phosphorylation is about 30% saturated at 200 $\mu W\ cm^{-2}$ (Wintermans, 1955; Tanner, Loos and Kandeler, 1966). Strehler (1953) found a maximum ATP content at 15% of the saturating light intensity of photosynthesis in *Chlorella*. We can not exclude, therefore, that photosynthetic formation of ATP and NADP in the intensity range up to 200 $\mu W\ cm^{-2}$ makes an increasingly important contribution to the energy supply.

Above 200 $\mu W\ cm^{-2}$ the increase in multiplication rate probably is due to the increase in carbon dioxide assimilation. The intensity dependency of the multiplication rate in media without sugar shown in fig. 4.1 was very similar to the photosynthesis curve (fig. 3.8). This is in agreement with the observation that the dry weight per frond is constant under these conditions (fig. 4.7). Addition of sugar to the medium increased the multiplication rate. Figure 4.1 shows that this increase was more pronounced at the lower than at the higher light intensities, whereas the rate of dry matter production was raised with a constant amount by sugar addition (fig. 3.10).

Kinetin exerted two effects. In the first place, it enhanced the effect of light on frond expansion and multiplication rate. In the second place, kinetin induced a change in the structure of the fronds. The intercellular spaces were enlarged, so that fresh and dry weight per cm^2 area were depressed. The cultures required less carbohydrates per cm^2 frond area per day notwithstanding their higher rate of expansion.

Owing to photosynthesis, dry matter production increased with light intensity (fig. 3.10). Sugar addition to the medium increased dry matter production at all light intensities with a constant amount. This suggests that sugar absorption from the medium was the limiting factor for non-photosynthetic dry matter production of *Lemna* cultures, and consequently also for the multiplication rate. However, the multiplication rate was hardly influenced by raising the sugar concentration above 1% (fig. 3.6), but starch accumulation increased (fig. 4.11). It appears therefore improbable that sugar absorption as such is a limiting factor. The bottleneck for sugar utilization in growth appears to be somewhere else in the metabolic pathways from sugar to cell constituents.

Apparently at increasing light intensity first phytochrome Pfr, then cytokinins, next intermediates of photosynthetic energy conversion, and finally the capacity to utilize the carbohydrates supplied by the medium or by photosynthesis limit the multiplication rate on a medium with 1% sugar.

5. THE INFLUENCE OF SHORT EXPOSURES TO RED LIGHT ON THE MULTIPLICATION RATE OF *LEMNA MINOR*

Brief illuminations stimulate some aspects of growth in *Lemna minor*. Since the stimulation is reversible by far red and the dose response relation for light of λ 658 nm was of the same order of magnitude as that found by BUTLER et al. (1964) for phytochrome photoconversion in vitro, it is probably a phytochrome mediated process. Spectrophotometric measurements of phytochrome in vivo in *Lemna minor* have shown that the active form of phytochrome Pfr disappears slowly during darkness. Therefore is was to be expected that illumination has to be repeated in order to maintain a certain Pfr level. In this chapter, the effects of the frequency of illumination and of the light dose on the multiplication rate are described.

5.1. THE INFLUENCE OF THE FREQUENCY OF ILLUMINATION

Lemna minor cultures were grown in the cabinets described in section 2.4.1 on standard medium with or without kinetin. The plants were illuminated for 470 seconds with red light once every 48 hours, once every 24 hours and once every 6 hours. Each illumination was preceded by a far red radiation of the same duration in order to remove Pfr if remnant from the previous illumination. Light was given at three intensities. The growth rate in continuous darkness was substracted from that in the light treatments; the resulting difference will be called 'light effect' (*LE*). In fig. 5.1 the *LE* of the different regimes of repetition of illumination are presented.

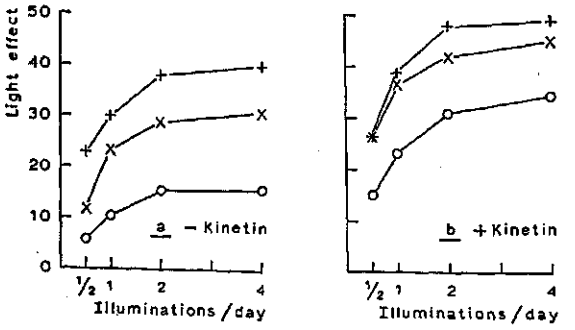


FIG. 5.1. Relation between the light effect *LE* (*MR* of light treated cultures minus *MR* of dark controls) and the number of illuminations per day ($^1/_2$ = 1 illumination in 2 days). Each exposure to red BC (470 seconds) was preceded by an exposure of 470 seconds to far red II of 5000 μW cm^{-2}. The medium contained 1.5% sugar. a = without kinetin, dark value of *MR* = 21; b = with 3 × 10^{-6} M kinetin, dark value of *MR* = 38. (+) = 6 × 10^5 μW sec cm^{-2}; (×) = 7 × 10^4 μW sec cm^{-2}; (○) = 4 × 10^3 μW sec cm^{-2}.

Comparison of fig. 5.1**b** with fig. 5.1**a** shows that the effect of light is much increased by kinetin. Under both conditions the growth rate is higher the more frequent the illuminations and the higher the light intensity. An increase in frequency was more effective than an increase in light intensity.

5.2. The effect of far red preceding a red radiation

As mentioned above, in these experiments the red radiation was always preceded by far red. It appeared desirable to examine the possible effect of this far red dose separately. A typical experiment is shown in fig. 5.2 and 5.3. No consistent effect of far red was found when the amount of red light per illumination was sufficient to saturate phytochrome conversion (6×10^5 μW sec cm^{-2}, upper two curves in figs. 5.2 and 5.3). Far red, given before a non-saturating dose of red (4×10^3 μW sec cm^{-2}, converting 30% of the Pr to Pfr) in a regime with dark periods of 6 hours or less, always resulted in a small reduction in multiplication rate. We attribute this reduction to removal of Pfr remaining from the previous red illumination. After dark periods longer than 6 hours, Pfr apparently had decreased to a very low level as a consequence of reversion and decay. Its concentration after a subsequent red illumination was, therefore, determined by the dose of red only.

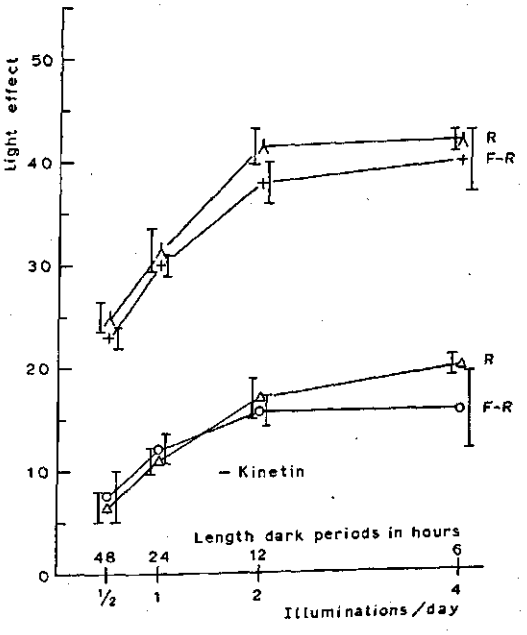


FIG. 5.2. The effect of far red II, 2.4×10^6 μW sec cm^{-2} in 470 seconds, given before each exposure to red BC light of 470 seconds in different cyclic regimes. Medium with 1.5% sugar. R = red light; F-R = red preceded by far red; (+) and (Δ) = 6×10^5 μW sec cm^{-2} red light dose, phytochrome conversion 80%; (Δ) and (○) = 4×10^3 μW sec cm^{-2}, phytochrome conversion 30%.

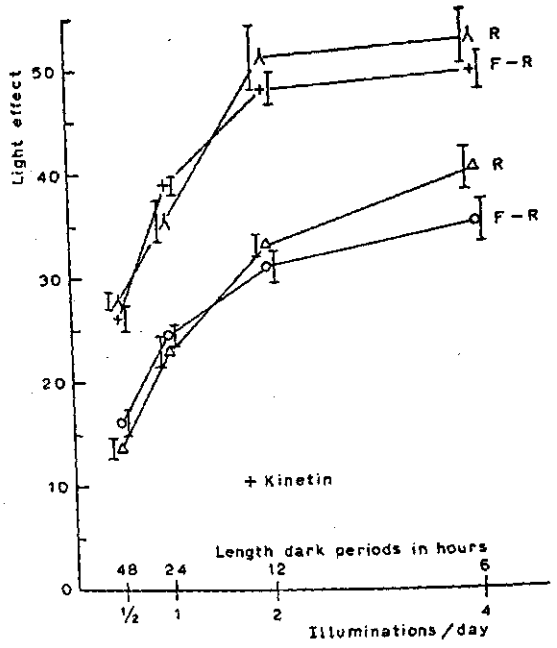


FIG. 5.3. Legend see fig. 5.2, but with 3×10^{-6} M kinetin in the medium.

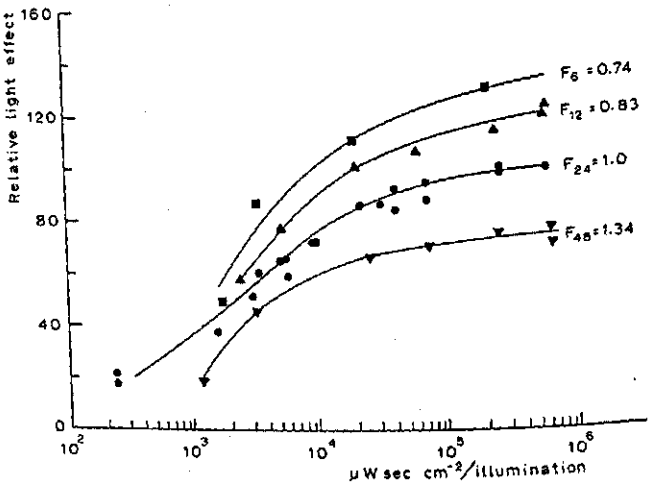


FIG. 5.4. Relation between amount of red BC light per illumination of 470 seconds and the relative light effect *LE* % (= *MR* of light treated cultures minus *MR* of dark controls expressed as percentage of *LE* value obtained with illuminations once every 24 hours at a dose of 6×10^5 μW sec cm^{-2}) at different light regimes. Every red illumination was preceded by 470 seconds far red II 5000 μW cm^{-2}. The medium contained 1.5% sugar and 3×10^{-6} M kinetin. *F* is a proportionality factor, converting the curves to the one with the 24 hour cycle, see fig. 5.5. The illuminations were given in cycles of (■) = 6 hours; (▲) = 12 hours; (●) = 24 hours; (▼) = 48 hours.

5.3. The relation between light dose and effect

The data of fig. 5.1 have been converted to percentages of the value observed at the highest dose of light in the 24 hours regime. These relative light effects, together with similar data from other experiments, are plotted against the log dose per illumination cycle in fig. 5.4. The curves for the different regimes of illumination obtained this way could be transformed into the 24 hours curve by multiplication of the values with a factor $F = LE_{24}/LE_p$ in which LE_{24} is the light effect at the highest intensity with a cycle length of 24 hours and LE_p that at the light regime in question. The results of these transformations are shown in fig. 5.5.

Figure 5.6 shows the relative light effect on the multiplication rate of cultures without kinetin as a function of dose per illumination. It differs from fig. 5.4 in that reduction of the cycle length below 12 hours had no effect.

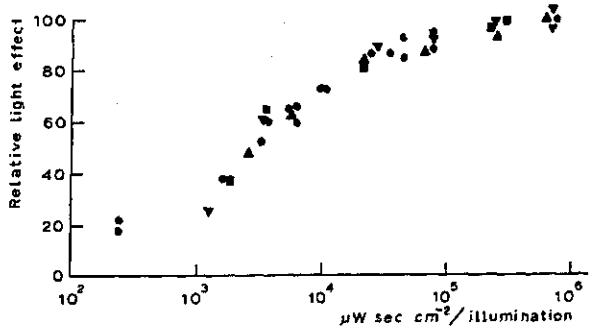


Fig. 5.5. Legend see fig. 5.4. but LE values are multiplied by the proportionality factors F.

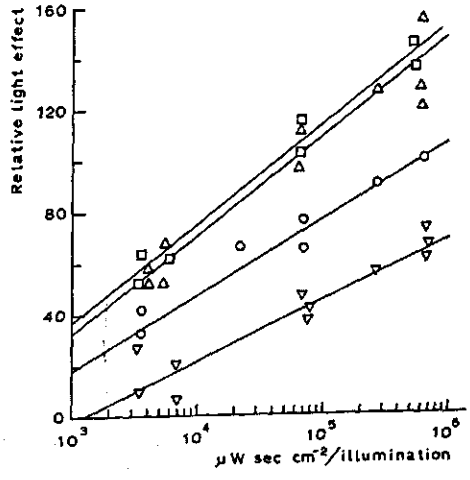


Fig. 5.6. Same as fig. 5.4, but without kinetin in the medium. Illumination cycles: (□) = 6 hours; (△) = 12 hours; (○) = 24 hours; (▽) = 48 hours.

5.4. Comparison of the effectivity of red BC light with that of 658 nm light

Comparison, in fig. 5.7, of the effect of red light of the BC lamp - filter combination with that of light transmitted by an interference filter of λ 658 nm (characterization of the light sources in table 2.2) shows that at light doses below about 10^4 μW sec cm^{-2}, the ratio of the efficiencies of the two light sources is constant (φ 658/φ BC = 2.4). The ratio of the efficiencies in phytochrome photoconversion, as calculated in section 2.4.3 (table 2.3) and measured with spectrophotometer (fig. 2.4), is lower (φ 658/φ BC = 1.6). In view of the large standard deviation of the averages in figure 5.7 not much attention is given to this difference.

At doses of 10^5 μW sec cm^{-2} of BC light, phytochrome conversion is light saturated (fig. 2.4). Fig. 5.7 shows, however, that the light effect is not saturated at this dose, and that the difference in efficiency between the two light sources diminishes as the dose of light is higher. This suggests that at high doses

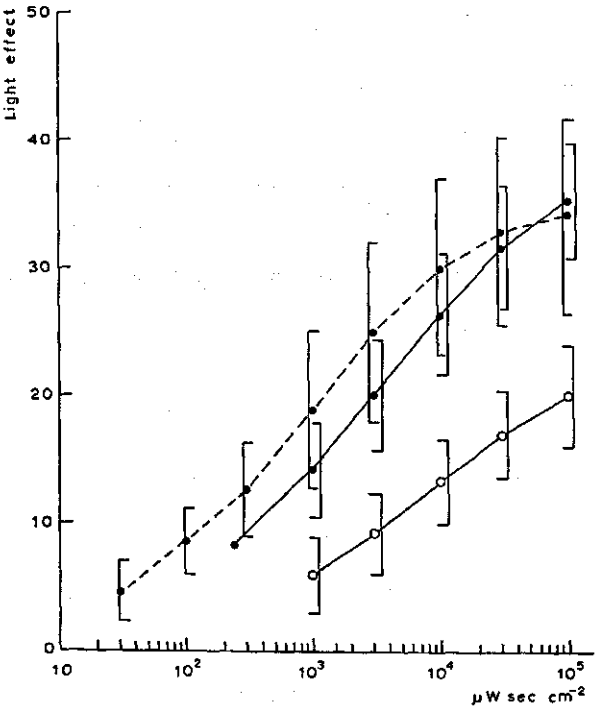


FIG. 5.7. Multiplication rate as a function of red light dose. In contrast to figs. 5.4 and 5.6, the multiplication rates are absolute values. (● – – ●) light source 658 (interference filter 658 nm), average of 5 experiments, medium with kinetin; (● — ●) light source BC, average of 9 experiments, medium with kinetin; (○ — ○) light source BC, average of 9 experiments, medium without kinetin. Vertical bars indicate the double standard deviation σ.

light stimulation is no longer a function of Pfr concentration only, and that another photoreaction, wether of phytochrome or of some other red absorbing pigment, is involved additionally.

5.5. Discussion

In an earlier publication (Rombach, 1965), we have presented evidence that in cultures illuminated once every 24 hours, the stimulating effect of red light was a function of the amount of Pfr generated by the illumination. Since the dose-response curves in fig. 5.4 are very similar in slope to the 24 hours curve, differing only by a proportionality factor, it appears reasonable to assume that the same holds for cultures illuminated in cycles of 6, 12 or 48 hours. In view of the observation, however, that the light effect on the multiplication rate is not saturated at an energy dose which saturates phytochrome conversion, other light dependent reactions, the nature of which is still obscure, may be involved in addition. In this context we may point to chapter 6, where it is demonstrated that part of the light effect is not reversible by far red, to section 6.2 and to the discussion in chapter 4, where it is suggested that intermediates of photosynthesis may cooperate with Pfr.

The multiplication rate is increased by illuminating the cultures more frequently. This could be related to Pfr decay and reversion. The effect of far red preceding the red illumination (figs. 5.2 and 5.3) favours such an explanation since it demonstrates a decrease of physiological active phytochrome in the plants as the dark period proceeds.

6. REVERSIBILITY OF THE LIGHT EFFECT BY FAR RED

6.1. INTRODUCTION

Far red has two kinds of effects on photomorphogenesis of plants: a brief irradiation following a red light exposure antagonizes the effect of the red, but in several cases an extended irradiation with far red alone has an effect in the same direction as a brief red illumination (see section 1.4.2).

Three types of far red were used in the experiments described in this chapter; called far red I, II and III. Far red I contains about 1.5% of its radiation in the wavelength band 690–710 nm, with far red II and III this percentage is only 0.3 (table 2.2). With far red I the photostationary state of phytochrome conversion gives 5% Pfr, with far red II this is 0.5% (table 2.3).

Since in the spectral regions concerned reactions involved in photosynthesis may take place, the following facts may be briefly recalled in mind. DELRIEU (1969) reported that at 700 nm the quantum efficiency for photosynthesis system I is about 30% of that at the maximum at 680 nm. In far red I, LINDEMAN (1972) measured 1.3% of the photosynthesis found in white light, and in far red II VAN RENSEN (1971) measured 10% of the photosynthesis obtained with incandescent light transmitted by a filter RG 8 (comparable with far red I). Light dependent phosphate fixation in far red II was, however, still 20% of that in white light. Although these data indicate that in green plants photosynthesis in far red II is only 0.13% of that in white light, photophosphorylation is much less reduced. As is mentioned in section 1.4.2, in etiolated beans photophosphorylation has been demonstrated after 12 hours of irradiation with far red (OELZE-KAROW and BUTLER, 1971). We have to keep in mind, therefore, that photosynthetic phosphorylation may contribute to the effects of red and far red irradiation on growth of *Lemna minor*.

6.2. THE EFFECT OF CONTINUOUS FAR RED AND MIXTURES OF RED AND FAR RED LIGHT

The effect of continuous far red II is presented in fig. 6.1**b**, closed and open circles, with and without kinetin respectively. The effect is greater, the greater the intensity of the far red. The same was found with continuous red (fig. 6.1**a**; fig. 4.2) or blue light (fig. 4.2) but with a higher efficiency than with far red.

When red light of the intensities of fig. 6.1**a** is admixed to the far red, the effect is higher than with the far red alone, but, in the absence of kinetin at least, lower than with the red light alone. This means that the effect of the red component is reduced by the far red component, which points to phytochrome Pfr as the effector of the photostimulation in red light. The reduction is still more

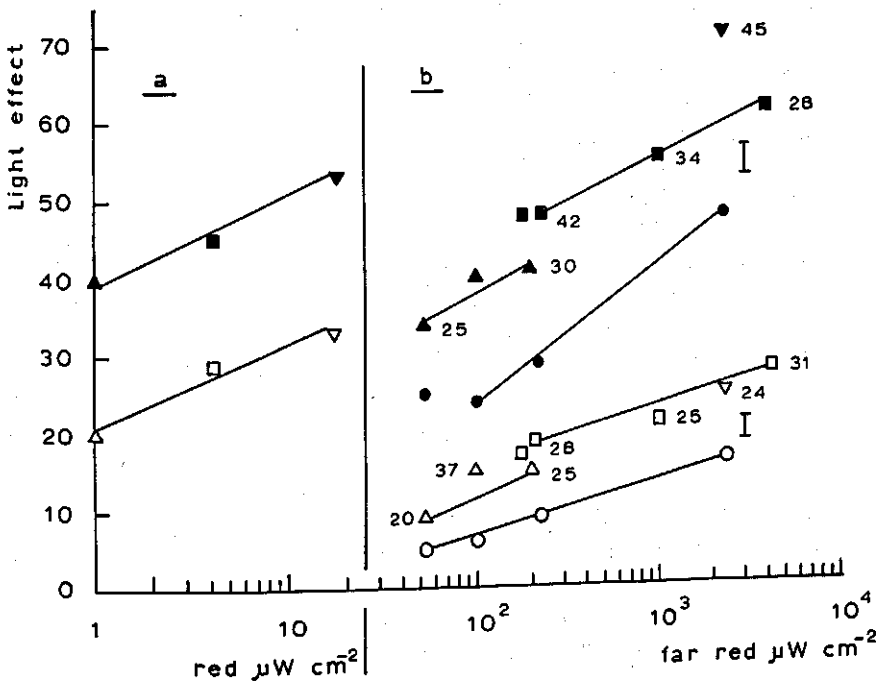


FIG. 6.1. **a**: Light effect *LE* (= *MR* of irradiated cultures minus *MR* of dark controls) as a function of the intensity of continuous red BC light. **b**: The same, but as a function of the intensity of far red II with or without admixtures of constant amounts of red BC light. Culture medium: with 1.5% sugar and 5×10^{-6} M thiamine. Open marks = without kinetin; closed marks = with 3×10^{-6} M kinetin. Red light, in fig. b in admixture to far red: (△) and (▲) = 1 μW cm^{-2} red; (□) and (■) = 5 μW cm^{-2} red; (▽) and (▼) = 18 μW cm^{-2} red, (○) and (●) = far red II alone. Numbers near the marks are percentages: see the text. Vertical bars indicate the double standard deviation $\bar{\sigma}$ of the mean. *MR* of dark controls: with kinetin 30; without kinetin 22.

evident when the increase in the multiplication rate brought about by mixing the red to the far red is examined. It then appears that only some 30% of the effect of the red alone is added to the effect of the far red component of the mixture, both in the absence and in the presence of kinetin. The actual percentages are indicated alongside the points in figure 6.1**b**. We may say that the effect of the red is reduced to about 30% by the far red component of the mixture.

Evidently, far red has an effect in the same direction as red, stimulating the multiplication rate, and at the same time a red antagonizing effect, much in the same way as found by DE LINT (1957) in inhibition of growth of the *Avena* coleoptile. The effect in the same direction as red, increasing as the far red intensity increases, can not be attributed to an increase of the Pfr fraction of the total phytochrome present, the photostationary Pfr level being independent of the radiation intensity at the far red intensities applied. As already mentioned, an effect via photosynthesis or via activation of phytochrome as suggested by

HARTMANN (1966) is more likely. On the other hand, adding red light to the far red results in a higher multiplication rate which, no doubt, is due to the fraction of phytochrome in Pfr being higher. The Pfr/P_{total} ratio in the light mixture is however still much lower than in the red alone, therefore only part of the effect of red alone is added to that of the far red.

The fraction of phytochrome in Pfr in plants illuminated continuously with mixtures of red and far red light can be calculated with equation 10, p. 24, or can be measured with a dual wavelength spectrophotometer. Both methods were applied and yielded about the same results. For practical reasons the measurements were made in plumules of *Pisum sativum* instead of in *Lemna minor*. In fig. 6.2**b** the Pfr fractions are noted alongside the fronds. Figure 6.2 contains the same points as fig. 6.1, but in view of the possibility that the Pfr fraction could be of more relevance to the physiological response than the addition of a constant amount of red light, the curves connect points of equal Pfr fractions instead of equal red admixtures. Figure 6.2**b** shows that in the presence of kinetin an increase in the Pfr fraction increases the steepness of the curve relating the light effect with far red intensity. This suggests a synergism between Pfr and the far red intensity effect in promotion of the multiplication rate in *Lemna minor*.

Measurements of the final size of the fronds in experiments with kinetin showed that in the mixtures of red and far red light the fronds were always

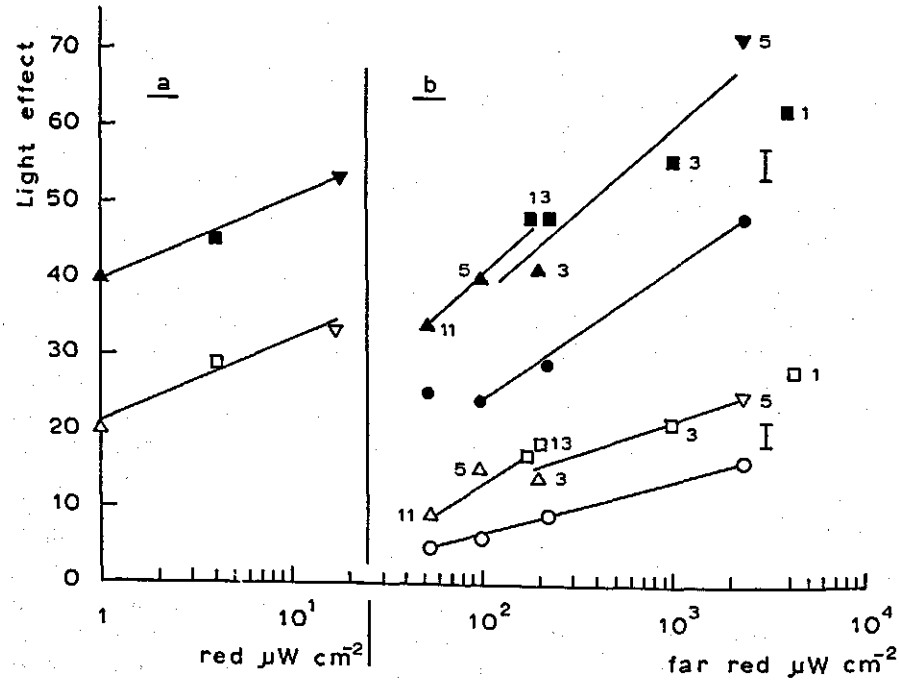


FIG. 6.2. The same as fig. 6.1, but the numbers near the marks indicate the photostationary Pfr level in percentage of total phytochrome.

smaller than in the red light alone. In the treatments with far red alone the frond size was only slightly larger than in darkness and was not promoted by an increase of the intensity of far red. Apparently this aspect was controlled by Pfr alone, it demonstrates once more phytochrome activity during continuous illumination.

6.3. The effect of far red given directly after red light

When far red was given directly after a brief illumination, the effect of the red illumination was partly reversed. The dose-response relation of this reversion (fig. 6.3) was obtained by exposing cultures to a saturating amount of red light once in 24 hours, and thereafter to various amounts of far red I or far red III. The effect of this radiation sequence is expressed as percentage of the effect of red light only. Far red III is 3 times more effective than far red I.

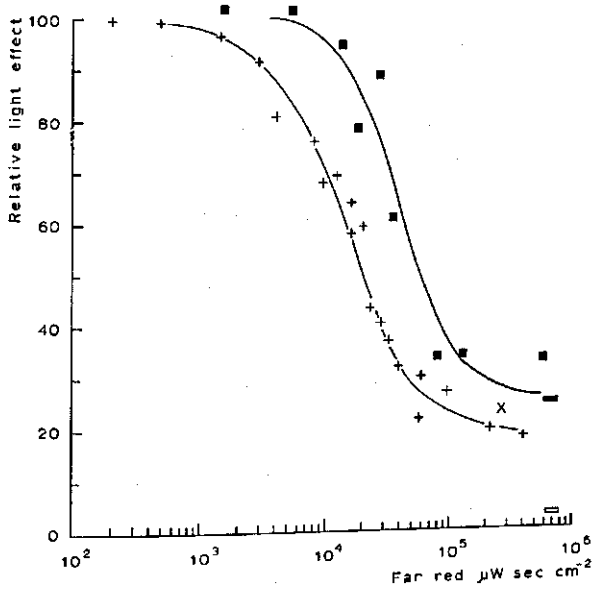


FIG. 6.3. Reduction of the relative light effect (% *LE*), in % of the *LE* of the red light alone, by different amounts of far red I or far red III applied immediately after a saturating amount of red light (10 minutes 500 μW cm^{-2} red BC once a day). Far red exposures 180 seconds or less at different intensities. Medium with 3 × 10^{-6} M kinetin and 1.5% sugar (■) = red followed by far red I; (+) = red followed by far red III; (■■) = far red I only; (□) = far red III only.

Figure 6.3 shows that far red I without preceding red illumination also has an effect on the multiplication rate. This is due to the photostationary state Pfr fraction in the far red. With far red I, this effect was 25% of the effect of a saturating brief red illumination, with far red III only 3%.

Reversion of the light effect by far red II (shown in figures 6.4 **a** and **b**, 6.9 and 6.10) or far red III (fig. 6.3) in experiments receiving one radiation treatment per day was never to a value below 18%, not even when the energy of the far red was increased. This is not due to the brief irradiations with far red itself, because far red II and III hardly stimulated growth when given alone. Evidently, this effect is due to the red light preceding the far red.

Thus, the light effect consists of a part which is reversible by far red, and of an irreversible part. The reversible part represents the 'classical' phytochrome effect. We cannot exclude that the far red non-reversible part is mediated by phytochrome too, as will be discussed in the sections 6.7.2 and 8.3. In the figures the far red non-reversible part is denoted by 'non- far red reversible part of the light effect'.

6.4.1. *The effect of the duration of the red illumination on the reversibility by far red*

If we assume that phytochrome in the form Pfr is active as soon it is formed by the absorption of light by Pr, part of the Pfr effect may have escaped from

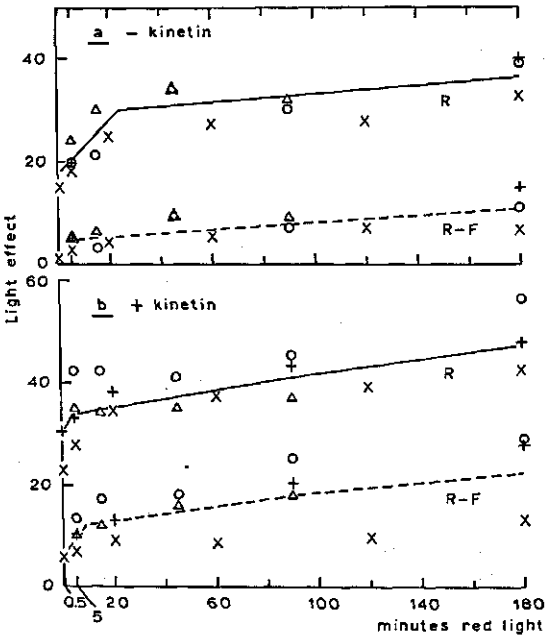


FIG. 6.4. The light effect (= *MR* of irradiated cultures minus *MR* of dark controls) as a function of the duration of exposures, given once a day, followed or not followed by far red II. Red BC light: 450 μW cm^{-2}, except for the exposures of 0.5 minute, when the intensity was 1000 μW cm^{-2}. Far red II: 10 minutes 1400 μW cm^{-2}. Medium with 1.5% sugar; **a**: without kinetin; **b**: with 3 × 10^{-6} M kinetin; (—— R)= red light only; (– – R – F)= red followed by far red. The different marks refer to different experiments.

far red reversion because the subsequent process, triggered by it, had already started before the end of the illumination. In that case, it can be expected that the far red non-reversible part will increase when the duration of the brief illumination is extended.

Experiments without kinetin (fig. 6.4**a**) as well as with kinetin (fig. 6.4**b**) show that the red light effect increases with the duration of the illumination.

In the absence of kinetin, the percentage of reversion by far red was almost the same after 5 or 180 minutes of red light (fig. 6.5**a**), so both the far red reversible and the far red non-reversible parts of the light effect were increased in the same proportion by the extension of the red illumination. This suggests that at irradiation periods longer than 5 minutes the increase in the light effect in plants growing without kinetin addition is due to the formation of a substrate essential for both the reversible and the irreversible parts of the red light effect.

With kinetin added, the increase in light effect resulting from extension of the exposure time was mainly due to an increase of the far red non-reversible part of the light effect (figs. 6.4**b** and 6.5**b**).

When the light intensity of the 180 minutes illuminations was so much reduced that the total amount of light per illumination was equal to that of the 5 minutes illuminations, the light effect and the far red non-reversible part of it were both reduced to the level found in the cultures illuminated 5 minutes a day,

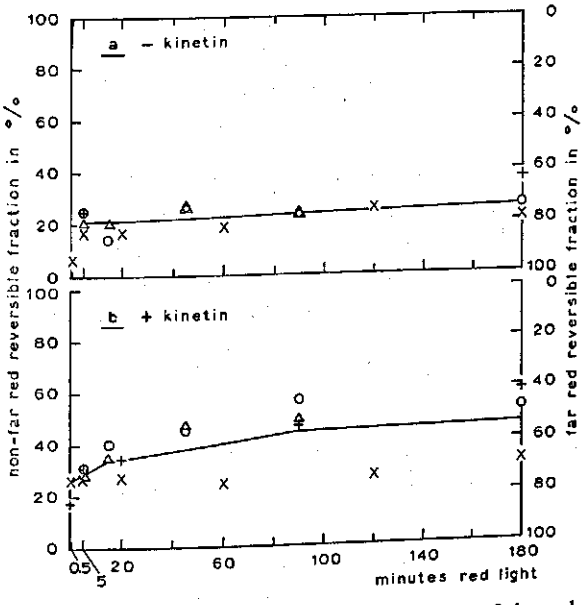


FIG. 6.5. The far red reversible part of the light effect (= *MR* of the red treated cultures minus *MR* of the red followed by far red treated cultures) and the far red non-reversible part of the light effect (= *MR* of the red followed by far red treated cultures minus the *MR* of the dark controls), expressed as percentage of the light effect of the red light alone. Same experiment as in figure 6.4. **a**: without kinetin, **b**: with kinetin.

TABLE 6.1. Effect of intensity and duration of daily repeated illuminations on the far red non-reversible part of the light effect on multiplication rate of *Lemna minor* cultures grown without (−K) or with 3×10^{-6} M kinetin (+K), with 1.5% sugar (1.5% S) or 3% sugar (3% S). Intensity of the red light: 1800 μW cm^{-2} (HR) or 50 μW cm^{-2} (1/36 R). The illumination times are indicated in minutes. When the red illuminations were followed by far red II (5 minutes, 5000 μW cm^{-2}), this was indicated by 5 min F.

Radiation treatment	*MR* illuminated minus *MR* dark cultures*		
	−K, 1.5 % S	+K, 1.5 % S	+K, 3 % S
180 min HR	40 ±2**	47 ±2**	51 ±1**
180 min HR 5 min F	15 ±1	27 ±2	21 ±2
180 min 1/36 R	25 ±2	37 ±1	37 ±1
180 min 1/36R 5 min F	5 ±3	11 ±2	12 ±2
5 min HR	20 ±3	33 ±1	34 ±2
5 min HR 5 min F	6 ±1	9 ±1	12 ±2

* Multiplication rate in darkness: −K 1.5% S: 21 ±1; +K 1.5% S: 31 ±3; +K 3% S: 26 ± 1
** Standard deviation $\bar{\sigma}$ of the mean.

as is shown in table 6.1. The low intensity red light saturates phytochrome conversion in 20 minutes, so during the remaining 160 minutes of the light period and during the subsequent dark period the Pfr level in cultures exposed to the low intensity light periods should be equal to that in cultures exposed to the high intensity light periods of 180 minutes. The increase in the far red non-reversible light effect in the high intensity treatment is therefore not ascribed to a difference in the Pfr level but to an other reaction which shows dependency on light intensity.

6.4.2. *The effect of temperature on the reversibility of the red light effect*

As discussed above, the non-reversibility of the red light effect by far red might be due to rapid action of Pfr. Low temperatures during the red light period might slow down this reaction resulting in a more complete reversibility.

Cultures were cooled to 4 °C with ice water before and during the red irradiation. Cooling down before, and warming up after each daily radiation treatment took 4 minutes each. The total radiation time for red + far red was

TABLE 6.2. Effect of temperature during irradiation on the far red non-reversible part of the red light effect. Cultures grown with 3×10^{-6} M kinetin and 1.5% sugar. Light treatment once a day with 600 μW cm^{-2} red BC light, followed, when indicated, by far red (quality II) 1400 μW cm^{-2}.

Light treatment	Multiplication rate	
	23 °C	5 °C
7 min red	70	65
7 min red, 10 min far red	38	37
10 min far red	30	–

17 minutes per day (table 6.2), so that during 25 minutes per day the plants were at a lower temperature than the 23 °C controls. The data of table 6.2 show that reversion of the red light effect by far red was not increased by the cold treatment. Therefore, no indication was obtained for a temperature controlled phytochrome reaction during illumination in this experiment.

6.4.3. *The effect of repetition of the red radiation on the reversion of the red light effect*

In table 6.3 the results of an experiment of far red reversion after 1 and after 4 times switching the phytochrome with red and far red radiation are given. These two treatments gave the same percent reversion. There appears to be no simple relation, therefore, between the far red non-reversible part of the light effect and the photoconversion as such.

TABLE 6.3. Effect of alternate red and far red irradiations on the far red non-reversible part of the red light effect on multiplication rate *MR*. The non-reversible part of the red light effect is expressed as percentage of the light effect obtained with 5 minutes red BC light. Red intensity: 260 μW cm^{-2}; far red II intensity: 1000 μW cm^{-2}. The light treatment was given once a day. The medium contained 1.5% sugar and 3×10^{-6} M kinetin. Illumination times in minutes, R = red BC light, F = far red II light.

Radiation treatment	*MR*	*LE**	*IPLE*** %
5 min R	60	37	
5 min R 10 min F	30	7	19
4 × (1 min R 1 min F) 5 min R	60	37	
4 × (1 min R 1 min F) 5 min R 10 min F	27	4	11
Darkness	23	-	

* *LE* = *MR* of the light treated cultures minus *MR* in the dark
***IPLE* = far red non-reversible part of the light effect

A different result was obtained if the red and the red- far red treatments were given with dark intervals of several hours. Then an increase of the far red non-reversible part of the light effect was obtained by increasing the number of light treatments per day. This is shown in figure 6.6. The total effect of the red light treatments increased with the number of illuminations per day (fig. 6.6 **a** and **b**); with 8 illuminations per day on a medium with kinetin and 1.5% sucrose, the multiplication rate reached the maximum of what was considered possible for heterotrophic growth (chapter 3, section 6). It therefore could be that under these conditions the multiplication rate of the cultures receiving red alone was limited by the carbohydrate metabolism, whereas the multiplication rate of the cultures receiving far red after red was not. This would reduce the difference in multiplication rate between cultures treated with red and with red followed by far red radiation and thus reduce the far red reversible part of the light effect. That in figure 6.6c in the presence of kinetin the far red reversibility is seen to decrease with increasing number of light treatments per day may be due to this limitation. To avoid this, the sucrose content of the medium was increased to

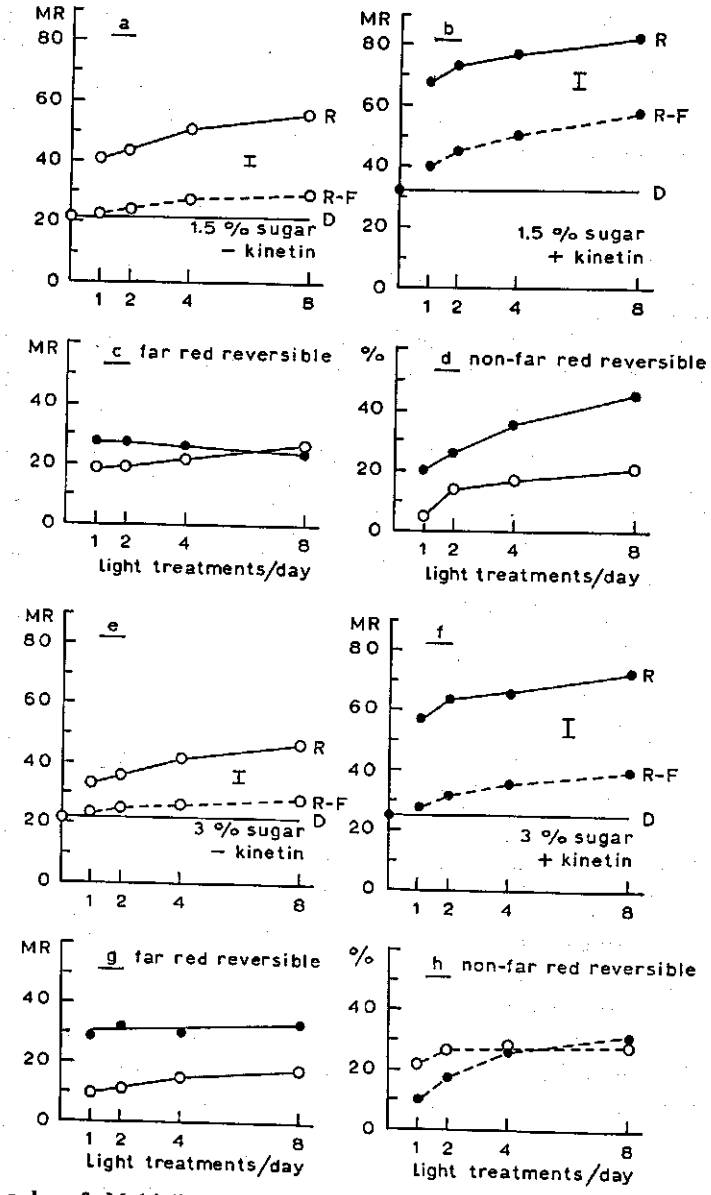


FIG. 6.6. a, b, e, f: Multiplication rate (*MR*) of cultures receiving cyclic treatments with red BC or red followed by far red II. Irradiations applied with different frequency of repetition. Red light 5 minutes 500 μW cm^{-2}; far red 10 minutes 1400 μW cm^{-2}; D = dark control; a, b, c and d with 1.5% sugar in the medium, e, f, g and h with 3% sugar in the medium; c and g: far red reversible part of the light effect (= *MR* of the red treated cultures minus *MR* of the red followed by far red treated cultures); d and h; far red non-reversible part of the light effect in percentage of the light effect of cultures treated with red only; (○) = without kinetin; (●) = with 3×10^{-6} M kinetin; (—R) = red light only; (– – – R-F) = red followed by far red. Vertical bars indicate twice the standard deviation $\bar{\sigma}$ of the mean.

3%. At this concentration a general reduction of growth rate is brought about which is due to the increase in osmotic value (the same result was obtained if mannitol or carbowax 6000 was added to the medium). Under this condition it is likely that growth rate at 8 illuminations per day was limited by the light treatment alone, but nevertheless almost no increase of the far red reversible part of the light effect was obtained when the number of illuminations per day was increased from 2 to 8 (fig. 6.6**g**).

In the figures 6.6**d** and **h** the far red non-reversible part of the light effect (= *MR* of red-far red treated cultures minus *MR* of cultures in continuous darkness) is expressed as percentage of the light effect of red illuminations only (= *MR* of red treated cultures minus *MR* in darkness). These figures show that the far red non-reversible part of the light effect increased with the number of illuminations per day. Relatively (figs. 6.6 **d** and **h**) and also in absolute sense (fig. 6.6 **a** compared with **b** and **e** with **f**) this increase was greater in cultures grown with kinetin than in those without.

The far red reversible part of the light effect (figs. 6.6c and **g**) was only in the absence of kinetin somewhat increased when the number of illuminations per day was increased. With kinetin added, the far red reversible effect was nearly optimal with one illumination per day.

The effect of far red alone, without preceding red, was 7% of the effect of red light alone when 8 irradiations per day were given.

6.4.4. *The effect of blue light on the far red non-reversible part of the light effect*

Phytochrome is an unusual plant pigment, in that its red absorption peak is much higher than its absorption maximum in the blue. Most plant pigments,

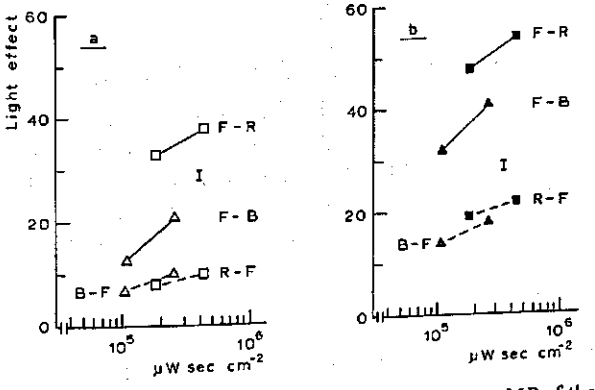


FIG. 6.7. Light effect (*LE*) (= *MR* of the light treated cultures minus *MR* of the dark controls) of cyclic treatments (cycle length 3 hours) with 285 or 660 seconds 640 μW cm^{-2} red light BC or 380 μW cm^{-2} blue E light. The energy content of these blue and red illuminations is indicated on the abscissa. The red and the blue illuminations were either preceded by 5 minutes of 2 000 μW cm^{-2} far red II (F–R or F–B), or followed by 5 minutes of 3 000 μW cm^{-2} far red II (R–F or B–F). a: without kinetin, 1.5% sugar; dark value *MR* = 20, far red only *MR* = 23. b: with 3 × 10^{-6} M kinetin, 3% sugar; dark value *MR* = 25, far red only *MR* = 28. Vertical bars indicate twice the standard deviation $\bar{\sigma}$ of the mean.

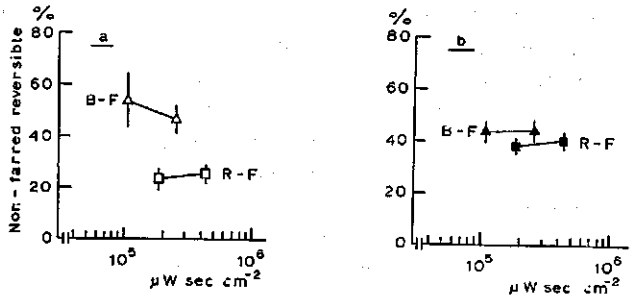


FIG. 6.8. The far red non-reversible part of the light effect (= *LE* of the cultures treated with red or blue light followed by far red) in percentage of the *LE* of the treatment with either red or blue, both preceded by far red II. Values are derived from fig. 6.7; a: without kinetin, 1.5% sugar; b: with 3×10^{-6} M kinetin, 3% sugar. Vertical bars indicate twice the standard deviations following from the $\bar{\sigma}$ values in fig. 6.7.

like the carotenoids, flavonoids, flavins etc. have their main absorption band at short wavelengths, even if they have additional bands in the red. If the far red non-reversible part of the light effect is mediated by a pigment other than phytochrome, there is the possibility that blue light is more effective than red light. Since the far red non-reversible part was expected to depend on the duration of the illuminations, exposures of 285 and 660 seconds were given. Figure 6.7 shows that the longer illuminations resulted in a larger light effect, but the far red non-reversible part remained the same in proportion (fig. 6.8). Figure 6.7 shows further that the light effect of the blue was much smaller than that of the red, but when far red followed the red or blue exposures, the effects of red and blue were almost equal. Figure 6.8 shows that without kinetin the total effect of the blue light was due for 50% to the far red non-reversible part of the light effect, that by red light for 25%. In an average of 4 experiments, the far red non-reversible part of the light effect with blue light was 21 $\pm$ 7.4% higher than with red light. In the presence of kinetin this difference was only 9.3 $\pm$ 5.8% (7 experiments). Evidently blue light favours the far red non-reversible part and red light the far red reversible part of the light effect. In the presence of kinetin the difference is less, mainly because the far red reversible part in the blue treated cultures is increased.

6.5 THE EFFECT OF FAR RED GIVEN AT DIFFERENT TIMES AFTER A RED ILLUMINATION

The increase in the multiplication rate by a brief red illumination of cultures otherwise kept in darkness was shown to be partly due to the formation of Pfr (chapter 5). If Pfr is removed by far red radiation before it has completed its action, the red light effect is reduced. In order to investigate the escape from control by phytochrome Pfr, several experiments were performed with a far red

irradiation at different moments after the red illumination, thus confining the action of phytochrome Pfr to dark intervals of different durations.

In these experiments red light was given for 5 minutes once every 24 hours; by varying the light intensity, different levels of Pfr were established. At different times after the red light, a saturating amount of far red radiation was given, removing the Pfr left over from the red illumination. The multiplication rate was measured after three weeks of growth in these regimes. The results of four experiments without kinetin are shown in figure 6.9, those of five experiments with kinetin in figure 6.10. The Pfr values given in these figures are derived from spectrophotometric measurements of phytochrome conversion in *Lemna minor* cultures grown for 7 days in continuous darkness. The data are discussed in the sections 6.5.1 and 6.5.2.

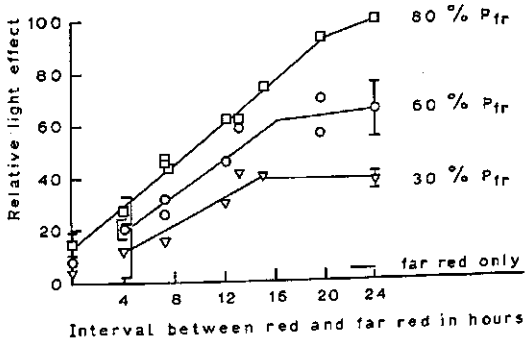


FIG. 6.9. The effect of the dark interval between red and far red on escape from far red reversion. The relative light effect, expressed as percentage of the light effect with the maximum red dose, of different amounts of red BC light, applied during 5 minutes once a day, followed by 5 minutes far red II, 1400 μW cm^{-2} after the dark periods indicated on the abscissa. Medium with 1.5% sugar, without kinetin. The percentages phytochrome conversion (% Pfr) are indicated alongside the curve. Doses of red light: (□) = 20 × 10^4 μW sec cm^{-2}; (○) = 1.0 × 10^4 μW sec cm^{-2}; (▽) = 0.3 × 10^4 μW sec cm^{-2}. Vertical bars indicate twice the standard deviation σ.

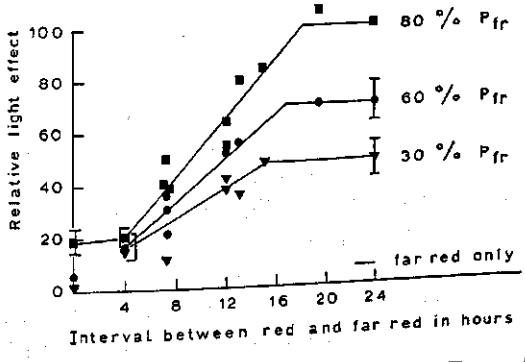


FIG. 6.10. Same as fig. 6.9, but cultures with 3 × 10^{-6} M kinetin. Doses of red light: (■) = 20 × 10^4 μW sec cm^{-2}; (●) = 1.0 × 10^4 μW sec cm^{-2}; (▼) = 0.3 × 10^4 μW sec cm^{-2}.

6.5.1. *Treatments with saturating amounts of red light*

The upper curves of figures 6.9 and 6.10 represent treatments with doses of red light that saturate phytochrome photoconversion. In both figures the light effect increased with the length of the time interval between the red and the far red illuminations. This could be expected when the red light effect has proceeded further the later the far red is applied. In this way the red light effect escapes from reversion by far red. In the cultures without kinetin this increase was almost linear up to 20 hours after the red illumination.

In cultures with kinetin 4 hours after illumination the reversibility was the same as immediately after illumination. A far red irradiation 20 hours after the red illumination had no effect on the multiplication rate in experiments with a 24 hours periodicity of illumination, but had a small effect when the periodicity of the illumination was increased to 32 hours (fig. 6.11). The latter observation indicates that 20 hours after an illumination, Pfr is still present and active in growth stimulation. This effect may be concealed in the 24 hours series by that of the next red illumination.

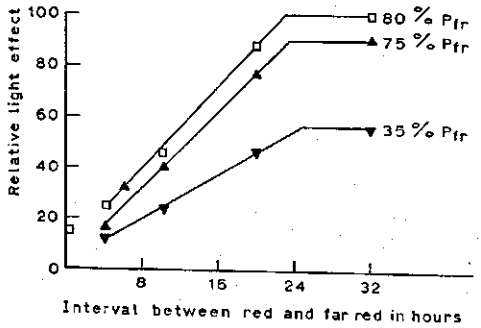


FIG. 6.11. Same as fig. 6.9, but cultures with 3×10^{-6} M kinetin and red light given with intervals of 32 hours. Doses of red light: (□) = 50×10^4 μW sec cm^{-2}; (▲) = 4.2×10^4 μW sec cm^{-2}; (▼) = 0.4×10^4 μW sec cm^{-2}.

6.5.2. *Treatments with non-saturating amounts of light*

The lower curves of figures 6.9, 6.10 and 6.11 represent treatments with amounts of red light not giving maximum conversion of phytochrome. The percentage of Pfr obtained with the different energy doses at the end of the illumination is indicated in the figures.

The figures show that the light effect depends on the period during which Pfr is present as well as on the Pfr level established by the illumination. Obviously, the magnitude of the light effect was always limited by the concentration of Pfr present, even during the first hours following the red illumination when Pfr content, as measured by ROMBACH and SPRUIT (1968), was highest.

6.6. Content of chlorophylls and carotenoids of plants exposed to brief illuminations

In the foregoing sections of this chapter it has been shown that the far red non-reversible part of the light effect increases as the exposures to red light are longer or more frequent. In view of the possibility that other photoprocesses participate in the stimulation of the multiplication rate, the contents of chlorophyll and carotenoids were measured. In cultures exposed once a day to illuminations of different duration, the chlorophyll content increased as soon as the exposure time was longer than 20 minutes (fig. 6.12). Far red after red reduced the chlorophyll content at the longer exposure times. Carotenoids responded in the same way as chlorophyll but the difference in content between dark grown and illuminated cultures was not as large (fig. 6.13).

For experiments with brief illuminations in different periodicity, the exposure times were adjusted so that the total exposure per day was 10 minutes for each treatment. To ensure maximum phytochrome photoconversion within the shortest exposures (25 seconds) white fluorescent light (Osram 25 Sa) was used, the intensity of the red light being too low. The photostationary Pfr level of this white light was found to be equal to that of the red.

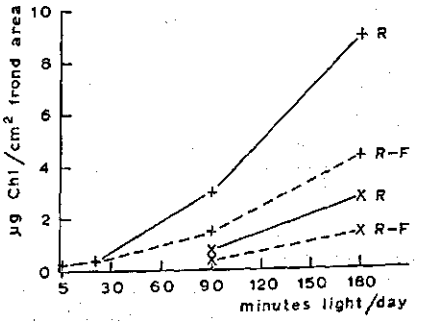


FIG. 6.12. Chlorophyll in cultures grown for 14 days in a regime of one exposure per day to red during the periods indicated, or to red followed by 10 minutes far red II. Light intensities: red 1800 µW cm^{-2}, far red 5000 µW cm^{-2}. Fresh weight 26 mg/cm^2 for plants exposed to red light only, 27 mg/cm^2 for plants treated with red followed by far red. Culture medium with 1.5% sugar and 3 × 10^{-6} M kinetin. (+) = chlorophyll a; (×) = chlorophyll b; (—R) = red light only; (- - - F-R) = red followed by far red.

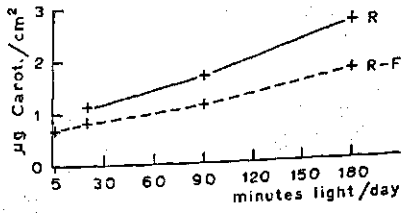


FIG. 6.13. Carotenoids, same experiment as that of fig. 6.12.

In these experiments the chlorophyll content increased proportionally to the number of illuminations per day (fig. 6.14). In contrast to what was found for the longer exposures to red light applied once per day, the chlorophyll content was not reduced by far red applied after each white illumination. Since experiments with red light had given a similar result, the non-reversibility is not due to the wavelength components below 600 nm in the white light. In these experiments the protochlorophyll content at the end of the dark periods was very low and hardly influenced by the light regime. An explanation for the difference in response to far red applied after long exposures (fig. 6.12) or after short exposures (fig. 6.14) could be that protochlorophyll synthesis during the longer exposures is limited by processes which are reinforced by Pfr presence during each preceding dark period. With short exposures almost no synthesis of protochlorophyll during each light period occurs, and each dark period is long enough for restoration of the low protochlorophyll level in spite of the absence of Pfr.

The content of carotenoids (fig. 6.15) also increased with the number of

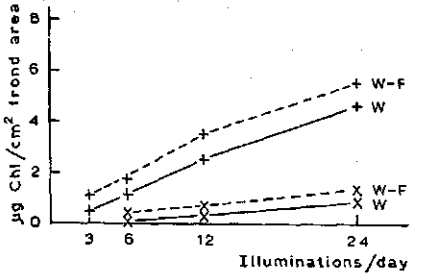


FIG. 6.14. Chlorophyll in cultures grown under cyclic illuminations with white light preceded or followed by far red. Light intensities: white (Osram L 140 W/25 Sa fluorescent lamps) 4500 µW cm^{-2}; far red II 5 minutes 5000 µW cm^{-2}. White exposures: 25 sec for illuminations once per hour, 50 sec for once per 2 hours, 115 sec for once per 4 hours and 250 sec for once per 8 hours. Culture medium with 3% sugar and 3 × 10^{-6} M kinetin. Fresh weight: 24 mg/cm² for plants exposed to white light only, 30 mg/cm² for plants treated with white followed by far red. (+) = chlorophyll a; (×) = chlorophyll b; (—W) = white light preceded by far red II; (– – W-F) = white light followed by far red II.

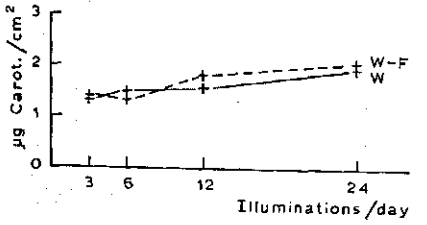


FIG. 6.15. Carotenoids, same experiment as that of fig. 6.14.

illuminations per day, but less than chlorophyll. Far red treatments subsequent to the white illuminations did increase the carotenoid level slightly, similar to what was found with the chlorophyll; this is due to a more dense structure of the fronds and is not found when the pigment content is expressed on fresh weight basis.

6.7. Conclusions and discussion

The experiments described in this chapter lead to the following conclusions:

1. Far red, applied continuously, has a stimulating effect on the multiplication rate, dependent upon the intensity.
2. Brief irradiations with far red, on the other hand, have almost no effect on the multiplication rate.
3. The stimulating effect on multiplication rate of low intensity red light is antagonized by simultaneous irradiation with far red.
4. Brief exposures to red light stimulate the multiplication rate, this effect can be reversed partly by far red applied immediately after the exposures to red light (fig. 6.3).
5. Reversion by far red of a brief red exposure was not complete, it appears that during 5 minutes red the process leading ultimately to an increased multiplication rate had made already some progress.
6. The total light effect increases with the number of illuminations per day. This is mainly due to an increase of the far red non-reversible part of the light effect, the reversible part of the light effect being much less influenced (fig. 6.6).
7. Blue light, in brief illuminations separated by dark periods of 3 hours, resulted in a light effect which was much smaller than that of a similar treatment with red light, the far red non-reversible part, however, was equal to that of the red illuminations (fig. 6.7).
8. Increasing the duration of each red illumination results in an increase in the far red non-reversible part of the light effect from 25% with light periods of 5 minutes to 45% with light periods of 90 minutes (fig. 6.5).
9. During darkness following a short red irradiation, the red light effect gradually 'escapes' from Pfr control. After the first 4 hours in darkness, this escape is not significantly different from that after 5 minutes; it increases almost linearly with time during the next 12 hours.
10. If the far red non-reversible part of the light effect is also considered as an escape from Pfr control, the experiments with different durations of illumination show that escape in light is much faster than in darkness. In red light, 45% of the effect becomes irreversible in 90 minutes (fig. 6.5); in darkness 8 hours are required to reach the same percentage (figs. 6.9 and 6.10).
11. From the escape experiments it was concluded that 20 hours after illumination Pfr was still active (fig. 6.11).
12. If the escape experiments were carried out with energy levels of red light converting less than 80% of the phytochrome to Pfr, the phytochrome

effect was reduced, no matter whether the Pfr was present for a short time or for a long time after the illumination.

13. Kinetin gives an increase of the light effect, mainly due to an increase of the far red non-reversible part (figs. 6.4 and 6.6).

The above observations lead to the conclusion that the stimulation of growth by light consists of two parts: one part is reversible by far red immediately after the illumination, the other part is not reversible by far red. The two parts will be discussed separately below.

6.7.1. *The reversible part of the light effect*

The far red reversible part of the increase of the multiplication rate by light most likely is due to Pfr, which stays active in the plants for at least 20 hours, in spite of the disappearance of Pfr by dark reversion and destruction observed spectrophotometrically. ROMBACH (1966) suggested this to be an indication for the existence of two Pfr fractions of different stability, of which the more stable fraction would be the physiologically active one, and the less stable would be the more abundant one.

Later measurements, with *Lemna minor* enriched in phytochrome by growing in continuous darkness for 3 or more days, showed that disappearance of Pfr was slower than originally estimated, the half life time of Pfr at 23 °C being 2.5 hours. If disappearance of Pfr continues at the same rate during 15 hours, its concentration after that period would be 1 % of the initial phytochrome concentration. According to figure 6.10, 20 % of the red light effect is still reversible by far red after a dark period of 15 hours. During the last 9 hours of a 24 hour cyclus 20 % of the total effect is therefore accomplished with the Pfr present during that period, i.e. 1 % or less of the amount present at time zero.

ROMBACH (1965) has derived a relation between the phytochrome conversion by light and the physiological effect of repeated illuminations on the multiplication rate of *Lemna minor*. He demonstrated that at very low Pfr levels the physiological effect increases sharply with the Pfr fraction of phytochrome, so that about 25 % of the effect of a saturating amount of light is obtained with an amount of red forming only 3 % Pfr. The effect of brief irradiations with far red I and II indicates the same: according to table 2.3 far red I gives 5 % Pfr; the light effect is 25 % (fig. 6.3); far red II yields 0.5 % Pfr which gives 3–5 % light effect (fig. 6.3). Therefore, ROMBACH's (1966) conclusion that in *L. minor* there are two Pfr fractions with different stability does not necessarily follow from our data.

The following data point in the same direction. In section 5.3 it was shown that far red applied before the red reduced the multiplication rate when photoconversion of phytochrome by the red light was partial and the dark periods were not much longer than 6 hours (in later experiments it was found that the dark periods had to be shorter than 8 hours). This indicates that at the end of a dark period of 6 hours sufficient Pfr is left to give, when added to the Pfr content established by the following red illumination, a for the plant sensible

higher Pfr level. After dark periods longer than 8 hours the Pfr level has decreased too much to produce this effect. This indicates that the decrease in Pfr level after an illumination is not a feature of spectrophotometry only, but can be observed in physiological experiments also. This means that from our experiments on multiplication rate in *Lemna minor* no significant indication could be obtained for a difference in stability between the physiologically active and the with spectrophotometer measured phytochrome.

The data with non-saturating light doses in figures 6.9, 6.10 and 6.11 show that there is no surplus of physiologically active Pfr in the plants, not even during the first hours after the illumination. According to fig. 6.10, a light dose of 10^4 μW sec cm^{-2}, giving 60% Pfr, results in 70% of the effect obtained with 80% Pfr, irrespective of the duration of the Pfr presence. This means that either all phytochrome is active, or that, if part of the phytochrome is inactive, it is not in free exchange with the part that is active in promoting multiplication rate (ROMBACH, 1966).

In most cases we have related the physiological response to the fraction of phytochrome in the Pfr form. The question whether a response is related to the Pfr/P_{total} ratio or to the Pfr concentration and the relation of this question to that of the active and the bulk phytochrome is discussed in section 8.3.

Figure 6.10 shows that the escape from far red reversion was often delayed for 4 hours. This implies that the Pfr present during the first 4 hours after the illumination gives no effect in addition to the far red non-reversible part of the light effect. It may be that substrates in the processes initiated by Pfr are exhausted by the far red non-reversible reaction and that Pfr has to be present for 4 hours for the initiation of the production of these substrates.

6.7.2. *The far red non-reversible part of the light effect*

A criterium for phytochrome involvement is the reversibility of a light reaction by far red. When an effect, or as in our case, part of a response is not reversible by far red, the probability of another pigment as a photoreceptor has to be considered. HARTMANN (1966), however, has developed a hypothesis according to which energy absorbed by phytochrome, aside from causing interconversion of the two forms of phytochrome, could be used in a reaction of Pfr with a substrate, leading to the response. Since the reaction supposed by HARTMANN (1966) depends on the intensity of the light and takes place during illumination, it is not reversible by far red at the end of the light period, only reactions controlled by Pfr during the dark period being reversible. Increase of the multiplication rate by continuous irradiation with far red II, as shown in fig. 6.1b, may point to such a reaction.

Whatever the photoreceptor pigment may be that brings about the far red non-reversible part of the light effect, it is likely that the effect is due to reactions with the pigment and catalysed by the pigment during illumination. In these reactions substrates, in the form of precursors in reaction chains and carriers of chemical energy, may be limiting factors.

In a 24 hours illumination cycle, processes leading to 25% of the total light

effect did proceed to a phase beyond Pfr control within a period of 5 minutes of red illumination. Substrate limitation may be the reason that during illuminations longer than 5 minutes further progress of the escape from phytochrome control is slowed down. If we follow this reasoning and look after the condition required for bringing about a far red non-reversible part of the light effect of about 45% in the presence of kinetin, we see from figure 6.5b that 90 minutes red light are required. Substrate production during 90 minutes red light is therefore sufficient for that part of the response. If the light is given in brief exposures, the substrate required for reactions during illumination has to be produced and accumulated during the dark periods. It then appears that, when Pfr is removed at the beginning of each dark period by an exposure to far red, a period of darkness of 8 times 3 hours is required for a far red non-reversible effect of 45%. If the process during the light period uses the same substrate as in darkness in the presence of Pfr, then the rate of escape from phytochrome control during the dark period may be determined by the rate of substrate production in darkness in the presence of Pfr. In that case, fig. 6.10 shows that a period of 8 hours is required to produce a sufficient amount for a far red non-reversibility of 45%. From these data the relative rate of substrate production per hour can be derived to be $^1/_{24}$ in darkness in the absence of Pfr, $^1/_8$ in darkness in the presence of Pfr, and $^1/_3$ in red light, all with kinetin in the medium.

The experiments with blue light in table 6.4 and figure 6.7 show that, when followed by far red, equal doses of blue and red light bring about equal far red non-reversible light effects. When, however, the Pfr is not removed at the beginning of the dark periods, the light effect of the red is much higher than that of the blue, in accordance with the higher fraction of phytochrome present as Pfr at the end of the red illumination. This means that the processes leading to the response to light are much more dependent on Pfr level in the dark than during the light period, the photostationary Pfr fraction in blue light (45%) being not limiting the processes during the light period. Apparently, during the illumination, a second activity of the light is involved. The effect of continuous far red, described in section 6.2 is perhaps caused by this activity also. Since the effect is intensity dependent in contrast to effects, like phytochrome photoconversion, dependent on the dose of light, an appropriate name could be: intensity effect.

Kinetin always results in an increase of the far red non-reversible part of the light effect. This may also be due to an increased supply of substrate during illumination.

The amount of chlorophyll in cultures receiving brief illuminations is proportional to the number of illuminations per day (fig. 6.14). With 8 illuminations per day the chlorophyll content was about 8% of the highest level in continuous light (fig. 4.12). With one illumination per day and exposure times longer than 20 minutes, the chlorophyll content increased with further extension of the illumination time. With an illumination of three hours red light per day the content was 30% of the highest level in continuous light.

Preliminary measurements of photosynthesis showed that as soon as chlorophyll was present in the *Lemna* cultures, they were capable of photosynthesis, as evidenced by an apparent decrease of the carbon dioxide output upon illumination. On the other hand, a concentration of 10^{-5} M N-(3,4-dichlorophenyl-N,N-dimethylurea (DCMU), reducing photosynthesis of green *Lemna* cultures to 10% of the controls, had almost no effect on the far red non-reversible part of the light effect. A concentration of 2×10^{-6} M carbonyl-cyanide-N-chlorophenyl-hydrazon (CCCP), an uncoupler of phosphorylation in mitochondria as well as in chloroplasts, inhibited photosynthesis in *Lemna* by 50%, but was less effective on growth rate, reducing both the far red non-reversible and the far red reversible part of the light effect to 75%. The two inhibitors together, 10^{-6} M CCCP + 4×10^{-6} M DCMU, reduced photosynthesis to 20% and both the far red reversible and far red non-reversible parts to 75%.

Although there can be little doubt that light is absorbed in chlorophyll and that light dependent reactions in the chloroplasts take place, the low sensitivity of the far red non-reversible growth stimulation to DCMU and CCCP renders it unlikely that photosynthesis system II is involved, or that the rate of phosphorylation is very critical. This suggests that, if photosynthesis is involved, it would be via the electron transport chain of photosystem I.

7. THE RATE OF CELL MULTIPLICATION AND THE SYNCHRONIZATION OF CELL DIVISION IN *LEMNA MINOR* TREATED WITH PERIODIC BRIEF ILLUMINATIONS

7.1. INTRODUCTION

In the preceding chapters it has been shown that brief illuminations increase the multiplication rate of *Lemna minor*, and that in light – dark cycles of 24 hours and longer the effect mainly was through Pfr. ROMBACH (1965, 1971) demonstrated that the light dose-response relation and the reversibility by far red for stimulation of the rate of frond expansion was similar to that of the multiplication rate. In chapter 4 it has been shown that this also holds for continuous light.

On the other hand, the main difference between fronds grown in darkness and in light was in their structure (section 4.3 fig. 4.6). From the literature it appeared that inhibition of leaf growth in dark grown plants was mainly by inhibition of expansion (sections 1.2.1 and 1.2.2). In this chapter it will be shown that in *Lemna minor* the rate of increase in cell number is also under control of light.

7.2. THE COLCHICINE METHOD

When in a population of cells with equal duration of the mitotic cycle the cells are arrested in mitosis by an agent without any inhibitory effect on other cell processes, the percentage of mitotic cells will increase until all cells are trapped in the mitotic phase. If the duration of the mitotic cycle is the same for every cell in a sample and cell division is not synchronized, every cell of the sample will divide once in a time interval equal to the length of the mitotic cycle T. If M is the percentage of cells in mitosis per hour, all cells ($= 100\%$) will have divided in $T = 100/M$ hours, so a mean value of T can be calculated if M is known. In tissues with partly synchronized cell division, mitotic activity is periodic: depending on the moment of observation, either a high or a low value for M will be found. To obtain a mean value from which T can be calculated M must be determined over a period containing maxima as well as minima in mitotic activity.

No inhibitor of mitosis is completely without action on other cellular processes. EVANS, NEARY and TONKINSON (1957) demonstrated that colchicine, which is very active in arresting mitosis, decreased the rate of accumulation of cells in metaphase after six hours of incubation, showing that processes in the cell division cycle prior to the metaphase also became affected. During these six hours, however, accumulation of cells in metaphase was proportional to time in colchicine treated plants. From the rate of accumulation during this

period the duration of the mitotic cycle was calculated.

Another technique for measuring the rate of cell division is by labeling the nuclei with tritiated thymidine, which is incorporated in DNA, and counting the labeled nuclei after autoradiography from high resolution stripping film (CLOWES, 1961). The colchicine method and the labeling method were compared by CLOWES (1961, 1962). With the labeling method the percentage of labeled nuclei increased linearly with time until 80% of the cells were labeled. The incubation with radioactive thymidine, however, influenced the rate of cell multiplication also, as showed up when the incubation with thymidine ^3H was extended for longer than 36 hours. Up to that time the cells of the requiescent centre hardly incorporated the label, but thereafter the rate of labeling increased, the cells being induced to divide by the prolonged treatment.

With the colchicine method (0.05% colchicine) accumulation of metaphase cells was linear with time for 6 hours. The length of the mitotic cycle, calculated from the rate of accumulation of metaphase cells during this period, agreed well with the results of the labeling method. In the present investigation the colchicine method was applied because the labeling technique requires very thin slicing or maceration of the meristem; slicing the primordia is difficult because of the smallness of the fronds, and maceration renders it impossible to study the meristematic growth of the tissue in situ. With the colchicine method the primordial fronds could be kept intact.

In preliminary experiments we observed that during the first 2 hours of colchicine treatment some cell division still took place. After that time no cells in anaphase could be found. The increase of cells in metaphase started already one hour after application of colchicine and was proportional with time during the subsequent 5 hours; after 7 hours no further increase did occur. In most experiments the accumulation in metaphase was measured between 2 and 6 hours of colchicine treatment.

The results obtained with colchicine concentrations of 0.025, 0.05 and 0.1% in water did not differ significantly. At the highest concentration chromosomes were somewhat more contracted and staining yielded more contrast and the response to colchicine appeared somewhat faster. Subsequent experiments were therefore made with 0.1% colchicine. Plants treated with this concentration for 24 hours did not show any lesions and resumed growth after being washed with water.

Cultures of *Lemna minor* were grown for 2–3 weeks on standard medium with kinetin in the cabinets described in section 2.4. The cultures received no light during this period, except for the illuminations with red light mentioned in the description of the experiments and for 10 minutes of very low intensity green safelight during sampling. At the end of this period, samples were transferred to the colchicine solution. After the incubation times indicated in the tables, parts of each sample were fixed.

Plants were fixed for 24 hours at 0°C in a mixture containing 75% ethanol and 25% glacial acetic acid; thereafter they were washed with and stored in 70% ethanol/water. For staining the chromosomes, whole plants were heated

during 1 minute to 80 °C in a saturated solution of carmine (MERCK, Darmstadt) in 50% acetic acid, thereafter the very young fronds of 0.17–0.47 mm were isolated from the reproductive pockets of adult fronds and mounted in a solution of 10 g gelatin, 10 g sorbitol, 50 ml acetic acid with carmine and 60 ml water (staining techniques taken from DARLINGTON and LACOUR, 1962).

Cells in mitosis were counted under the microscope in field areas of 0.14 × 0.09 mm² in the upper epidermis of the meristematic parts of the fronds. The number of cells in mitosis is expressed as percentage of the number of cells in the examined area. Fronds smaller than 0.5 mm were completely meristematic, in larger fronds the meristematic part is confined to the basal area (fig. 7.1). In some experiments, cell numbers were counted from photomicrographs. The average number of cells in an area of 0.14 × 0.09 mm² was 490 ± 50;

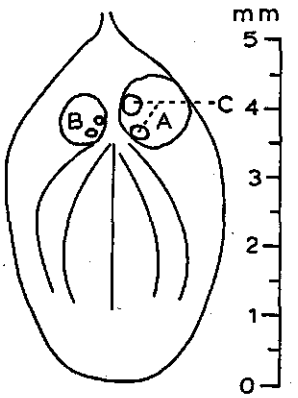


FIG. 7.1. Location of the meristematic fronds A and B in an adult frond. The epidermis of frond A has rapidly expanding cells, cell division being restricted to small areas around the second order fronds C. Frond B consists entirely of dividing cells and is at the maximum size used for counting mitotic cells.

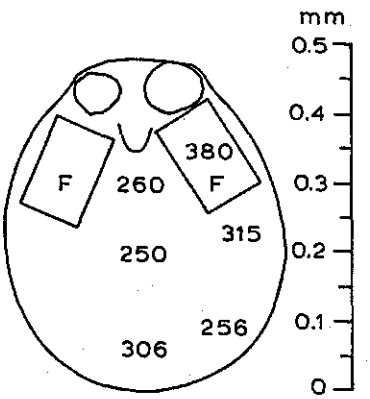


FIG. 7.2. Frond B of fig. 7.1. The rectangle F gives the site of the maximum number of epidermis cells per unit of area, this site was selected for counting. The number of epidermal cells per area of 0.01 mm² at different sites at the upper side of the fronds are indicated in the figure.

with fronds larger than 0.3 mm the location influences cell number per area (fig. 7.2). No dividing cells were found in fronds protruding from the reproductive pockets.

7.3. The mitotic activity

7.3.1. *The mitotic activity in fronds exposed to periodic brief illuminations*

7.3.1.1. 24 hours periodicity

Cultures were divided in two groups, one group received a red illumination every day at 8.40 h, the other group 12 hours later. After 20 days, samples from both groups were put on colchicine at regularly spaced moments over a period of 30 hours. The percentages of cells in mitosis are presented in tables 7.1 and 7.2

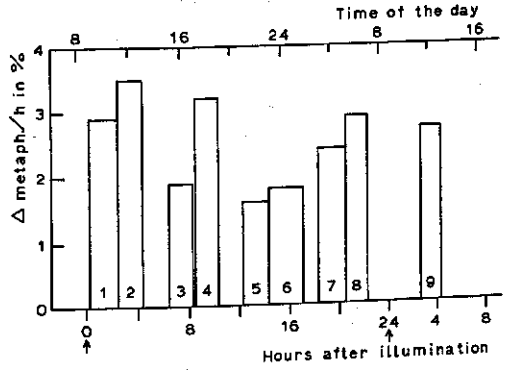


FIG. 7.3. Metaphase accumulation per hour at different moments during an illumination cycle in *Lemna minor* treated with colchicine. The plants received 470 seconds 600 $\mu W\ cm^{-2}$ red BC light with a periodicity of 24 hours. Moment of illumination: indicated by arrow. Numbers within the bars refer to numbers in column 1 of table 7.1. The width of the bars indicates the time lapse between fixation of part 1 and part 2 of the sample.

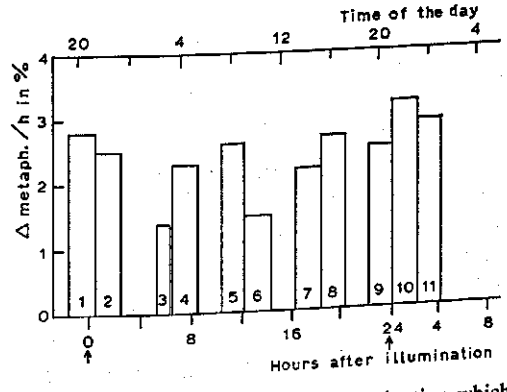


FIG. 7.4. Same as fig. 7.3 except for the moment of illumination which was 12 hours later. Data of table 7.2.

TABLE 7.1. Accumulation of cells in metaphase in colchicine treated *Lemna minor* in a 24 hours illumination cycle. Cultures were grown with 1.5% sugar and 3×10^{-6} M kinetin, they obtained at 8.40 each morning 500 $\mu W\ cm^{-2}$ red BC light for 470 seconds. After 2 weeks, samples were taken at different moments in the 24 hours cycle and fixed in 3 parts, after 0, 2 and 4 hours colchicine treatment.

1 Sample number	1 time of colchicine application	2 Part of sample	3 Time of fixation	4 Number of observations	5 Cells in metaphase %	5 σ	6 Accumulation of metaphases %/hour	6 σ
1	6.50	2	9.00	11	4.3	0.8		
		3	11.07	13	10.4	1.6	2.9	0.45
2	9.00	1	11.17	9	2.7	1.1		
		2	11.10	13	5.1	1.3		
		3	13.03	11	11.6	3.2	3.5	0.85
3	13.10	1	13.06	8	1.8	0.7		
		2	15.04	10	4.0	1.2		
		3	17.03	14	7.7	1.5	1.9	0.5
4	15.07	1	17.09	11	1.5	0.6		
		2	17.06	17	3.5	1.0		
		3	19.03	14	9.8	1.3	3.2	0.5
5	19.09	2	21.04	9	3.8	0.9		
		3	23.05	10	6.9	1.4	1.6	0.5
6	21.09	1	23.11	9	2.1	0.5		
		2	23.08	11	3.5	0.7		
		3	1.51	13	8.2	0.9	1.8	0.2
7	1.59	1	1.54	10	1.9	0.7		
		2	3.01	12	1.5	0.5		
		3	5.07	18	6.5	1.3	2.4	0.3
8	3.15	1	5.12	10	1.9	0.5		
		2	5.10	14	4.2	0.7		
		3	7.02	13	9.6	1.6	2.9	0.4
		1	7.06	10	2.6	0.9		
9	8.50	1	9.12	12	2.2	0.7		
		2	11.10	12	5.1	1.0		
		3	13.00	10	10.1	2.1	2.7	0.5
		1	13.03	15	2.2	0.5		

column 5. In plants not treated with colchicine (part 1 of the samples indicated in column 2) one half of the mitotic cells were in metaphase, the other half in anaphase. After 2 hours colchicine treatment only very few cells in anaphase were found. The rate of accumulation of metaphases at different moments of the illumination cycle is given in figures 7.3 and 7.4. The figures show minima at the 5th and 13th hour after illumination in both series. If the time scales of figs. 7.3 and 7.4 are shifted so that the times of illumination coincide, the position of the maxima and minima in the two figures coincide also. Probably, the positions of the maxima and minima were set by the brief illumination. In fig. 7.5 the data of figs. 7.3 and 7.4 are combined.

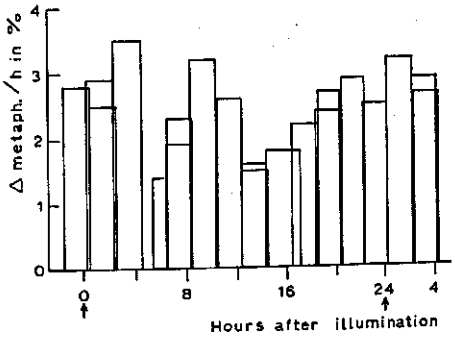


FIG. 7.5. Combination of the data from fig. 7.3 and 7.4.

7.3.1.2. 48 hours periodicity

The treatment was similar to that with the 24 hours series, except that the incubation time with colchicine was longer.

The results are presented in table 7.3 and fig. 7.6. Following illumination the number of cells in mitosis first increased somewhat, but fell considerably after 13 hours, like in the 24 hours experiments. Approximately 24 hours after illumination a maximum was found in the accumulation rate of cells arrested in mitosis. This maximum was followed by a gradual decrease, suggesting the exhaustion of a growth factor which was restored by illumination.

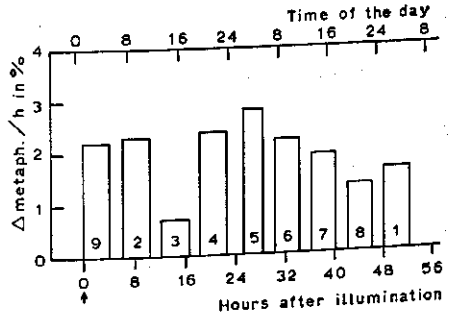


FIG. 7.6. The same as figure 7.3 but the plants received 470 seconds red BC light once in 48 hours. The numbers within the bars refer to the numbers in column 1 of table 3.

7.3.1.3. 32 hours periodicity

In this experiment the illumination regime of one part of the cultures differed half a cycle with the other part, like the two series given the 24 hours periodicity of illumination, but in the present experiment is was assumed beforehand, that they could be treated as one population. In this way data from the second half of the illumination cycle were obtained at the same time as from the first half, thus reducing a 36 hours working day to 20 hours.

TABLE 7.2. Accumulation of cells in metaphase in colchicine treated *Lemna minor* in a 24 hours illumination cycle. Cultures were grown with 1.5% sugar and 3×10^{-6} M kinetin, they obtained at 20.40 p.m. 500 μW cm^{-2} red BC light for 470 seconds. Sampling as in table 7.1.

1 Sample number and time of colchicine application		2 Part of sample	3 Time of fixation	4 Number of observations	5 Cells in metaphase		6 Accumulation of metaphases	
					%	σ	%/hour	σ
1	17.13	2	19.12	9	6.6	1.3		
		3	21.13	13	12.3	1.7	2.8	0.5
2	19.16	1	21.20	13	2.6	0.8		
		2	21.16	9	5.5	1.2		
		3	23.16	13	10.5	3.5	2.5	1.1
3	23.24	1	23.20	10	1.3	0.4		
		2	2.02	9	6.4	1.1		
		3	3.04	12	7.8	1.2	1.4	0.9
4	2.05	1	3.13	12	2.1	0.7		
		2	3.10	15	2.2	0.7		
		3	5.16	14	7.1	1.0	2.3	0.5
5	5.23	1	5.20	11	2.1	0.7		
		2	7.10	14	2.7	0.6		
		3	9.02	12	7.7	2.2	2.6	0.4
6	7.14	1	9.08	10	1.5	0.5		
		2	9.05	25	3.3	1.2		
		3	11.14	13	6.6	2.4	1.5	0.2
7	11.21	1	11.17	10	1.7	0.5		
		2	13.06	15	2.4	0.5		
		3	15.12	11	6.7	1.1	2.2	0.3
8	13.10	1	15.17	13	1.7	0.5		
		2	15.15	12	3.4	1.1		
		3	17.06	14	8.4	1.6	2.7	0.5
9	17.12	1	17.09	11	2.7	1.1		
		2	19.03	14	3.6	0.9		
		3	20.59	13	8.4	1.4	2.5	0.4
10	19.09	1	21.05	15	2.5	0.5		
		2	21.01	12	3.8	0.9		
		3	23.03	14	10.3	2.0	3.2	0.5
11	21.08	2	23.06	12	3.5	1.1		
		3	0.55	15	8.8	1.3	2.9	0.4

TABLE 7.3. Accumulation of cells in metaphase in colchicine treated *Lemna minor* in a 48 hours illumination cycle. Cultures were grown with 1.5% sugar and 3×10^{-6} M kinetin, they obtained at 1.00 a.m. on alternate days 500 $\mu W\ cm^{-2}$ red BC light for 470 seconds. After 3 weeks, samples were taken at different moments in the 48 hours cycle and fixed in three parts, after 0, 2 and 6 hours colchicine treatment.

1 Sample number and time of colchicine application	2 Part of sample	3 Time of fixation	4 Number of observations	5 Cells in metaphase		6 Accumulation of metaphases	
				%	σ	%/hour	σ
1 23.00 28–7–63	2	1.01	10	2.8	0.7	1.6	0.2
	3	5.06	6	9.2	0.9		
2 5.00 29–7–63	1	5.03	9	1.9	0.9	2.3	0.4
	2	7.01	12	3.5	1.5		
	3	11.22	12	13.5	3.5		
3 11.26	1	11.29	11	2.0	0.6	0.7	0.3
	2	13.13	10	2.5	0.7		
	3	17.16	14	5.5	2.6		
4 17.23	1	17.20	12	1.3	0.5	2.4	0.4
	2	19.17	9	3.8	1.3		
	3	23.27	12	13.7	3.0		
5 23.35	1	23.32	11	2.6	0.5	2.8	0.4
	2	2.10	11	5.7	1.6		
	3	5.27	12	14.8	2.3		
6 5.33 30–7–63	1	5.30	12	2.1	0.5	2.2	0.2
	2	7.17	14	2.8	0.9		
	3	11.14	19	11.7	1.8		
7 11.31	1	11.28	11	1.5	0.5	1.9	0.3
	2	13.12	12	3.0	1.1		
	3	17.15	12	10.6	2.2		
8 17.23	1	17.21	11	1.6	0.5	1.3	0.4
	2	19.12	9	2.5	0.7		
	3	23.11	10	7.7	2.6		
9 23.17	1	23.14	11	1.7	0.6	2.2	0.3
	2	0.59	11	1.9	0.7		
	3	5.03	11	10.7	2.0		

As appears from the first two sample numbers, after one hour colchicine treatment the percentage of cells in metaphase was doubled (table 7.4, column 6) but also the number of cells in anaphase was increased (column 5). This shows that an incubation time in colchicine of one hour was not yet sufficient for a complete arrest of mitosis in the metaphase, so that some escape to the anaphase occurred. Furthermore also the anaphase was arrested to some degree. The accumulation rate of mitotic cells was calculated from cells in metaphase only.

TABLE 7.4. Accumulation of metaphase cells in colchicine treated *Lemna minor* in a 32 hours illumination cycle. Cultures grown 21 days with 1.5% sugar and 3×10^{-6} M kinetin. They obtained 500 $\mu W\ cm^{-2}$ red BC light for 230 seconds. On day of sampling numbers 1–6 (column 1) received the illumination at 10.10 a.m., the numbers 7–12 16 hours later. After 3 weeks, samples were taken at different moments in the 32 hours cycle and fixed after 0, 1 and 4 hours colchicine treatment.

1 Sample number	1 time of colchicine application	2 Part of sample	3 Time of fixation	4 Number of observations	5 Cells in anaphase %	6 Cells in metaphase %	7 Accumulation of metaphases %/hour	7 σ
1	6.25	1	6.25	17	0.6	0.7		
		2	7.40	13	0.8	1.5		
		3	10.03	15	0.0	6.2	2.0	0.3
2	9.15	1	9.15	17	0.4	0.4		
		2	10.13	14	0.6	0.8		
		3	13.09	17	0.0	5.3	1.6	0.2
3	12.10	2	13.15	12	0.6	1.0		
		3	16.10	16	0.0	5.4	1.6	0.2
4	15.08	2	16.20	11	0.5	1.2		
		3	19.07	18	0.0	5.8	1.8	0.3
5	18.12	2	19.16	14	0.6	0.9		
		3	22.10	18	0.0	6.7	2.0	0.3
6	21.09	2	22.16	15	1.2	1.5		
		3	1.14	16	0.0	10.2	2.9	0.3
7	6.25	2	7.35	14	0.9	1.3		
		3	10.05	12	0.0	9.3	3.1	0.3
8	9.10	2	10.20	13	0.7	1.4		
		3	13.04	11	0.0	10.3	3.3	0.4
9	12.05	2	13.13	16	0.8	1.4		
		3	16.07	13	0.0	10.6	3.2	0.3
10	15.04	2	16.15	11	1.5	1.5		
		3	19.04	32	0.0	8.2	2.4	0.3
11	18.07	2	19.11	16	1.3	1.5		
		3	22.10	18	0.0	6.7	2.0	0.3
12	21.04	2	22.24	15	1.0	1.8		
		3	1.11	13	0.0	6.5	1.7	0.3

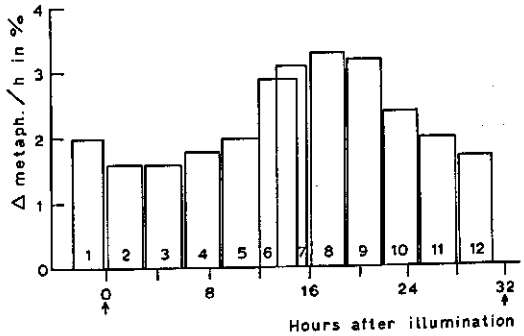


FIG. 7.7. The same as figure 7.3 but the plants received 230 seconds red BC light with a periodicity of 32 hours. The numbers in the bars refer to sample numbers in column 1 of table 7.4.

Fig. 7.7 shows the percentage of cells trapped per hour in metaphase in samples taken at different moments during the illumination cycle. A broad maximum of mitotic activity appears in the middle of the dark period. The minimum at 13 hours after the illumination, observed in the experiments with a periodicity of illumination of 24 hours or 48 hours, did not show up here.

7.4. THE CELLULAR MULTIPLICATION RATE

In the meristematic tissues growth proceeds by multiplication of cell number for which the same relation holds as for the increase in frond number:

$$dn/dt = G_c n_o \tag{13}$$

$$n_t = n_o e^{G_c t}$$

$$G_c = (1/t) \ln (n_t/n_o) \tag{14}$$

Herein n_o = number of cells at time t_o, n_t = number of cells at time t, G_c = the rate constant for cell multiplication. The cellular multiplication rate MR_{cells} is defined, in analogy of the multiplication rate of fronds (section 2.3.1) as $434 \times G_c$, with the time t expressed in days. The cell numbers n_o and n_t have not been determined directly, but the time required for doubling the cells (generation time T) was derived from the rate of accumulation of cells arrested in mitosis (section 7.2): $T = 100/M$. Since T is expressed in hours and G_c in equation 14 in days, T must be divided by 24. This substituted in equation 14 gives:

$$G_c = (M/4.16) \ln 2; \quad G_c = 0.166\, M; \quad MR_{cells} = 72\, M. \tag{15}$$

The figures 7.3, 7.4 and 7.5 show that cell division was partly synchronized.

TABLE 7.5. The effect of different periodicities of illumination on the rate of cell division and the multiplication rate of fronds. Experiments of tables 7.1, 7.2, 7.3 and 7.4. Rate of cell division expressed in multiplication rate of cells and in duration of mitotic cycle T.

1 Lenght of illumination cycle in hours	2 Increase in metaphases %/hour	3 Duration of the mitotic cycle T in hours	4 Cells passing through mitosis during one illumination cycle in %	5 MR_{cells}	6 MR_{fronds}	7 Ratio MR_{cells}/MR_{fronds}
24	2.43	41.2	58.3	175	61	2.9
32	2.27	44.0	72.6	163	57	2.9
48	1.98	50.8	95.0	143	52	2.7

Since the values of M varied considerably, it was necessary to derive a mean value representative for the mitotic cycle from the different values observed.

From the similarity of figs. 7.3 and 7.4 and from the good agreement of the overlapping data from the two separate phases of the 32 hours illumination cycle (fig. 7.6) it is likely that the time course of mitotic activity and therefore also the mean value of M, in each subsequent illumination cycle was the same. The values for the different illumination cycles were calculated from the figures 7.5, 7.6 and 7.7 by dividing the area between the abscissa from the first to the second illumination and a smooth line connecting the tops of the bars by the length of the illumination cycle. The results are given in table 7.5, column 2. The values in column 3, 4 and 5 are derived from column 2. Column 4 shows that an increase of the interval between the illuminations resulted in a lower rate of cell division. It can be expected that there is some relation between the multiplication rate of fronds and the multiplication rate of cells in the meristems. Therefore, the ratio between cellular multiplication rate and frond multiplication rate is given in column 7.

7.5. THE INFLUENCE OF THE PHYTOCHROME SYSTEM ON THE CELLULAR MULTIPLICATION RATE

In two experiments, the influence of different intensities of red and far red radiation on the accumulation rate of metaphase cells in colchicine treated plants was studied. The first experiment was made with an illumination periodicity of 32 hours. According to fig. 7.7 the mitotic activity under this periodicity of illumination was much lower during the first 12 hours after illumination than it was in the period from 12 to 22 hours after illumination. Therefore, a determination of mitotic activity was carried out in both periods.

Table 7.6 shows that stimulation by red light was greater as the energy was increased, and also that far red counteracted the effect of a preceding red illumination. In column 4 of table 7.6 an estimate is given of the generation time using the values of column 3. In view of the great differences in accumula-

TABLE 7.6. The effect of different illumination conditions on the accumulation rate of cells in metaphase in colchicine treated *Lemna minor* fronds. Before sampling: cultures grown 2 weeks with 1.5% sugar and 3×10^{-6}M kinetin in a 32 hours illumination cycle with red BC light for 400 seconds. Sampling and colchicine treatment in two intervals: group I from 8 to 12 hours after illumination, group II from 13 to 18 hours after illumination. For the calculation of the rate of cell division the accumulation rates in the two groups are averaged. The standard deviation of the values in column 3: $\sigma = 0.3$.

1 Illumination schedule and light dose in 10^3 μW cm^{-2}	2 Time group	3 Increase in metaphases %/hour	4 Generation time T in hours	5 MR_{cells}	6 MR_{fronds}	7 Ratio MR_{cells}/MR_{fronds}
500 red	I	2.0	50	14	53	2.7
	II	2.1				
6.6 red	I	1.4	60	120	48	2.5
	II	1.9				
0.25 red	I	1.0	85	85	31	2.7
	II	1.3				
500 red, directly followed by 2000 far red II	I	1.1	90	80	30	2.7
	II	1.1				
500 red, 4 hrs later followed by 2000 far red II	II	1.4	75	97	32	3.0
500 red, 10 hrs later followed by 2000 far red	II	1.7	60	120	43	2.8
2000 far red II	II	0.9	110	60	25	2.4

tion rate at different moments of the illumination cycle it is not possible to obtain a good approximation of the generation time from only one or two values. Nevertheless the ratio of cellular multiplication rate and of frond multiplication rate is roughly the same as in table 7.5.

A second experiment was performed with plants illuminated once per day with red or far red radiation with interference filters. The colchicine treatment started directly after the last illumination and lasted 6 hours in total, the lapse between the second and the third sampling being 4 hours. According to fig. 7.5, this time interval comprises a maximum as well as a minimum in mitotic activity.

Table 7.7 gives the values resulting from the different treatments. They show the same trend as those in table 7.6: the percentage of cells trapped in metaphase was greater the greater the amount of light, and this effect was reduced by far red to an extent determined by the amount of far red energy. The estimation of the generation times (column 3) was made in the same way and is subject to the same objections as the corresponding values in table 7.6. The values for the ratio of cellular multiplication rate to frond multiplication rate (column 6) in this experiment were smaller than in the other experiments,

TABLE 7.7. The effect of different energy doses, applied in a periodicity of 24 hours, on the rate of cell division, and the elongation and the multiplication rate of fronds in *Lemna minor* grown with 1.5% sugar and 3×10^{-6} M kinetin. Sampling and colchicine treatment for the measurements of cell division during a period of 6 hours after the last illumination. Red light: lamp-filter combination 658, far red light: lamp filter combination III.

1 Illumination schedule and light dose in 10^3 μW sec cm^{-2}	2 Increase in metaphases % hour $\sigma = 0.3$	3 Estimated generation time in hours T	4 MR_{cells}	5 MR_{fronds}	6 Ratio MR_{cells}/MR_{fronds}	7 Increase in length of fronds mm/day $\bar{\sigma} = 5\%$	8 $10^3 \times$ ratio increase in length/ MR_{fronds}
10 far red, followed by							
79 red	1.8	55	132	54	2.4	0.44	8.1
3 red	1.4	71	102	49	2.1	0.41	8.3
0.3 red	1.2	83	87	37	2.3	0.28	7.6
79 red, followed by							
7.5 far red	1.2	83	37	43	2.0	0.38	8.8
1.9 far red	1.3	77	93	51	1.8	0.42	8.2
10 far red only	0.8	125	57	22	2.6	0.18	8.2

except for the cultures receiving far red only, so perhaps the fraction of cells entering mitosis during the colchicine treatment was below the mean value.

In this experiment also the daily increase in length of the expanding fronds was measured, the results are given in column 7 of table 7.7. In column 8, the ratio of the daily increase in length of the fronds over the multiplication rate is given, yielding values similar to those of figure 4.3.

7.6. DURATION OF THE GROWTH PHASES OF A FROND

The growth of a frond consists of cell multiplication annex with plasmatic growth, followed by cell expansion, the latter two phenomena being accompanied by dry weight increase. In this section we have tried to analize the increase of a single frond from initial stages to its ultimate size. It turned out that in cultures receiving 5 minutes red light once a day, growing with a multiplication rate MR_{fronds} of 70, it takes 14 days for a frond to grow out from 0.05 mm to 1 mm (this is the size of a frond just protruding from the reproductive pocket) and an additional 8 days to reach its ultimate size. This outcome will be explained below.

7.6.1. *The rate of meristematic growth and the rate of expansion of fronds*

Area increase of a meristematic frond is due to cell multiplication and for

a small part to a gradual increase in cell diameter, the cells of a frond of 0.5 mm diameter being 20 % larger than those of a frond of 0.05 mm (fig. 7.2). Since this increase in cell diameter is spread over many days, its contribution to the relative rate of area increase will be neglected. Then the relative rate of area increase is equal to the relative increase in the number of epidermal cells:

$$G_A = G_c$$

Since the frond area is proportional to the number of epidermal cells, equation 14 changes into:

$$G_A = (1/t) \ln (A_t/A_o) \tag{16}$$

In the tables 7.5, 7.6 and 7.7 the cellular multiplication rates are given as MR_{cells}, for obtaining G_c or G_A these values must be divided by 434 (see section 7.4).

Growth of fronds protruding from the reproductive pockets proceeds by cell extension and by the development of intercellular cavities; in these aspects it differs from growth during the meristematic phase. To distinguish it from the G_A of the meristematic frond, the relative rate of area increase of the expanding frond will be indicated by G_{Ae}.

Young fronds, until they have attained half the final length, have the form of circular discs. The increase in length of the expanding fronds is linear with time until the fronds are fullgrown. This implies that over a period of time t, the radius R of a frond increases from R_o to $R_o + k_r t$, in which k_r is the increase in radius per unit of time. The area of the expanding frond at the beginning of the growth period is:

$$Ae_o = \pi R_o^2 \tag{17}$$

at the end of the growth period of t days the area will be

$$Ae_t = \pi(R_o + k_r t)^2 \tag{18}$$

It follows that the increase in frond area is neither exponential nor linear with time, but follows an intermediate course. Therefore the relative rate of area increase

$$G_{Ae} = (1/Ae)(dAe/dt) \tag{19}$$

cannot be constant and will be lower the larger the area of the growing frond. The value of dAe/dt follows from equation 18:

$$dAe/dt = 2\pi k_r(R_o + k_r t) \tag{20}$$

substitution gives

$$G_{Ae} = 2k_r/(R_o + k_r t) \tag{21}$$

it follows that the relative rate of area increase of a frond with radius R_o is:

$$G_{Ae}(R_o) = 2k_r/R_o \tag{22}$$

Table 7.8 repeates in columns 2 and 3 the data of table 7.7 on frond elongation and cellular multiplication rate. From the data on frond elongation the relative rate of area increase of a frond with a radius of 0.75 mm is derived (column 4). The cellular multiplication rates are corrected in such a way that the ratio of MR_{cells} over MR_{fronds} is 2.7, this ratio being found in the tables 7.5 and 7.6 for different treatments. In column 5 these multiplication rates, divided by 434, are presented as the relative rate of area increase of the meristematic fronds.

TABLE 7.8. Relative rate of increase in area of meristematic and of expanding fronds under different conditions of illumination, derived from data on cell multiplication and frond elongation in table 7.7. Medium with 1.5% sugar and 3×10^{-6} M kinetin. Light treatments once per day red 658 light (interference filter 658 nm) in 3 minutes, far red: III (interference filter 740 nm) in 1 minute. $2k_r$ = Increase in diameter of fronds larger than 1 mm; MR_{cells} = value of table 7.7, corrected as described in the text, $G_{Ae(0.75)}$ = relative rate of area increase of fronds of 1.5 mm diameter; G_A = relative rate of area increase of the meristematic frond = $MR_{cells}/434$.

1 Illumination schedule, light dose in 10^3 μW sec cm^{-2}	2 $2k_r$ mm/day	3 MR_{cells}	4 $G_{Ae(0.75)}$	5 G_A
10 far red – 79 red	0.44	145	0.59	0.33
10 far red – 3 red	0.41	132	0.55	0.30
10 far red – 0.3 red	0.28	100	0.37	0.23
79 red – 7.5 far red	0.38	116	0.51	0.26
79 red – 1.9 far red	0.42	137	0.56	0.32
10 far red only	0.18	59	0.24	0.14

When the values for the increase in area of the expanding fronds in column 3 are compared with those of the meristematic fronds in column 4 it turns out that the relative rate of area increase at the beginning of the phase of frond expansion is much greater than that of fronds in the phase of meristematic growth. The thickness of the fronds does not change much, the fronds being 10 to 13 layers thick in the phase of meristematic growth as well as in the phase of expansion growth, when the intercellular cavities are formed. The rates of area increase, therefore, can be considered equal to the rates of increase in cell volume and to the rates of increase in fresh weight of the fronds. It would be interesting to know whether the increase in rate of growth at the beginning of frond expansion is accompanied by a proportional increase in metabolic activity and protein synthesis or that it is due to an increased uptake of water and to cell wall extension only.

7.6.2. *The duration of the meristematic phase and the elongation phase of frond growth*

The time it takes for a meristematic frond to grow out from one size to the other can be calculated from equation 16. For a culture receiving a brief illumination once a day, table 7.5 gives a cellular multiplication rate $MR_{cells} = 180$, for fronds of this culture $G_A = 180/434 = 0.414$. The time t required for growth of these fronds from 0.05 mm to 0.5 mm diameter is:

$$0.414\ t = \ln (0.5/0.05)^2$$
$$t = 11.1 \text{ days}$$

During the time that the diameter of the frond increases from 0.5 to 1.0 mm, the cells of the epidermis increase in width, the intercellular cavities in the mesophyll begin to develop, and the rate of increase changes to that of the expanding frond. Fronds smaller than 1 mm diameter do not protrude from the reproductive pockets and could therefore not be measured directly. We had to satisfy ourselves with an estimation and assumed that over this period the relative rate of area increase will be intermediate between that of the meristematic frond and that of an expanding frond of 1.5 mm. In cultures receiving a brief illumination once a day an increase of 0.5 mm per day was found in several experiments, for a frond of 1.5 mm diameter this gives a rate of area increase $G_{Ae} = 0.66$ mm²/mm² day; since the G_A of the meristematic frond is 0.414 mm²/mm² day, we have chosen an intermediate value of 0.54 mm²/mm² day. Assuming a nearly exponential growth during this phase of frond expansion, we find for the time required for growth from 0.5 to 1.0 mm diameter:

$$0.54\ t = \ln (0.25/0.0625)$$
$$t = 2.6 \text{ days.}$$

These calculations show that a frond primordium of 0.05 mm diameter requires 11 + 2.6 days before it protrudes from the reproductive pocket. With a daily increase in length of 0.5 mm it takes 8 days before a frond of 1 mm has attained the length of a fullgrown frond (5 mm), so the total period of growth from 0.05 to 5 mm takes 22 days in cultures illuminated 5 minutes once a day.

The time course of growth of a frond, as calculated above for sizes below 1 mm and measured directly above 1 mm length, is shown in fig. 7.8. The length is on a logarithmic scale, so linearity in the figure means a constant growth rate factor G_A. During the first days of expansion growth there is a temporary increase in growth rate, during that period G_{Ae} is larger than G_A during the preceding period. Above 1 mm the length of the frond increases in direct proportion to time, which leads to a decrease in the growth rate factor G_{Ae}.

The ratio between multiplication rate and increase in length per day was found to be constant in most cases (fig. 4.3, table 7.7) as well as the ratio between the relative rate of cell multiplication over frond multiplication (tables

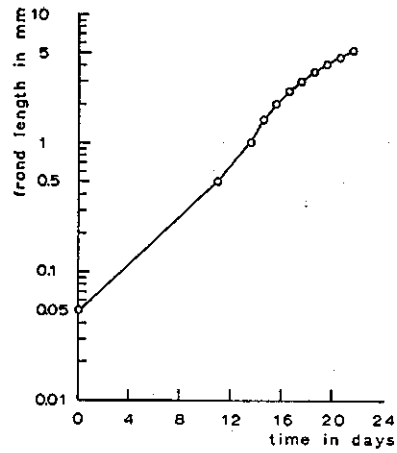


FIG. 7.8. Growth in length of a frond of *Lemna minor* illuminated once a day during 5 minutes with 500 μW cm^{-2} red BC light. Medium with 1,5% sugar and 3 $\times$ 10^{-6} M kinetin. The values below 0.5 mm are derived from the cellular multiplication rate, the values above 1 mm from direct measurements.

7.5 and 7.6); the time it takes for a frond to grow from one size to the other is inversely proportional to the multiplication rate.

The multiplication rate of cultures grown in continuous darkness in the presence of kinetin is half that of plants illuminated once per day, so the time required for growth from 0.05 to 5 mm is twice that in the repeatedly illuminated cultures, viz. 43 days. This means that the period of growth of a frond is long in comparison with the duration of an experiment, which was in most cases 3 weeks.

7.7. DISCUSSION

In section 7.3.1.1 it has been shown that during an illumination cycle maxima in mitotic activity alternate with minima. The correspondence between the figures 7.3 and 7.4 indicates that the timing of mitosis was influenced by the illumination programme, but an absolute synchronization of mitosis was not observed. In the three periodicities of illumination applied, the length of the mitotic cycle always exceeded the length of the illumination cycle; it is possible that this inequality influences the position of the maxima and minima of mitotic acitivity. How timing of cell division was influenced by the illumination programme is not clear: some part of the cell division cycle must have been more susceptible to the processes induced by the illumination than other parts. The participation of phytochrome in the induction of these processes is evident: Pfr shortens the duration of the division cycle and increases the rate of cell multiplication to the same degree as it accelerates frond multiplication and frond expansion.

Tables 7.5 and 7.6 show a cellular multiplication rate 2.7–3.0 times higher than the multiplication rate of fronds. Since the phase of cell division comprises only part of the generation time of a complete frond, the multiplication rate of cells in the meristematic parts is much higher than the multiplication rate of fronds. In cultures under optimal conditions (continuous illumination with 3000 μW cm^{-2} white light) a multiplication rate $MR_{\text{fronds}} = 180$ was found. This value, multiplied by 2.7 gives $MR_{\text{cells}} = 500$, which corresponds with a generation time of 14 hours for the meristematic cells. This value is of the same magnitude as that found by WRIGHT (1961) for cell multiplication in the wheat coleoptile 30 hours after germination, and is among the highest found in the literature for the cellular multiplication rates in meristems. When the difference in distance between roots, photosynthesizing organs and the meristem is considered, the transport system in the land plants must be remarkable effective to enable a meristematic growth rate as high as that in *Lemna minor.*

In completely heterotrophic light - stimulated cultures of *Lemna minor* a multiplication rate $MR_{\text{fronds}} = 70$ was found. This would correspond with a cellular multiplication rate of 190 and with a cellular generation time of 39 hours. Rates of $MR = 140$ (suspension culture of *Daucus carota*, WETHERELL, 1969) and $MR = 220$ (suspension culture of roots and cotyledons of *Phaseolus vulgaris*, DENG FONG LIAU and BOLL, 1970) are reported for heterotrophic tissue cultures. Apparently meristematic growth rate in light stimulated heterotrophic cultures of *Lemna minor* does not differ much from that in tissue cultures in the best conditions. This could be an indication that the absence of roots, stems and leaves in the tissue cultures has no great influence on the growth potentialities.

8. GENERAL DISCUSSION

8.1. Aspects of nutrition

In autotrophic plants the main effect of light on growth is by photosynthetic carbon assimilation. By feeding the plants with sugar, growth of *Lemna* cultures can be made independent of photosynthesis, so that the photostimulus effects of light become apparent. By photostimulation the multiplication rate and the fresh weight production were increased to the limit of the capacity of carbohydrate assimilation from the medium, but dry weight production was not stimulated. This indicates a constant rate of sugar uptake from the medium independent of both light intensity and fresh weight production. The sugar uptake from the medium remained essentially the same, even at higher light intensities, when photosynthesis contributed to the energy balance.

The constant rate of sugar consumption per frond suggests that sugar uptake from the medium is always at full capacity. Sugar uptake by carbohydrate-starved cultures during the first hours of incubation on a sucrose medium is much higher than the sugar uptake of sucrose - grown cultures. Therefore it is unlikely that the capacity of the sugar transport mechanism from the outside to the inside limits the rate of sugar uptake. Apparently sugar utilization is limited by processes of basal sugar metabolism and is not influenced by photostimulation or photosynthesis.

The sugar taken up from the medium was used for frond multiplication and for dry weight accumulation in the fronds. Both illumination and addition of kinetin caused a shift of the sugar utilization in the direction of frond multiplication.

Addition of kinetin stimulated the multiplication rate to about 170% of the control, both in darkness and in cultures receiving brief illuminations at regular intervals of time. Probably the effect is due to an overall stimulation of metabolic processes. Evidence for such a stimulation in *Lemna minor* in the light is provided by Trewavas (1970, 1972), who reported stimulation of RNA turnover and of protein synthesis by benzyl-amino-purine.

In continuous light, the effect of kinetin on the multiplication rate is smaller the higher the light intensity, disappearing at intensities above 200 $\mu W\ cm^{-2}$. It might be supposed that cytokinin production in *Lemna minor* is correlated with light intensity, and that above 200 $\mu W\ cm^{-2}$ the endogenous cytokinin production saturates the requirement for frond multiplication, but this is contradicted by the observations presented in the figures 3.5, 4.4 and 4.8, showing that above 200 $\mu W\ cm^{-2}$ the effect of kinetin on frond size and dry weight percentage is not smaller than at the lower light intensities.

On the other hand, Bezemer-Sybrandy (1969) reported that, while the natural cytokinin isopentenyl-adenosine isolated from a hydrolysate of tRNA from yeast had the same effect as kinetin, the hydrolysate of tRNA isolated

from *Lemna minor* stimulated frond multiplication without having any influence on frond size, dry weight percentage or root growth. Therefore, it might be that the effect of added kinetin on the multiplication rate is similar to that of the endogenous cytokinin but that it acts more strongly on frond size, dry weight percentage and inhibition of root growth. The endogenous cytokinins of *Lemna minor*, however, have not yet been identified. According to KENDE and SITTON (1967), in *Helianthus annuus* the growing root tips are sites of cytokinin production, the cytokinins being transported with bleeding sap. SHYBAOKA and THIMANN (1970) reported that in *Pisum* seedlings only the bleeding sap of light grown plants contained cytokinins. This may also be the case with *Lemna minor*. Root growth was not evaluated in our experiments, but incidental observations indicated that in *Lemna minor* cultures on a medium with sugar at light intensities below the compensation point the rate of root elongation was very low; it increased in proportion to the light intensity. A relation of the endogenous cytokinin level to root growth is therefore worth studying.

In trees an indirect relation between the growth of roots and light intensity was demonstrated by WASSINK and RICHARDSON (1951) but the relation between the growth of roots and that of leaves is complex as was shown by VAN DER LEK (1934) and RICHARDSON (1957). In our experiments the decrease in the effect of kinetin at light intensities above 100 $\mu W\ cm^{-2}$ is less pronounced in blue than in red light, which would mean that the production of endogenous cytokinin is not simply a function of photosynthesis.

In GORHAM's (1950) experiments yeast extract proved to be indispensable for growth in long periods of darkness. HILLMAN (1957) found that kinetin stimulated the growth rate in darkness and suggested that the growth stimulation by yeast extract was due to kinetin analogs liberated from the nucleic acids of the yeast extract during autoclaving. TASSERON-DE JONG and VELDSTRA (1971) on the other hand observed that addition of cytokinins reduced the longevity of *Lemna minor* in darkness. We found that this reduction could be counteracted by the yeast extract or by thiamine. The thiazole component of thiamine, 4-methyl-5-(β hydroxyethyl)-thiazole could substitute completely for the thiamine.

In the presence of thiamine, casein hydrolysate could be omitted from the medium, but in the absence of thiamine casein hydrolysate promoted the multiplication rate and the longevity of the cultures. This ties in with the conclusion of BORNKAMM (1970) that yeast extract is required for nitrate assimilation in *Lemna minor* in continuous darkness. From our experiments it appears that thiamine is the active factor, whose synthesis is impaired in darkness. The endogenous concentration of thiamine in cultures transferred from the light to continuous darkness is sufficient for at most a ninefold increase in plant number.

A brief illumination once a day prevents the onset of thiamine deficiency. This effect is reversible by far red, which points to control by Pfr.

8.2. Quantitative aspects of the development of a frond

In chapter 4 we described the relation between the multiplication rate of fronds and frond expansion. A new frond appeared when the mother frond had attained a specific size. The values of fig. 4.4 show that the ratio between the rate of frond elongation and the multiplication rate under different conditions varies between 8 and 12 and is therefore fairly constant. The same ratio appears again in table 7.7. This ratio was determined in several experiments with and without kinetin, under continuous light as well as with brief illuminations. No effect of variations between the experiments was found; in most cases the variation between experiments was greater than that between different treatments within the same experiment. A much lower value was found only in plant material previously kept in darkness for a long time, so that apparently the ratio could be altered at least temporarily by the previous conditions of the cultures.

Cleland and Briggs (1967) found that in *Lemna gibba* strain G3 only fronds smaller than 0.07 mm could be induced to flower by photoperiodic treatment. Apparently the environmental conditions during an early phase of growth determine subsequent development; this may be the same with *Lemna minor*. In section 7.6.2 it has been shown that the period of meristematic growth of a frond is long (about 2 weeks in plants illuminated 5 minutes once a day) compared with the duration of the experiment (3 weeks). Therefore part of the fronds existing at the end of the experiment were initiated under conditions different from those during the experiment. In our experiments, the effect of light on the multiplication rate was not influenced by the condition under which the plants of the inoculum were grown. The rates of frond elongation, however, are more likely to be influenced by the pre-experimental condition, the multiplication rate being measured over a long period of time, whereas frond elongation was measured over a period of 6 days and, therefore, concerned fronds which in some of the treatments may have been in a specific, perhaps especially sensitive phase of meristematic growth before or at the moment of transfer to the experimental conditions.

From the experiments on the rate of cell division described in chapter 7 it appears that the ratio of the cellular multiplication rate to the multiplication rate of fronds is also constant. Thus it would seem that if the multiplication rate of a culture is given, the cellular multiplication rate and the rate of frond expansion can be estimated.

Apparently photostimulation affects all these growth processes more or less to the same degree. In experiments to be published elsewhere we found that photostimulation of frond elongation could be prevented by actinomycin D, indicating that here transcription of DNA is involved. From the above it would appear, however, that no special gene is involved unless this gene were to activate all the other genes involved in the process of frond development and multiplication. Also in *Populus americana*, grown under different light intensities and temperatures, the whole pattern of development, and not only parts

of it, proved to be accelerated or retarded by changing the environmental conditions (PIETERS, 1974).

8.3. THE PHOTOSTIMULATION OF GROWTH

Rates of cell division, frond elongation, and frond multiplication were stimulated by light intensities far below the level of compensation of respiration by photosynthesis, and also under light regimes consisting of brief exposures allowing the formation of only small quantities of chlorophyll.

The participation of phytochrome in the stimulation by brief red light exposures is indicated by the reversion of the stimulation by far red in short exposures subsequent to the red ones. That phytochrome is also involved in the photostimulation by continuous red light of low intensity is concluded from the observation that blue light has much less effect (fig. 4.2) and that far red in admixture to red reduced the stimulation of the multiplication rate brought about by the red component when given alone (fig. 6.1).

Spectrophotometric determinations, however, showed that in continuous red light of 1 $\mu W\,cm^{-2}$ or higher the phytochrome content is below the detection level, whereas in plants grown in continuous far red I, establishing a photostationary state of 5% Pfr (table 2.3), the total phytochrome concentration is 7 times that level. Therefore it is questionable whether the Pfr concentration in continuous red light is higher than in a light mixture where 5% of the phytochrome is Pfr. This means that in photostimulation of the multiplication rate in *Lemna minor* it is very probably not the concentration that is decisive but the Pfr/P_{total} ratio established by the illumination. A similar conclusion was reached in many other investigations with plants in which the phytochrome level had been manipulated by a suitable pre-treatment, e.g. those by KLEIN et al. (1967), by FOX and HILLMAN (1968 a and b), KENDRICK and HILLMAN (1972) and by RAVEN and SHROPSHIRE (1975).

The Pfr/P_{total} ratio is found to decline rapidly during darkness after an illumination, owing to reversion and to decay of Pfr as well as to de novo synthesis of Pr. The physiological activity of Pfr, however, is not found to decline rapidly. In pea stem sections of pre-illuminated plants, by applying, 8 hours after the first, a second illumination consisting of light establishing various photostationary states, HILLMAN (1965) could demonstrate that the plant responded in a way that could only be understood when the Pfr/P_{total} ratio of the phytochrome active in the response was much higher than the ratio measured with the spectrophotometer (the *Pisum* paradox).

These experiments suggest that etiolated plants contain a high amount of inactive phytochrome, decaying, when in the Pfr form, at the rate observed in the spectrophotometer (bulk phytochrome) and a small amount of physiologically active phytochrome, when in the Pfr form decaying at the rate observed in physiological experiments.

The rate of decay of the physiologically active form of Pfr is not always found to be slower than the rate measured with the spectrophotometer. An example of exact correspondence of the spectrophotometrically and the physiologically observed rates of decay is found by OELZE-KAROW and MOHR (1973) in the threshold response of the lipoxygenase synthesis to the Pfr/P_{total} ratio (or to Pfr concentration?). The experiments described in section 6.7.1 suggest that in *Lemna minor* the rate of decay of physiologically active Pfr is also not very different from that measured with the spectrophotometer.

These data from the literature suggest that the Pfr/P_{total} ratio of a separate part of phytochrome is responsible for the physiological effects of the red - far - red - reaction - system. RAVEN and SPRUIT (1973), e.g., have proposed the idea of the migration of Pfr to small compartments to explain the very high light sensitivity of de-etiolation phenomena in chlorophyll synthesis.

If the Pfr/P_{total} ratio controls a physiological reaction, the primary reaction in which Pfr is involved would resemble reactions controlled by the redox potential or by acidity.

It is conceivable, however, that the amount of physiologically active phytochrome in the plants rich in spectrophotometric phytochrome might be the same as in plants pretreated so as to be low in phytochrome. Then the concentration of active phytochrome in *Lemna minor* grown in continuous red light might be the same as that in continuous far red. If this holds, the relative concentration of the physiologically active Pfr does not differ from the Pfr/P_{total} ratio. Therefore, on the basis of the data either from own experiments or from those reported in literature it is not possible to decide whether phytochrome control is exerted by the Pfr/P_{total} ratio in the physiologically active part of phytochrome or by the phytochrome concentration in this part. Further analysis must wait until the amount of physiologically active phytochrome can be determined directly.

In light regimes consisting of brief illuminations, reversibility of the light stimulation by far red depends on the light regime. The reversibility was greater the longer the dark periods between the red illuminations and the shorter the illumination time.

With periods of 3 hours once a day, the reversibility of the light effect decreased as the light intensity was increased (table 6.1): the far red non-reversible part of the light effect proved to be dependent on the light intensity. As explained in section 6.4.1, this is not due to an increase in Pfr level. The light reactions involved in this reaction are probably active during the light periods only, whereas the far red reversible responses continue during the whole period that phytochrome Pfr is present. To decide, the photoproducts would have to be identified and measured, or else, changes in absorption spectra of the pigments involved would have to be demonstrated. This we have not yet done.

Addition of kinetin especially increased the far red non-reversible part of the light effect on the multiplication rate. This may be due to an increase in the availability of substrate during the illuminations, either by stimulation of

overall metabolism (TREWAVAS, 1970, 1972) or by a more rapid transport of substrates, a supposition resulting from the observations of LIVNE and GRAZIANI (1972) on the increase in water permeability of leaf discs by addition of kinetin.

Continuous far red light antagonizes the red when given in admixture, but when given alone, it stimulates the multiplication rate in relation to darkness (section 6.2). This stimulation is stronger the higher the far red intensity. Since photoconversion of phytochrome at the light intensities applied is rapid, this far red effect cannot be ascribed to a higher photostationary Pfr level; its dependence on radiation intensity suggests a relation to the far red non-reversible effect of red illumination.

The photoreceptors of the far red non-reversible processes leading to stimulation of the multiplication rate are so far unknown. The experiments described in section 6.4.4 demonstrate that blue light is more effective in the far red non-reversible part of the light effect than in the far red reversible part; this suggests a pigment with more absorption in the blue than is found in phytochrome. An action spectrum of the far red non-reversible part of the light stimulation could give considerable information. Since the phytochrome photostationary state has an influence (section 6.2), the plants would have to be irradiated simultaneously with the wavelength band to be tested and with a sufficient intensity of red or far red light to maintain the Pfr at the desired level. A similar process was followed by HARTMANN (1966). Since the intensity of these admixtures has to be kept low as compared with that of the monochromatic light whose action is being tested, action spectra can best be obtained in wavelength regions which yield a photostationary state of Pfr not too far from the one desired or regions of low absorption of phytochrome.

The intensity dependency of the far red non-reversible part of the light effect suggests a high energy reaction, as has been found for many morphogenetic processes in various plants. In most cases the action spectra of high energy reactions show peaks in the blue and the far red part of the spectrum (WASSINK et al., 1950; CURRY and WASSINK, 1956; MOHR and WEHRUNG, 1960; DE LINT, 1961; EVANS et al., 1965; HARTMANN, 1967).

Differences between the far red and the blue effects have been found by many investigators. EVANS et al. (1965) showed that the far red peak disappears with age in the growth inhibition of hypocotyl elongation of seedlings of *Lactuca sativa*. ELLIOT (1975), giving an action spectrum of leaf expansion in *Pisum sativum* seedlings pre-illuminated in such a way that these were insensitive to red light, found a peak only in the blue (maximum effect was obtained when the blue light was combined with yellow light). Further evidence for the existence of at least two pigment systems in the intensity dependent light reaction is given by GRILL and VINCE (1966) and by ENGELSMA (1967a and b) for processes leading to anthocyanin synthesis, and by MEYER (1968) for inhibition of hypocotyl growth of gherkin seedlings.

Photostimulation of various physiological processes via blue light absorbing pigments without effects in the red or far red was observed long before the effects of red and far red were recognized (e.g. KONINGSBERGER, 1922). Action spectra are often similar to that of phototropism; the light requirement is often low. Recent examples are the action spectra for decrease in protoplasmic viscosity in *Elodea densa* (VIRGIN, 1954), the acceleration of protoplasma streaming in *Elodea canadensis* (SEITZ, 1964), the low- and the high-intensity chloroplast movements in *Lemna trisulca* (ZURZYCKI, 1962), the high-intensity chloroplast movement in *Vaucheria sessilis* (HAUPT and SCHÖNFELD, 1962) and the stimulation of endogenous respiration in *Chlorella* (KOWALLIK, 1967). The action spectrum of the high energy inhibition of elongation of the hypocotyl of *Lactuca sativa* seedlings (HARTMANN, 1967) was very similar to these spectra in the blue part of the spectrum. A somewhat different action spectrum, with peaks at about 420 and 580 nm, was found for cell division in root cultures of wheat by BJÖRN et al. (1963) and for light stimulation of the heterotrophic growth of *Chlorella* by KARLANDER and KRAUSS (1966).

These action spectra show that blue light absorbing pigments in plants may have a profound influence on metabolism and photomorphogenesis. Much progress has recently been made by POFF and BUTLER (1974) and by MUÑOZ and BUTLER (1975) in the analysis of photomorphogenetic responses in fungi with action spectra similar to that of phototropism in plants. They showed that the primary reaction here is probably the interaction of a photo-activated flavin with a cytochrome. We foresee that this finding will be followed in the near future by an analysis of the blue sensitive reactions in higher plants as well.

Not alone interactions of processes induced by light absorbed in different pigments may complicate the evaluation of a light treatment. Also the possible effects on leaf development by radiation received by the stem and petiole tissue must be taken into account, as was found by DE GREEF and CAUBERGS (1972) and OELZE-KAROW and MOHR (1974). Such effects might influence the results even in *Lemna minor* fed via the nutrient medium and with only a few cell layers of connective tissue between the mother and daughter fronds.

The far red non-reversible light process may act by increasing the concentration of a substrate for a reaction regulated by phytochrome, as was demonstrated by GRILL (1967) for the effect of blue light on the accumulation of anthocyanin in turnip seedlings, or by increasing the rate of a primary reaction of Pfr, as proposed by HARTMANN (1966) and SCHÄFER (1974).

With regard to *Lemna minor*, substrate production in light and darkness and a possible involvement of photosynthesis herein is discussed in section 6.7.3. An effect of light intensity on the rate of phytochrome reactions cannot be excluded; to distinguish between these reactions identification of the photo products and measurement of the kinetics of their behaviour during illumination is required.

SUMMARY

Axenic cultures of *Lemna minor* L. were grown on a medium containing sugars and amino acids. In continuous darkness the growth rate was one-tenth of the maximum in continuous light. In darkness early death revealed a thiamine deficiency; this deficiency was counteracted, without increasing the multiplication rate, by feeding the plants thiamine or its thiazole component. The rate of cell division, the rate of frond expansion, and the frond multiplication rate were increased by illumination; at low intensities this increase was in the nature of a photostimulus effect. The increase in growth rate was accompanied by a decrease in dry weight. The sugar uptake from the medium per frond per day was constant, independent of the light intensity and multiplication rate. At intensities of above 100 μW cm^{-2} continuous red light, the multiplication rate increased in proportion to the rate of photosynthesis.

Periodic brief illuminations yielded a stimulation of the frond multiplication rate of up to 4 times the dark value. Red illuminations of 2 minutes every 48 hours were sufficient to prevent thiamine deficiency; this effect of the red illuminations was reversible by far red. Stimulation of the multiplication rate was likewise reversible by far red; the effect of continuous low intensity red light was reduced by a simultaneous far red irradiation, while the effect of brief illuminations was reduced by far red following the red. From this it was concluded that phytochrome is a photoreceptor for these light effects. The results of experiments with different far red and red illumination schedules suggested that in plants with much phytochrome only a small part of it is active but that the Pfr level in this part decreases at a rate not very different from that of the bulk phytochrome as measured by the spectrophotometer.

Part of the light effect was not reversible by far red. This part increased by increasing the length of the illumination periods, the light intensity, and the number of illuminations per day. In this increase blue light was much more effective than it was in the far red reversible part of the light effect. This indicates a photoreaction different from phytochrome photoconversion.

Kinetin specially increased the far red non-reversible part of the photostimulation. Together with the intensity dependency of this part of the stimulation, this gave rise to the supposition that the non-reversible part of the light effect was limited by the supply of substrates in phytochrome controlled reactions during the light periods. Thus it would seem plausible to assume that the far red non-reversible part is built up during the red and the blue light periods, whereas the reversible part is developed during the intervening dark periods.

SAMENVATTING

De groeisnelheid van aseptisch op een medium met mineralen, suikers en aminozuren heterotroof gekweekt eendekroos is in continu donker slechts een tiende van de maximale groeisnelheid in licht. In het donker vertonen de planten thiamine deficientie, zij sterven voortijdig af. Door het toedienen van de thiazool-component van thiamine is dit te voorkomen, de groeisnelheid in het donker neemt hierdoor niet toe. Belichting leidt tot verhoging van de groeisnelheid, gemeten als snelheid van celdeling, lengtegroei en vermenigvuldiging der plantjes. Bij lage lichtintensiteit heeft deze verhoging een fotostimuluskarakter: de verhoging gaat ten koste van het drooggewicht van de plant. De suikeropname uit het medium per plant en per tijdseenheid wordt niet door de belichting en evenmin door de groeisnelheid beïnvloed. Bij een intensiteit van meer dan 100 μW cm^{-2} is verdere toename van de groeisnelheid evenredig aan de snelheid van de fotosynthese.

Een fotostimulering van de groei, tot 4 keer de donkerwaarde, of tot 40% van die in continu licht, treedt ook op wanneer periodiek korte tijd belicht wordt. De thiamine deficientie in het donker is reeds met een periodieke belichting van 2 minuten rood licht eens in de 48 uur op te heffen. Dit effect blijft achterwege als iedere belichting gevolgd wordt door ver-rood; het rood effect is dus omkeerbaar met ver-rood. Dit is ook met de groeistimulering het geval: bij continue belichting met rood van geringe intensiteit vermindert een gelijktijdige bestraling met ver-rood de groeibevordering door het rode licht, bij kort durende rode belichtingen gebeurt dit wanneer een korte bestraling met ver-rood op de rode belichting volgt. Uit het rood – ver-rood antagonisme wordt geconcludeerd dat het effect is toe te schrijven aan phytochroom; deze conclusie wordt gesteund door de waarneming dat de werkzaamheid van rood licht van de golflengte 660 nm belangrijk groter is dan die van rood licht met een bredere spectrale samenstelling of dan die van blauw licht.

Uit proeven met verschillende belichtingsregimes met rood en ver-rood werden aanwijzingen verkregen dat slechts een zeer gering gedeelte van het phytochroom werkzaam is en dat in het donker de Pfr fractie van dit werkzame gedeelte afneemt met een snelheid die niet veel verschilt van die welke bekend is uit spectrofotometrische bepalingen, die betrekking hebben op de grote massa van het aanwezige phytochroom.

De reductie van het lichteffect door ver-rood is geringer naarmate de donkerperioden tussen de belichtingen korter zijn of naarmate de belichtingen langer duren en de lichtintensiteit hoger is. Hieruit wordt afgeleid dat het totale effect van een belichting uit een met ver-rood omkeerbaar deel en een met ver-rood niet omkeerbaar deel bestaat. Aannemelijk is dat het niet omkeerbare deel tijdens de belichting ontstaat en het door ver-rood omkeerbare deel tijdens de donkerperioden tot stand komt.

Wij hebben aanwijzingen dat blauw licht ten aanzien van het niet omkeerbare

deel vrijwel even werkzaam is als rood licht. Ingegaan is op de vraag of het niet omkeerbare deel eveneens door phytochroom wordt bewerkstelligd en in hoeverre andere fotoreacties, wellicht behorende tot de fotosynthese, erbij zijn betrokken. Door toedienen van kinetine neemt speciaal het niet omkeerbare gedeelte van het lichteffect sterk toe. Het effect van kinetine en de afhankelijkheid van de lichtintensiteit doen vermoeden dat het niet met ver-rood omkeerbare deel van de lichtwerking gelimiteerd is door de voorziening met stoffen die tijdens de belichting aan phytochroom-reacties deelnemen als substraten.

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Curriculum vitae

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