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The Department of Plant Biology, 290 Panama Street, Stanford, California 94305, devotes its attention to the study of photosynthesis, the means by which plants manufacture organic matter, and to the physiological and biochemical mechanisms that underlie their functional diversity and adaptations.

Research at the Department of Embryology, 115 West University Parkway, Baltimore, Maryland 21210, is directed toward a better understanding of the molecular and cellular mechanisms underlying differentiation, growth, and morphogenesis and the manner in which these processes are coordinated in a number of developing systems, both normal and abnormal. The Carnegie Embryological Collection is now housed in the Carnegie Laboratories of Embryology, University of California, Davis, California 95616.
Director

William R. Brites

Stanford, California

Department of Plant Biology
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INTRODUCTION

During the past decade, a significant portion of the Department of Plant Biology's efforts has been devoted to learning how desert species tolerate the environmental extremes to which they are exposed. Until relatively recently, these studies have concentrated on high-temperature stress. Thus Björkman, Berry, and their associates have learned a great deal about the kinds of photosynthetic carbon pathways in plants adapted to high temperature, the components in a membrane that enable it to remain functional at high temperatures, and finally how those plants that can grow at wide ranges of temperature modify their biochemistry in response to changing temperature—a process known as acclimation.

The past year has brought a significant change in emphasis. Of the reports that follow, only one deals with acclimation and only two with plants from arid regions, of which neither addresses problems of temperature stress. However, there are three articles dealing with water stress and five with photoinhibition (damage to photosynthetic capacity occurring when more light is absorbed by a leaf than can be used by normal photosynthetic reactions). Photoinhibition can be induced by any factor restricting utilization of the reducing power generated by light-driven electron transport. Low temperature, limitation in CO₂ availability, and water stress can in combination with high light intensity all lead to photoinhibition and, as will become clear below, all ultimately produce similar kinds of damage. Since in arid regions any or all of these factors may obtain, a knowledge of their specific effects is crucial to complement our already extensive knowledge of high-temperature effects. Such considerations make the shift in research direction both logical and timely.

In singling out these studies on the effects of stress, there is no intention to slight other areas of research in the Department. Occasionally, however, there is a turning point in the Department's research or the emergence of a new theme which merits special mention. This year, the stress research provides such a case.

Photosynthesis

Our present computer capabilities have made certain kinds of spectral analysis almost routine for studying a wide range of problems. Brown and Schoch used the RESOL program (which resolves complex spectra into simple components) to gain substantial new information about the spectral components associated with the two photosystems involved in green plant photosynthesis. From chloroplasts of wheat and pea, they isolated both the light-harvesting chlorophyll-protein complex associated with photosystem II and the photosystem I pigment complex, and studied their spectra. These spectra could readily be modeled with the same four components that have served for spectra of intact chloroplasts in the past. However, the sum of components from the two preparations did not match the whole chloroplast spectrum. The results suggest the presence of a previously undescribed component, absorbing at 685 nm, which was apparently lost during the extraction procedure.

It is well known that when plants are grown under intermittent light—for example, 15 minutes of light every three hours—they form very little chlorophyll b and none of the light-harvesting chlorophyll-protein complex associated with photosystem II. It was therefore of considerable interest to compare spectra from chloroplasts of...
such plants with spectra from plants grown under normal light conditions. Schoch and Brown analyzed such spectra with the techniques just mentioned. They found that even without chlorophyll $b$ (which absorbs maximally in vivo at 650 nm) there was still a substantial amount of 650-nm absorption. This absorption must therefore arise from lower vibrational levels of the longer wavelength forms of chlorophyll $a$ still present. Intermittent light reduced the magnitude of both the 661-nm and 678-nm components by the same proportion, strengthening the hypothesis that both of these bands belong to the same pigment moiety, a form of chlorophyll $a$.

Although this work does not appear below in a formal report, Brown has made considerable progress in developing and extending techniques for isolation and characterization of a variety of chlorophyll-protein complexes, both from higher plants and from a series of microalgae not previously studied. Brown carried out this work while on sabbatical leave in Australia during the academic year 1980-1981. These new techniques, along with new experimental material for the various complexes, should substantially enhance her efforts to characterize these complexes in as unaltered a form as possible and to learn how they interact.

This past year there have been two other studies of the intrinsic properties of chloroplast photosynthetic machinery. It is now well known that light is required to activate several enzymes involved in carbon dioxide assimilation. Satoh has extended our knowledge of such photoactivation processes by showing that light is required to activate the enzyme ferredoxin NADP-reductase, a key enzyme in the transfer of electrons from photosystem I to NADP$^+$. Thus photoactivation, known to be required for several of the enzymes that catalyze photosynthetic carbon fixation, is also required of at least one enzyme mediating photosynthetic electron transport as well. The reaction probably involves a reduction of the enzyme.

Melis and Harvey continued studies started last year on the ratio of the two photosystem reaction centers, PS II/PS I, in plants grown in different spectral environments. Melis and Brown had previously shown, contrary to expectations and a great deal of dogma, that this ratio need not be unity but could be higher or lower. Melis and Harvey have now shown that any environment enriched for far-red light (which is preferentially absorbed by photosystem I) shows a significant increase in the number of photosystem II reaction centers relative to those of photosystem I. Such a shift would help keep the two photoreactions in balance in a far-red-enriched environment. Indeed, plants that grow in naturally shaded environments receive light enriched in far-red wavelengths (because of absorption of red light by chlorophyll in the plants overhead); as predicted, Melis and Harvey found enrichment in photosystem II reaction centers in such plants. Chloroplasts having a high ratio of photosystem II to photosystem I reaction centers appear relatively enriched in granal as opposed to stromal membranes. Thus differentiation at the photosystem level in response to far-red enrichment may be accompanied by morphological differentiation.

Over the years, there has been considerable debate about the role of chloroplasts in the guard cells of stomata. Recent reports have indicated that guard cells are unable to fix carbon dioxide photosynthetically. Since guard cells are the valves that regulate gas exchange in higher plants and since they are strongly affected by light, it is extremely important to determine the photoreactions of which they are capable. Zeiger, Armond, and Melis managed for the first time to obtain reasonable chloroplast preparations from guard cells without contamination by...
mesophyll chloroplasts. They simply used albino portions of the variegated leaves of *Chlorophytum comosum*. Zeiger and colleagues were then able to show that these chloroplasts have both photosystem I and photosystem II pigments; there is also an intact electron transport pathway between the two photosystems. Melis and Zeiger also obtained indirect evidence that guard cell chloroplasts can make ATP, and that CO₂ can apparently lead to ATP consumption. How CO₂ leads to such photosynthetic energy consumption remains a mystery, since guard cells evidently do not carry out conventional photosynthetic CO₂-fixation reactions.

As mentioned above, one way to learn about the effects of a given stress is to find out how the successful plants cope with it, an approach which has been very fruitful in temperature studies. To obtain good examples of resistance to water stress, Fork and Oquist might have traveled the well-known road to Death Valley. They chose instead some examples nearer at hand: mosses and liverworts inhabiting the surfaces of trunks and branches in the redwood forests near Stanford, and the red alga *Porphyra*, which lives on intertidal rocks along the seacoast. The mosses and liverworts become thoroughly desiccated during the long summer dry season, but can function again rapidly upon rehydration; *Porphyra* can become dehydrated with each low tide, and in the daytime, can be simultaneously scorched by the sun.

Fluorescence studies carried out at 77 K indicated that all of these plants lose the emission from photosystem II preferentially on desiccation. As reported last year, *Porphyra* simply diverts energy absorbed by photosystem II to photosystem I, as seen by a large increase in photosystem I fluorescence. When an alga isolated from the lichen *Cladonia implexa* was similarly studied, it revealed a small diversion of energy from photosystem II to I but mostly just a loss of photosystem II fluorescence. In the mosses and liverworts studied, loss of photosystem II fluorescence alone was observed with no evidence of energy diversion. In all cases, apparent damage to photosystem II was more severe when drying occurred in the light, suggesting that photosystem II is more susceptible to photodamage than photosystem I. For plants that normally become desiccated in deep shade, an energy diversion mechanism to protect photosystem II from photodamage is evidently not required, and the effects of desiccation are reversible.

Oquist and Fork also watched fluorescence changes at room temperature in drying *Porphyra*, since *Porphyra*, unlike most species, shows substantial photosystem I emission at room temperature. The room-temperature studies amply support conclusions from low-temperature investigations: upon drying, *Porphyra* shows a dramatic increase in energy transfer from photosystem II to I, and damage to photosystem II is significantly less if drying is carried out in the dark.

With a supply of *Porphyra* on hand, Fork, Oquist, and Hoch used fluorescence techniques to provide careful documentation that the long-wavelength fluorescence band from this alga, seen at room temperature, really does arise from photosystem I. If so, such documentation is important, since one can then use fluorescence techniques and physiological temperatures to study a host of reactions associated with photosystem I instead of descending to 77 K.

In a departure from their desiccation studies, Fork and Oquist followed the dark reoxidation of the primary electron acceptor for photosystem II at 77 K (by studying fluorescence). The dark reoxidation was biphasic, and could be resolved into two components with very different rate constants. This suggests that the photosystem II centers possess some heterogeneity. Since the light-induced reduction of the...
centers is also known to be biphasic, suggesting heterogeneity, the results of Fork and Öquist nicely complement the existing literature.

In the sole study dealing with thermal acclimation, Öquist and Fork, with Schoch and Malmberg, followed the relative ease with which various photosynthetic pigment-protein complexes could be solubilized by treating with detergent the membranes from the thermophilic alga *Synechococcus lividus* grown at two different temperatures. Attempted solubilization at a series of different temperatures revealed several effects of growth temperature. Most prominently, the photosystem I antenna pigment complex, CP$_{a1}$, was quite inaccessible to detergent below the phase transition temperature for the membrane lipids. Fork had previously shown that this phase transition temperature was higher when the plants were grown at the higher temperature.

The next five reports deal with different manifestations of the phenomenon of photoinhibition. In the first of these studies, Satoh and Fork investigated the effects of anaerobiosis on the photosynthetic chloroplasts from the green alga *Bryopsis*. Chloroplasts in excellent condition can readily be obtained from this organism, making it a particularly favorable material for studies of injury effects. It is known that oxygen can serve as a competing electron acceptor for photosynthetically generated electrons. Satoh and Fork reasoned that if this was so, oxygen could actually act as a protectant when conditions leading to photoinhibition prevailed. This prediction was verified by the demonstration that even under very low light fluence rates, photoinhibitory damage could be detected if oxygen was excluded.

Last year, Powles, Berry, and Björkman showed that at low temperatures, for example at 6°C, high light intensity led to photoinhibition in the chilling-sensitive bean, *Phaseolus vulgaris*. This year, Fork, Öquist, and Powles investigated the effects of such photoinhibitory treatment on photosystems I and II. Photoinhibitory treatment clearly led to the accumulation of inactive photosystem II reaction centers. The phenomenon was detectable both with intact leaves (fluorescence measured from the upper leaf surface) and with isolated chloroplasts. However, the fluorescence measurements of the leaves showed the effect much more dramatically, indicating that chloroplasts in the upper surface of the leaf are the most damaged. The extracted chloroplasts, of course, represent chloroplasts from throughout the leaf, not just the upper surface.

**Physiological Ecology**

The distinction between *Photosynthesis* and *Physiological Ecology* at this point represents mostly a change in laboratories (and in experimental plants). In both areas, the experimental approach still includes fluorescence measurements; both groups ask, as an underlying question, what are the consequences of stress?

There is even a substantial overlap of authorship: Björkman, Powles, Fork, and Öquist investigated the effects of water stress on photosynthetic performance of the shrub *Nerium oleander* in full sunlight and under shaded conditions. They found that water stress damaged whole-chain electron transport far more than it damaged photosystem I electron transport, and they verified that there was a strong effect of water stress on photosystem II. Fluorescence measurements indicated that water stress leads to photoinhibitory inactivation of photosystem II reaction centers precisely as it does for bean under chilling conditions (see above).

The final two papers on photoinhibition take a rather different turn. The mobile laboratory also took a different turn, heading not for Death Valley but
for San Mateo County Redwood Memorial Park, on the west side of the Santa Cruz Mountains. There, on the forest floor, in a deeply shaded environment, can be found a variety of small plants, among them *Oxalis oregana*. The leaves of this species consist of three leaflets, each attached to a vertical petiole by its own pulvinus. By changing the relative turgor in different groups of cells, the pulvini regulate the position of the leaflets with respect to the petiole over a 90° range. Normally, the light intensity incident on these plants is about 0.5% that of full sunlight. However, occasional sunflecks pass over them, and may persist for an hour or more. On arrival of the sunfleck, the light intensity may increase as much as 200-fold.

Björkman and Powles found that when such a jump occurs, the leaves fold rapidly downward, exposing only a small area to the full sunlight. The folding response was remarkably rapid—the lag period was as brief as six seconds, and folding was complete within six minutes. Recovery after passage of the sunfleck was slower, showing about a 10-minute lag and requiring another 35 minutes for restoration of the original horizontal leaflet position.

Light intensities of 15–30% of full sunlight are sufficient to trigger the folding response. Above the threshold intensities, the lag period becomes shorter and the angular rate of position change more rapid. It is the pulvini themselves that sense the changes in incident light intensity, and the effective wavelengths are in the blue. Perhaps the photoreceptor is similar to those for solar tracking by leaves and for corn phototropism discussed elsewhere in this Report.

By making gas exchange measurements with the mobile laboratory, Powles and Björkman were able to demonstrate convincingly that the folding reaction forestalled photoinhibitory damage. If the *Oxalis* leaves were constrained from folding when a sunfleck passed by, substantial photoinhibition occurred, requiring several hours for recovery. Curiously, the folding reaction scarcely affected photosynthesis rates. Photosynthesis in these shade plants is normally saturated at very low light intensities. Hence, even when they are folded and presenting only a very small area to the sun, there is still sufficient light in the sunfleck to saturate their photosynthetic machinery.

Two other studies complete the year's contributions in physiological ecology. In the first of these, Seemann, Tepperman, and Berry report on continuation of work described last year on the photosynthetic capacities of some of the annual plants from Death Valley. These plants by and large have photosynthetic capacities well above those of most temperate species: species of *Camissonia* show the highest values recorded to date. In most cases, the very high photosynthetic capacity could be accounted for by unusually high levels of the enzymes required for fixing CO$_2$ into organic matter, most notably the enzyme that initially binds the CO$_2$ for subsequent photosynthetic steps, ribulose-1,5-bisphosphate carboxylase-oxygenase. (After years of struggling with difficult and uneuphonious acronyms for this enzyme, workers have finally suggested the nickname “Rubisco.” At least it is pronounceable.) With *Camissonia*, however, there is another important difference. The Rubisco from *Camissonia* has a specific activity significantly higher than that of any other species studied. Thus, not only is there more of it, but it is more efficient. The basis for this greater efficiency is at present unknown.

The final study in this section concerns the phenomenon of sun-tracking. The leaves of certain species have the capacity to follow the sun with great precision, hence maximizing the interception of incoming solar energy.
Koller has been studying solar tracking in two such species—*Lavatera cretica* and *Malva parviflora*—by means of a tracking device which permits him to sustain a given angle for incident light with respect to the leaf surface and to study the tracking as a steady-state phenomenon. Leaves of both species can undergo reorientation at a high velocity for as much as an hour, and the rates can far exceed the earth's rotation (15° per hour). Koller has recorded rates as high as 90° per hour, and has found that leaves can continue to coast for some time in darkness following driving illumination. The stimulus itself was shown earlier by Koller and Schwartz to be perceived not by the pulvinus itself, as is the case for the leaf-folding *Oxalis* response, but rather by the major leaf veins. Some influence is then transmitted down the veins to the pulvinus, which is located where the leaf blade is attached to the petiole. Turgor changes in the pulvinus mediate most of the leaf movement. There is evidence, however, that the petiole itself can also participate; functional differentiation of pulvinus from petiole is hence incomplete. The leaves can reorient either toward or away from the stem, and both processes probably involve active transport phenomena. Leaf tracking is yet another process regulated by a blue light photoreceptor.

**Molecular Biology**

Several years ago, a Trustees' Visiting Committee referred to the tools of plant molecular biology as highly promising but still in need of substantial sharpening. Comparison of the reports from the Molecular Biology group five years ago with those in this Report will indicate immediately the dramatic extent to which sharpening has occurred. Murray and Thompson, continuing their detailed analysis of the pea's genome structure, have inquired as to the nature of the DNA sequences in the neighborhood of those sequences that actually code for messenger RNA. Surprisingly, the coding sequences are enriched in DNA regions where there is a paucity of highly repetitive DNA, and are depleted in regions wherein highly repetitive elements abound. Those repeats which co-isolate with gene sequences in these experiments have low copy numbers and show significantly lower sequence divergence than the average repeat. Murray and Thompson have thus been able to gain fairly specific information about the DNA sequences close to those regions actually functioning as genes in the sense of coding for proteins, though the DNA constituting the genes in peas is probably less than 1% of the total!

Murray and Thompson also showed that when coding sequences are being expressed, they are in a special conformation with respect to the associated protein complex. This renders the coding sequences more susceptible to degradation by certain DNA-hydrolyzing enzymes. While completely unexpected on the basis of studies with yeast, this observation confirms for plants what is already fairly well documented for animal systems, and it demonstrates for the first time that such an altered conformation may persist in a truly quiescent tissue. (The source of material was unhydrated wheat germ.)

Everett, Jorgensen, and Thompson have used cloning techniques to follow the appearance and increase in amount of two messenger RNAs during the greening of pea buds. These two species appear gradually over a period of about 72 hours in the light, after an initial lag of over 12 hours. Prior treatment with a small amount of red light eliminated this lag, so the level of the two RNAs in plants so treated is as high after 24 hours in the light as in controls after 72 hours. Since the red light effect is reversible by far red, the phenomenon is evidently under phyto-
chrome control. The function of the proteins produced from these messengers is unknown, but it is reasonable to expect that they may somehow be involved in photosynthesis.

Cuellar and Thompson report on the fine structural organization of repetitive DNA from pea. They employed three clones produced during studies described last year; the clones contain common repetitive elements, but these are interspersed with completely unrelated sequences. Furthermore, certain portions of the total pea DNA hybridize with only one of the clones, indicating that this clone contains sequences occurring in neither of the other two. Cross-hybridization experiments indicated that where homology exists between clones, the regions of homology are short. Furthermore, each clone contains substantially more repetitive DNA than that found in the common elements. Sequences in genomic DNA related to those in the clones show a range of repetition frequencies and both a tandem and interspersed arrangement. These experiments demonstrate elegant fine tuning to document for specific fragments of DNA organizational properties until now deducible only indirectly from studies of the whole genome or at best large fractions of it.

Finally, Palmer and Thompson have produced some detailed studies on the linear organization of the chloroplast genomes from several species. Mung bean chloroplast DNA, like that of many other plants studied in several different laboratories, possesses two copies of an ~23,000-base segment of DNA, one of which is inverted with respect to the other. It turns out that both pea and broad bean lack this inverted repeat. The linear sequences of homologous regions of DNA between pea and mung bean are very different, with one sequence badly scrambled with respect to the other. By contrast, the linear order of sequences in spinach, cucumber, and petunia—containing the inverted repeat—is remarkably similar to that in mung bean, despite the much greater evolutionary distance of these plants from mung bean. Broad bean shows scrambling not only with respect to mung bean, spinach, cucumber, and petunia, but also with respect to pea. Evidently, the inverted repeat confers some evolutionary stability to the linear arrangement of genetic material in the chloroplast DNA. In the absence of the inverted repeat, all sorts of inversions and translocations have been allowed to occur. The way in which this stability is conferred by the inverted repeat is completely unknown. It is clear, however, that the chloroplast genome may provide a very sharp tool for the study of plant evolution.

**Photomorphogenesis**

Although our formal discussion of research on photomorphogenesis begins here, we have already mentioned blue light photoreceptors twice and phytochrome once. This seeming anomaly indicates two things: (1) the artificiality of the current divisions of the Department, and (2) more important, the kind of cross-fertilization that frequently occurs between divisions. The studies of photoinhibition provide another striking example of this phenomenon.

Gorton has continued her efforts to unravel the complexities of phytochrome responses in light-grown plants. She showed previously that responses of corn to end-of-day far-red light exhibited certain anomalies not explainable by traditional models of phytochrome action. This year she extended these studies to several other plants, including oat, mung bean, and sunflower. The anomalies, which appeared in the oat studies but not in those with sunflower or mung bean, are evidently not ubiquitous. Nevertheless, they are forcing significant changes in our concept of phytochrome action, and they
will have to be accounted for in any future models.

Mandoli has continued her studies of the responses of dark-grown oat seedlings to red light by investigating in detail the site(s) of photosensitivity for the responses of the mesocotyl below the node and the coleoptile above. Surprisingly, she found maximum sensitivity to red light not where most of the phytochrome is found, namely in the node itself and in the coleoptile tip, but rather just below the node (for the mesocotyl response) or both just below and just above the node (for the coleoptile response). She also discovered that there is significant light piping through both organs, with fairly sharp attenuation across the node. By quantifying this light piping, she was able to account in part for the dose-response curve on the basis of reactions of the two photoreceptive sites to light directly absorbed at the sites as well as that piped there from other regions of the seedling. These findings are of considerable importance, particularly in view of the magnitude of the piping and the fact that the possibility of light piping has been virtually ignored in all previous studies.

Walton and Shinkle have made significant progress in understanding the mechanism by which the corn mesocotyl responds to low fluences of red light. Walton had already shown that red light strongly reduced the amount of the glucan synthetase localized in the Golgi in corn mesocotyls. In the present work, he and Shinkle have shown that the fluence-response curve for reduction of the glucan synthetase activity was almost identical to that for mesocotyl growth suppression. Both effects were somewhat reversible by far-red light, and both could be partially potentiated by far-red light alone. Auxin, which Vanderhoef and Briggs showed some years ago would antidote the growth suppression caused by red light, also antidotes the decline in glucan synthetase activity. Hence both growth and glucan synthetase activity may well be modulated by a red-light-regulated supply of auxin.

Leong, continuing his detailed characterization of the light-sensitive flavin–cytochrome b complex in corn membrane preparations, has shown its association with a particular ATPase, quantified the amount of flavin, and measured the midpoint potential of the cytochrome. Since the system is almost certainly located in the plasma membrane, knowledge of these properties should provide considerable insight into the functional nature of the membrane system limiting the plant cell.

Until this year, there was little evidence linking this pigment complex with photoreception for phototropism in corn, save that both light-induced cytochrome reduction and phototropism were inducible by blue light. Recently, however, Leong has made considerable progress by using a new class of herbicides based on a diphenyl ether structure. He showed that these compounds (which require light for their herbicidal action) significantly enhance photoreduction of the cytochrome. They also significantly sensitize the phototropic response of etiolated oat seedlings! These experiments substantially strengthen the case that the flavin–cytochrome complex does indeed serve as the photoreceptor for phototropism. Further, the herbicides provide a specific probe which may help us to elucidate the function of this light-sensitive electron transport chain in the plant plasma membrane.

The final report brings us full circle, back to the computer and the RESOL program. Schoch, Gorton, and Briggs reinvestigated the dramatic spectral changes, first described by Shibata in this Department some 25 years ago, which occur after protochlorophyll phototransformation in the leaves of dark-grown seedlings. These changes involve a shift in the absorption maximum of the newly formed chlorophyll from about 684 nm to about 670 nm.
Schoch and her co-workers were able to show (1) that carotenoids, despite their extensive presence in the proplastids, were in no way involved in the shift, (2) that reduction of the double bonds of the long aliphatic tail of chlorophyll was likewise not involved, and (3) that the shift probably did not involve proteolytic cleavage of the apoprotein. While we are still unaware of the physical or chemical changes behind the shift, we can eliminate two possibilities and consider a third unlikely. The findings briefly described in these few paragraphs are far better documented in the reports that follow, and virtually all of them either have appeared or will soon appear in the published literature. They represent a fine record of high-caliber productivity.

Winslow R. Briggs

SPECTRAL ANALYSES OF CHLOROPHYLL-PROTEIN COMPLEXES

Jeanette S. Brown and Siegrid Schoch

Investigation of the state of chlorophyll in photosynthetic membranes is of major interest to the researchers of this Department. Some years ago, French (French et al., 1972) proposed that chlorophyll a exists in four major and several minor different states in vivo. This hypothesis was based on the results of curve analyses of absorption spectra of many plant species. For the most part, the spectra were from particles prepared by forcing chloroplasts or algae through the needle-valve of a French pressure cell, a treatment which does not usually alter the nature of the pigment. Procedures have recently been developed to isolate the more elementary chlorophyll-protein complexes yet retaining the pigments in their native state.

Our analyses of complex spectra have been greatly facilitated this year by two major improvements. A shaft encoder and microprocessor connected to our Cary 17 spectrophotometer make possible the transfer of absorption spectra to a Hewlett-Packard computer system. In addition, Mr. Glenn Ford has slightly modified the RESOL (Year Book 67, 536-546) program to run on this computer. It takes only 15-20 min to perform a complete analysis of a spectrum having 2048 digital points and a maximum of nine components, allowing ten iterations. Because each curve deconvolution is relatively brief and inexpensive, we are able to analyze each spectrum more carefully and to compare many more spectra than previously (Year Book 70, 487-495).

With these improvements in analytical procedures, we have begun a program to analyze spectra of native chlorophyll-protein complexes from many sources. We expect to test French's original hypothesis further and to extend our knowledge of the way chlorophyll functions in photosynthesis.

We isolated a light-harvesting chlorophyll a-b-protein (LHCP) complex and photosystem I (PS I) complex from both wheat and pea chloroplasts using Triton X-100 according to the procedure of Burke et al. (1978). Details of these experiments and results of the curve analyses of the absorption spectra are given in Brown and Schoch (1981).

All of the spectra could best be fitted with the same four major component bands found by French et al. (1972). As also observed by these same authors, the half-band widths of the components in the PS I complexes were consistently greater (by 1–3 nm) than in the LHCP. The amounts of the 660-nm and 678-nm components were propor-
tionately the same in all the spectra, suggesting that exciton interaction may cause an absorption band splitting between these two components.

The addition of a narrow band at 675–676 nm comprising about 1% of the total absorption remarkably improved the overall curve fitting of the LHCP and chloroplast spectra without changing the other bands, but it did not improve the analysis of the PS I spectra. The significance or possible function of this small component is unknown.

It is known from other kinds of studies (Thornber et al., 1977) that LHCP comprises about 50–60% of the total chlorophyll in higher plants, and the PS I fraction about 10–25%. Although individual analyses of the wheat or pea LHCP and PS I spectra, compared to analyses of spectra of chloroplast particles, indicated no destruction of chlorophyll forms by the fractionation procedure, we could not fit the chloroplast spectra with the sum of spectra of these two fractions in any reasonable proportion. This result indicated that at least one major chlorophyll fraction was not recovered by our procedure. We calculated the shape of this missing fraction, which has a major component band absorbing near 684 nm. Other circumstantial evidence (Delepelaire and Chua, 1979; Waldron and Anderson, 1979) suggests that this missing fraction may contain the chlorophyll a antenna of photosystem II. These results indicate that spectral analysis of isolated chlorophyll-protein complexes will continue to aid in understanding the state of chlorophyll in photosynthetic membranes.

REFERENCES

COMPARATIVE SPECTROSCOPY OF CHLOROPHYLL a IN DAYLIGHT- AND INTERMITTENT-LIGHT-GROWN PLANTS
Siegrid Schoch and Jeanette S. Brown

Striking differences have been observed in the fluorescence emission and excitation spectra of chlorophyll a between normally greened plants and plants grown under light-limiting conditions (Year Book 79, 176–179; Davis et al., 1976; Strasser and Butler, 1977a,b; Mullet et al., 1980). Plants developed under light-limiting conditions form very little chlorophyll b and essentially none of the light-harvesting chlorophyll a-b protein (LHCP) (Argyroudi-Akoyunoglou and Akoyunoglou, 1970; Ryberg et al., 1980). Both photosystems are fully active in these plants, and they offer the possibility of measuring the spectroscopic properties of chlorophyll a without interference from the normally large proportion of LHCP. Last year, we compared the fluorescence of fully green, daylight-grown (DL) and intermittent-light-grown (Iml), young wheat and pea plants. Now, with the new computer connection to the Cary spectrophotometer (see Brown and Schoch, this Report), we have extended these studies to absorbance measurements.

Wheat seedlings were cultivated in the same manner as last year except that the Iml-grown plants were illuminated for 15 min out of 3 hours
darkness instead of 2 hours. This change decreased the possibility of LHCP formation even more. Chloroplasts were also prepared in the same manner as last year from both DL-grown and ImL-grown, seven-day-old seedlings.

Absorption spectra were measured at 89 K in a Cary spectrophotometer equipped with a scattering transmission attachment and Dewar cell holder (Brown and Schoch, 1981). Computer analysis of these spectra was also carried out, as described elsewhere (Brown and Schoch, 1981).

Resolved spectra of DL-grown and ImL-grown wheat chloroplasts are shown in Fig. 1. The data from these two analyses are given in the first two columns of Table 1. The sum of components listed in Table 1 for the DL chloroplasts gave an excellent, relatively unique fit to the experimental spectrum, but several sets of somewhat different components could be used to fit the ImL spectrum with nearly the same precision. The last column of Table 1 lists the components resolved from a spectrum of wheat LHCP prepared from green chloroplasts (Brown and Schoch, 1981).

Figure 2 shows a difference spectrum of DL-minus-ImL spectra. These spectra were normalized before subtraction to make the greatest difference near 650 nm, the chlorophyll b region, because we know that the amount of chlorophyll b is indeed much lower in the ImL chloroplasts. With this qualification, the spectra had equal absorbance at 668 nm. The green chloroplasts had more absorption near 660, 678, and 690 nm, as well as in the chlorophyll b region, whereas the ImL plastids showed a unique peak at 683 nm.

Although the choice is admittedly somewhat subjective, we prefer to compare the third analysis (Table 1, column 4) of the ImL spectra with that of the DL chloroplasts because a relatively close (error \( \times 16 \)) fit was obtained with only seven components.

The significant differences were as follows:

1. The component thought to represent chlorophyll b near 650 nm is not as reduced in the ImL spectra (from 21 to 17) as might be expected. If the 639-nm band is included with the 650-nm band, the difference becomes greater (from 41 to 26 or from 55 to 26) when compared with the chlorophyll b-enriched LHCP. The results suggest that up to half of the 650-nm component in LHCP-containing chloroplasts may include absorption by lower vibrational levels of the longer-wavelength chlorophyll a components as well as by chlorophyll b.

2. The proportions of the 661-nm and 678-nm components were the same in DL and ImL spectra, while the 670-nm component increased in the ImL spectrum. This observation supports the suggestion of exciton interaction between the 661-nm and 678-nm forms of chlorophyll (Brown and Schoch, 1981). Also, as predicted, the addition of a small 676-nm band did not improve the fit in ImL spectra.

3. A 683-nm component in the ImL spectrum is prominent in the difference spectrum (Fig. 2) and is different from the 683 band in normal green spectra. As shown in Table 1, this ImL 683 band has a much narrower width (by 5 nm) and is over 50% more Lorentzian in shape. A similar 683-nm component was previously observed in spectra of an isolated chlorophyll a–protein complex from Euglena (Year Book 73, 694–706). Fluorescence excitation spectra of ImL chloroplasts but not of DL chloroplasts (Year Book 79, 176–179) show a peak near 683 nm. Circumstantial evidence links this 683 component with photosystem II (Delepelaire and Chua, 1979). Perhaps this special band is masked by antenna chlorophyll in fully greened plastids.

4. There is little or no absorption beyond 685-nm by ImL-grown plants. Also, the 689-nm component in the ImL spectrum was 87% Lorentzian
**TABLE 1. RESOL Analyses of Daylight-Grown ($\text{Chl } a/b = 2.6$) and ImL-Grown ($\text{Chl } a/b = 6.4$) Wheat Chloroplast Spectra**

<table>
<thead>
<tr>
<th>Component Band Maxima</th>
<th>650-720 nm</th>
<th>630-720 nm</th>
<th>630-720 nm</th>
<th>LHCP</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Daylight</td>
<td>ImL</td>
<td>ImL</td>
<td>ImL</td>
</tr>
<tr>
<td></td>
<td>S.E. = 0.24</td>
<td>S.E. = 0.25</td>
<td>S.E. = 0.28</td>
<td>S.E. = 0.29</td>
</tr>
<tr>
<td></td>
<td>Error $\times 19$</td>
<td>Error $\times 13$</td>
<td>Error $\times 16$</td>
<td>Error $\times 16$</td>
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<tr>
<td>639</td>
<td>20 (83)</td>
<td>14</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>649-651</td>
<td>21 (88)</td>
<td>14</td>
<td>16 (100)</td>
<td>14</td>
</tr>
<tr>
<td>661-662</td>
<td>23 (91)</td>
<td>13</td>
<td>22 (100)</td>
<td>14</td>
</tr>
<tr>
<td>670-671</td>
<td>26 (92)</td>
<td>12</td>
<td>28 (83)</td>
<td>12</td>
</tr>
<tr>
<td>676</td>
<td>1 (100)</td>
<td>4</td>
<td>2 (100)</td>
<td>6</td>
</tr>
<tr>
<td>678-679</td>
<td>29 (98)</td>
<td>12</td>
<td>30 (100)</td>
<td>13</td>
</tr>
<tr>
<td>683-684</td>
<td>14 (100)</td>
<td>13</td>
<td>11 (88)</td>
<td>9</td>
</tr>
<tr>
<td>689-693</td>
<td>4 (100)</td>
<td>12</td>
<td>4 (84)</td>
<td>9</td>
</tr>
<tr>
<td>700</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>705</td>
<td>4 (100)</td>
<td>17</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

*The S.E. as % of peak height, error amplification factor, and wavelength range are given for each analysis. The area of each component is expressed as % of the total area under the 660-705 nm bands. The (%G) is the percentage Gaussian shape of a Gaussian-Lorentzian mixture. Band maxima and widths in nm.*
compared to 100% Gaussian in the DL spectrum. This difference in and/or lack of long-wavelength-absorbing forms of chlorophyll can account for the decrease in long-wavelength fluorescence emission observed earlier (Year Book 79, 176-179; Davis et al., 1976; Mullet et al., 1980).

Fig. 1. Absorption spectra at 89 K of wheat chloroplasts grown under daylight (A) or intermittent light (B). The measured data are plotted as points, while the line through them is the sum of the component curves, the characteristics of which are given in Table 1. The error of fit at each point is shown below each spectrum on a scale with the designated magnification.
Fig. 2. Differences between absorption spectra of green and ImL chloroplasts from wheat. The spectra were normalized at 668 nm before subtraction.

These four major differences, found by comparing detailed analyses of chlorophyll spectra of the same plant species grown under different light conditions, show the value of such studies for our eventual understanding of the native state of chlorophyll and how it functions in photosynthesis.

REFERENCES


FLUORESCENCE INDUCTION AND PHOTOACTIVATION OF FERREDOXIN-NADP+ REDUCTASE IN Bryopsis CHLOROPLASTS

Kazuhiko Satoh

It is well known that when dark-adapted algal cells or leaves are suddenly illuminated with strong light, the yield of chlorophyll fluorescence shows several transients before it reaches a steady-state level (Kautsky and Appel, 1960; Govindjee and Papa­georgiou, 1971; Katoh et al., 1975). The most pronounced transient in the induction of chlorophyll fluorescence is the DPS, which occurs concomitantly with the induction of cytochrome f photooxidation. Satoh et al. (1977) explained this DPS transient and the induction of cytochrome f oxidation as reflecting a photoactivation on the reducing side of photosystem I.

Recently, Satoh and Katoh (1980), using intact spinach chloroplasts, observed that nitrite also decreased the DPS transient. Nitrite accepts electrons from reduced ferredoxin through nitrite reductase (Heber and Purczeld, 1977). The results indicate that the dark inactivated site is on the reducing side of ferredoxin, because nitrite reduction bypassed the inactivated site. Satoh and Katoh (1980) also showed that oxalacetate and 3-phosphoglycerate, both of which accept electrons from NADPH, had little effect on the DPS transient. From measurements of the activity of glyceraldehyde 3-phosphate dehydrogenase and malic dehydrogenase, they concluded that the regulation site was located before the reduction of NADP+. Ferredoxin-NADP+ reductase is the only enzyme occurring between ferredoxin and NADP+ and, therefore, ferredoxin-NADP+ reductase might be the enzyme subject to changes of activity during a dark-light transition.

In this work, we used isolated intact
chloroplasts from *Bryopsis* and established conditions under which the envelopes of the chloroplasts became leaky without affecting the DPS$_1$ transient. Under such conditions, we were able to observe the change in the activity of the enzyme ferredoxin-NADP$^+$ reductase during the dark-to-light transition of the chloroplasts. We also found that reduction of the enzyme might induce the photoactivation and that oxygen acted as a principal electron acceptor after the photoactivation had taken place.

**RESULTS**

Studying the cause of the DPS$_1$ transient was difficult because the transient could be observed only in cells or in intact chloroplasts which had barriers to almost all ions and even small molecules. Therefore, we tried to get leaky chloroplasts by changing the osmolarity of the reaction medium. Figure 3 shows the rates of CO$_2$ fixation and NADP$^+$-Hill reaction at various concentrations of sorbitol in the reaction mixture. In the case of the NADP$^+$-Hill reaction, 1 mM NADP$^+$ was added to the reaction mixture and absorbance changes at 340 nm were recorded. At 1.0 M sorbitol, *Bryopsis* chloroplasts showed a high rate of CO$_2$ fixation but no NADP$^+$-Hill reaction. This is because NADP$^+$ cannot penetrate into the chloroplasts in intact *Bryopsis* chloroplasts. The photo-reduction of intrinsic NADP$^+$ was probably too small to be measured by our technique. Upon lowering the concentration of sorbitol, the rate of the CO$_2$ fixation reaction was decreased. Concomitantly, the rate of the NADP$^+$-Hill reaction increased. At 0.25 M sorbitol, the CO$_2$ fixation was greatly depressed and the rate of NADP$^+$-Hill reaction reached a relatively high level. The increase of NADP$^+$-Hill reaction and the decrease of the CO$_2$ fixation show that the envelopes of the chloroplasts became leaky only to small molecules such as NADP$^+$, and that large molecules such as ferredoxin may have remained in the chloroplasts, thus explaining the high rate of the NADP$^+$-Hill reaction at low concentrations of sorbitol.

Figure 4 shows the time course of chlorophyll fluorescence at various concentrations of sorbitol. The marked DPS$_1$ transient remained unaffected at a concentration of 0.25 M sorbitol (Fig. 4b) but it became less pronounced at concentrations of 0.1 M or less (Fig. 4c,d). The persistence of the DPS$_1$ transient (despite the loss of the CO$_2$ fixation at low concentrations of sorbitol) supports the suggestion that the CO$_2$ fixation reaction is not required for the DPS$_1$ transient.

Figure 4 shows that addition of 1 mM NADP$^+$ had no significant effect on the DPS$_1$ transient at 0.25 M sorbitol, suggesting that the availability of NADP$^+$ is not a requirement for the DPS$_1$. Only when the sorbitol concentration was lowered below 0.1 M did the effect of NADP$^+$ become noticeable (Fig. 4f,g). In the absence of sorbitol, NADP$^+$ markedly decreased the extent of chlorophyll fluorescence, indicating that the functional integrity of the system may have been damaged.

Ferredoxin-NADP$^+$ reductase is known to catalyze the reduction of DCIP by NADPH in the dark (Shin, 1971). Therefore, if this enzyme changes its activity in the dark and light conditions, the diaphorase activity may also change according to the dark-to-light transition of the chloroplasts. The diaphorase activities were measured at two sorbitol concentrations, 0.25 M and 0 M. At 0.25 M, a marked difference in the rate was observed in dark- and light-adapted chloroplasts. But at 0 M sorbitol, little difference was observed (Table 2).

To elucidate the mechanism of the photoactivation, we measured the effects of incubation of the chloroplasts in the dark with reductants at 0.25 M sorbitol concentration (Fig. 5). The reductants were removed by two cen-
Fig. 3. Effects of concentration of sorbitol in the reaction medium on the rate of photosynthesis and NADP⁺-Hill reaction in *Bryopsis* chloroplasts. The reaction mixture contained in 2 ml, 50 mM HEPES (pH 7.5), 11 mM MgCl₂, 1 mM MnCl₂, 2 mM NaNO₃, 2 mM EDTA, and various concentrations of sorbitol, as indicated in the figure. Chlorophyll concentrations were 26.2 μg/ml in the photosynthesis measurements (circles) and 10.5 μg/ml in the NADP⁺-Hill reaction measurements (triangles).

The time course of chlorophyll fluorescence in the chloroplasts that had been incubated with ascorbate (Fig. 5b) was similar to that in control chloroplasts which had been incubated with no reductant (Fig. 5a). Incubation of the chloroplasts with dithiothreitol slightly decreased the DPS₁ transient (Fig. 5c). A dramatic effect was obtained by the addition of dithionite (Fig. 5d). Incubation of the chloroplasts with dithionite largely eliminated the DPS₁ transient of chlorophyll fluorescence, indicating that the photoactivation process had already taken place. These results show that the reduction of a certain substance, which can be reduced by dithionite and partly by dithiothreitol but not by ascorbate (i.e., ferredoxin-NADP⁺ re-
TABLE 2. Diaphorase Activities in Dark- and Light-Adapted Chloroplasts.

<table>
<thead>
<tr>
<th>Sorbitol Concentration</th>
<th>Dark-Adapted</th>
<th>Light-Adapted</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25 M</td>
<td>3.0 (±0.5)*</td>
<td>5.5 (±0.9)</td>
</tr>
<tr>
<td>0.25 M</td>
<td>7.4 (±1.1)</td>
<td>7.8 (±1.0)</td>
</tr>
</tbody>
</table>

*Numbers in parentheses are standard deviations. Each value is the average of five measurements. Chlorophyll concentration was 14.8 μg/ml. Other conditions were the same as in Fig. 3.

Fig. 4. Effects of concentration of sorbitol and addition of NADP+ on the DPS1 transient of chlorophyll fluorescence: (a) 1.0 M sorbitol; (b) and (e) 0.25 M sorbitol; (c) and (f) 0.1 M sorbitol; (d) and (g) 0 M sorbitol. 1.0 mM NADP+ was also added in (e), (f), and (g). The intensity of the excitation light was 2.0 × 10^4 erg cm^-2 s^-1. Chlorophyll concentration was 5.41 μg/ml. Other conditions were the same as in Fig. 3.

Fig. 5. Time courses of chlorophyll fluorescence after incubation of the chloroplasts with ascorbate, dithiothreitol, or dithionite at 0.25 M sorbitol. Chloroplasts were incubated for 5 min with no reductant (a), with 10 mM ascorbate (b), with 10 mM dithiothreitol (c), or with 5 mM dithionite (d). The incubation medium contained 0.25 M sorbitol, 50 mM HEPES (pH 7.5), 2 mM EDTA, 11 mM MgCl2, 1 mM MnCl2, 2 mM NaN3. The reductants were removed by washing the chloroplasts with two centrifugations (see text). Chlorophyll concentrations were 2.86, 2.44, 2.59, and 2.31 μg/ml for curves a, b, c, and d, respectively. Other conditions were the same as in Fig. 4.

Fig. 3. (a) 1.0 M sorbitol; (b) and (e) 0.25 M sorbitol; (c) and (f) 0.1 M sorbitol; (d) and (g) 0 M sorbitol. 1.0 mM NADP+ was added in (e), (f), and (g). The intensity of the excitation light was 2.0 × 10^4 erg cm^-2 s^-1. Chlorophyll concentration was 5.41 μg/ml. Other conditions were the same as in Fig. 3.

The photoactivation process is also known to be related to oxygen. In the absence of oxygen, the DPS1 transient was inhibited (Bannister and Rice, 1968). Oxygen may participate in the photoactivation process in two ways. First, it may act as a mediator; in other words, oxygen may be necessary for the process itself. Second, it may act as an electron acceptor after the photoactivation process has taken place. In this case oxygen is not necessary for the process itself. Therefore, we tested whether the photoactivation process would occur in the absence of oxygen. Chloroplasts were preilluminated for 10 sec in the absence of oxygen (in the presence of 5 mM dithionite) and then washed twice by centrifugation to remove the dithionite. The fluorescence...
time course of the chloroplasts showed no DPS₁ transient, indicating that the photoactivation process had already taken place (data not shown). Addition of dithionite without preillumination had little effect on the DPS₁ transient in 1.0 M sorbitol. These data show clearly that oxygen is not necessary for the photoactivation process itself, thus eliminating the first possibility.

In order to test whether oxygen acts as an electron acceptor after chloroplasts are photoactivated, the yield of chlorophyll fluorescence was measured under anaerobic conditions after photoactivation. In the presence of oxygen and after the photoactivation, electrons flow rapidly, Q remains oxidized and, therefore, the fluorescence yield remains low (Fig. 6b). In the absence of oxygen, however, the extent of chlorophyll fluorescence reached higher levels, showing that Q was largely reduced in photoactivated chloroplasts (Fig. 6c). The extent and time course of this fluorescence change was the same as those for nonphotoactivated chloroplasts under anaerobic conditions, indicating that the difference between the yields of chlorophyll fluorescence in Fig. 6b and 6c is not due to the quenching of chlorophyll fluorescence by oxygen (data not shown). These data show that even after the photoactivation of ferredoxin-NADP⁺ reductase, electrons do not flow rapidly under anaerobic conditions, and that oxygen is acting as an electron acceptor.

REFERENCES

The use of a sensitive spectrophotometric method has recently allowed workers to determine the stoichiometric ratio of PS II to PS I reaction centers in different photosynthetic membranes (Melis and Brown, 1980). It was found that considerable differences in this ratio existed between membranes from the grana and stroma regions of chloroplasts. Grana membranes contained most of the chlorophyll b, photosystem II centers, and plastoquinone of the chloroplast. Stroma membranes contained primarily photosystem I reaction centers. In the present investigation, we used different spectral qualities of light during plant growth to probe the relationship between certain chloroplast structural and functional parameters. We found that the quality of the absorbed light controlled the relative concentrations of system II and system I reaction center complexes and, in addition, it controlled the chloroplast membrane differentiation into grana and stroma lamellae.

Results

A first group of experiments was conducted by growing *Pisum* plants in the laboratory under continuous illumination of different spectral qualities. Table 3 compares the results obtained with *Pisum* chloroplasts developed in the greenhouse (control), under far-red-deficient (cool white fluorescent) light, or far-red-enriched (incandescent) light. Greenhouse and far-red-deficient plants showed minor quantitative differences in the Chl a/Chl b ratio, and in their Q and P_{700} contents. However, chloroplasts developed in far-red-enriched light showed a significant quantitative difference from those developed in far-red-deficient light in the Q and P_{700} contents: on a chlorophyll basis we consistently measured an enrichment in the far-red-developed chloroplasts in the amount of Q present and a simultaneous decrease in the concentration of P_{700}. Such alterations shifted the Q/P_{700} ratio from the average value of 1.8 to 2.8, representing an overall change of approximately 60%.

Figure 7 compares, in cross section, the ultrastructure of *Pisum* chloroplasts developed under far-red-deficient and far-red-enriched light. There is a considerable difference in the relative abundance and size of grana and stroma lamellae in the two samples. Under far-red-deficient light the chloroplasts appeared to have thinner grana stacks and more extended stroma thylakoids (Fig. 7, upper). Far-red-enriched light generally yielded a higher density of thicker grana (Fig. 7, lower).

<table>
<thead>
<tr>
<th></th>
<th>Chl a/Chl b</th>
<th>Q, µmol</th>
<th>P_{700}, µmol</th>
<th>Q/P_{700}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Greenhouse</td>
<td>2.50 ± 0.05</td>
<td>0.42 ± 0.06</td>
<td>0.23 ± 0.01</td>
<td>1.83</td>
</tr>
<tr>
<td>Fluorescent</td>
<td>2.96 ± 0.05</td>
<td>0.40 ± 0.06</td>
<td>0.22 ± 0.01</td>
<td>1.82</td>
</tr>
<tr>
<td>Incandescent</td>
<td>2.64 ± 0.06</td>
<td>0.49 ± 0.06</td>
<td>0.17 ± 0.01</td>
<td>2.83</td>
</tr>
</tbody>
</table>

*The amounts of Q and P_{700} in µmol correspond to 100 µmol chlorophyll (a + b). The chloroplasts were developed either in the greenhouse, by far-red-deficient cool white fluorescent light, or by far-red-enriched incandescent light.*
Fig. 7. Electron micrographs of *Pisum* chloroplasts greened under far-red-deficient (upper) and under far-red-enriched light (lower).
Fig. 8. Electron micrographs of *Asarum* (upper) and *Polystichum* (lower) chloroplasts showing the extended grana stacks, high membrane density, and decreased stroma volume, typical of shade-adapted plants.
Since grana thylakoids are enriched in Q (Melis and Brown, 1980), the above structural changes are in agreement with the light quality-induced changes in Q and P_{700} contents.

A second group of experiments involved shade species, which in nature occur only in the lower vegetation level of densely shaded habitats. An important feature of shade plants is their chloroplast size and ultrastructure (Björkman, 1973; Boardman et al., 1974). Figure 8 shows the chloroplast ultrastructure of the typical shade species *Asarum* and the fern *Polystichum*, revealing the large well-developed grana stacks, which in many cases extend across the entire chloroplast body. A concomitant decrease in the relative number or length of the intergrana stroma lamellae was not always apparent in the shade species we examined. However, the density of the membrane phase (grana and stroma thylakoids) was always higher than in chloroplasts from plants exposed to full sunlight, occupying almost the entire chloroplast volume and thus resulting in a drastic reduction of the relative stroma volume (Boardman et al., 1974). This result correlates with the decreased levels of ribulose bisphosphate carboxylase and other soluble proteins (Björkman, 1968) in such chloroplasts. Interestingly, in the three shade species examined, the light-saturated uncoupled rates of electron transport were low, ranging between 100 and 250 \( \mu \text{eq mg Chl}^{-1} \text{ h}^{-1} \) (Boardman et al., 1974; Boardman, 1977). Table 4 also shows that in the shade species *Asarum*, *Polystichum*, and *Tolmiea* the Chl a/Chl b ratios were considerably lower and the stoichiometric ratios of Q/P_{700} were generally higher than that of sun-adapted plants. (Compare with data in Table 3.) In these chloroplasts there is a parallel increase in Chl b, PS II content, and thylakoid membrane density in the chloroplast volume.

Since a shade habitat is an environment of low light intensity but is enriched in far-red light (Björkman, 1973), it may be observed that chloroplasts from the above shade species show the combined effect of the low light intensity on the Chl a/Chl b ratio (Boardman, 1977) superimposed on the light quality effect on the reaction center ratio Q/P_{700}.

Under predominantly far-red-enriched illumination, PS I reaction centers turn over electrons faster than their PS II counterparts. The increased PS II/PS I ratio observed under these conditions may serve to offset this energetic imbalance and to maintain a balanced electron flow through the electron transport chain.

The quality of light during chloroplast growth apparently controls both the differentiation of the membrane phase into areas of grana stacks and stroma thylakoids and the stoichiometric amounts of PS II and PS I reaction centers. It may be concluded that in oxygen-evolving organisms, PS II and PS I constitute integral thylakoid membrane–protein complexes that are structurally and, therefore, stoichiometrically independent of each other. We hypothesize that the structural differentiation of the chloroplast membranes into grana regions occurs, at

**Table 4. Chlorophyll a to Chlorophyll b Ratios, Q Content, and P_{700} Content in the Shade-Adapted Species *Asarum candatum*, *Polystichum munitum*, and *Tolmiea menziesii***

<table>
<thead>
<tr>
<th></th>
<th>Chl a/Chl b</th>
<th>Q, ( \mu \text{mol} )</th>
<th>P_{700}, ( \mu \text{mol} )</th>
<th>Q/P_{700}</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Asarum</em></td>
<td>2.32 ± 0.4</td>
<td>0.52 ± 0.07</td>
<td>0.19 ± 0.01</td>
<td>2.74</td>
</tr>
<tr>
<td><em>Polystichum</em></td>
<td>2.29 ± 0.05</td>
<td>0.62 ± 0.07</td>
<td>0.16 ± 0.01</td>
<td>3.88</td>
</tr>
<tr>
<td><em>Tolmiea</em></td>
<td>2.31 ± 0.05</td>
<td>0.46 ± 0.07</td>
<td>0.19 ± 0.01</td>
<td>2.42</td>
</tr>
</tbody>
</table>

*The amounts of Q and P_{700} in moles correspond to 100 mol chlorophyll (a + b).
least in part, in order to facilitate the centralization and relative concentration increase of PS II reaction centers. This subject is discussed more fully elsewhere (Melis and Harvey, 1981).

REFERENCES


FLUORESCENCE PROPERTIES OF GUARD CELL CHLOROPLASTS: EVIDENCE FOR LINEAR ELECTRON TRANSPORT AND LIGHT-HARVESTING PIGMENTS OF PHOTOSYSTEMS I AND II

Eduardo Zeiger,* Paul Armond, and Anastasios Melis

Chloroplasts are a central structural feature of stomatal guard cells (Meidner and Mansfield, 1968). Many investigators have suggested ways in which these chloroplasts might be crucial for stomatal functioning (Zelitch, 1969; Hsiao, 1976), but most hypotheses have proven untenable in the face of new discoveries or have not received conclusive experimental support. A better understanding of the physiological and biochemical properties of guard cell chloroplasts is crucial for a definition of their role in stomatal movements. Further progress depends largely on our ability to obtain pure preparations of guard cells and to monitor the photosynthetic activity of their chloroplasts in vivo.

Here, we used albino portions of variegated leaves from Chlorophytum comosum as a source of uncontaminated guard cell chloroplasts and studied their pigment content by fluorescence spectroscopy. Our findings indicate that guard cell chloroplasts contain light-harvesting pigments of both PS I and PS II. The fluorescence induction kinetics of guard cell and mesophyll chloroplasts are also presented. Our results support the notion that guard cell chloroplasts operate a linear electron transport system. We postulate that a central role of guard cell chloroplasts is to provide light-dependent energy to sustain the active ion transport required for stomatal opening during the day.

RESULTS

Emission Spectra of Guard Cell Chloroplasts. Low-temperature (77 K) emission spectra were obtained in a fluorospectrophotometer from samples frozen in liquid N2. Because of the high sensitivity of this technique, it was crucial to use preparations of guard cell chloroplasts free from contaminating mesophyll. The albino portions of variegated leaves from C. comosum provided an adequate source. An albino leaf segment, previously examined under fluorescence microscopy to ensure the absence of mesophyll chloroplasts, was mounted in a metal holder so that its abaxial surface faced the incident light beam, and was then immersed in liquid N2. The emission spectrum (Fig. 9) shows peaks near 686 and 740 nm, peaks usually attributed to pigments from PSII and PS I, respectively (Satoh and Butler, 1978). The spectrum from a suspension of chloroplast iso-
isolated from a green portion of the same leaf shows identical peaks (Fig. 9). We suggest that guard cell chloroplasts of *Chlorophytum* have pigments of both PS I and PS II.

**Variable Fluorescence of Guard Cell Chloroplasts.** The fluorescence induction curve of higher plant chloroplasts reflects the light-dependent transition of PS II from a weakly fluorescent condition ($F_o$) when all photochemical centers of PS II are open and capable of performing a charge separation, to a more strongly fluorescence state ($F_m$) as these centers become closed. In terms of the quencher theory (Duysens and Sweers, 1963), the fluorescence rise is the consequence of photoreduction of the primary electron acceptor $Q$ of PS II to its $Q^-$ forms (Melis and Duysens, 1979; Van Gorkom, 1974). According to the linear electron transport scheme, electrons accumulate on $Q$ after the reduction of the plastoquinone pool and the other electron acceptors by PS II. Consequently, in dark-adapted chloroplasts, the kinetics of the fluorescence induction provide an indicator of the accumulation of electrons in a number of carriers located between the two photosystems.

Figure 10a shows the light-induced fluorescence rise curve in dark-adapted isolated mesophyll chloroplasts and guard cells from *C. comosum*. Both mesophyll and guard cell chloroplasts showed the typical kinetic transition from $F_o$ to $F_m$ through the intermediary fluorescence level ($F_{pl}$) (Forbush and Kok, 1968; Kautsky et al., 1960; Malkin and Kok, 1966). The two time courses were similar, suggesting that PS II mediated the reduction of a pool of electron acceptors in both cases. With both preparations, irradiation at 700 nm was capable of restoring the fluorescence levels to their dark-
adapted state (data not shown), indicating that PS I was acting as a sink for electrons accumulated by PS II.

The presence of a functional PS II in guard cell chloroplasts is further demonstrated by measurements of the variable fluorescence induction kinetics in the presence of the PS II inhibitor DCMU. Inasmuch as DCMU prevents the reoxidation of Q* by the plastoquinone pool (Duysens and Sweers, 1963), fluorescence induction in its presence occurs in a much shorter period of time. Figure 10b shows that chloroplasts from both the mesophyll and guard cells of C. comosum respond to the inhibitor as predicted. Under these experimental conditions, the variable fluorescence induction reflects the reduction of the primary electron acceptor of PS II only.

These data indicate that guard cell chloroplasts have a functional PS II connected through an intermediate plastoquinone pool to PS I. A comparison of the areas confined by the ordinate, the Fm level, and the induction curve, in the presence and absence of DCMU, provides an estimate of the relative size of the plastoquinone pool (Thorne and Boardman, 1971). Our estimate (data not shown) indicates that the pool sizes in both types of chloroplasts are similar. These results are discussed more fully elsewhere (Zeiger et al., 1981).

REFERENCES


FLUORESCENCE PROPERTIES OF GUARD CELL CHLOROPLASTS: EVIDENCE FOR CO2 MODULATION OF PHOTOPHOSPHORYLATION

Anastasios Melis and Eduardo Zeiger

High-resolution chlorophyll a fluorescence was employed in the study of kinetic transients from mesophyll and guard cell chloroplasts of Chlorophytum comosum. Like their mesophyll counterparts, guard cell chloroplasts showed the OPS transient (Papageorgiou, 1975), a manifestation of linear electron transport (Bannister and Rice, 1968), and the slower MT transition reflecting chloroplast photophosphorylation. The MT transition of guard cell chloroplasts was sensitive to CO2, providing the first evidence for a specific response of the guard cell chloroplast to CO2.

RESULTS

Fluorescence Transients of Mesophyll and Guard Cell Chloroplasts from Intact Tissue. Figure 11 compares the in vivo chlorophyll fluorescence transients from dark-adapted mesophyll and guard cell chloroplasts. Mesophyll chloroplasts showed the typical tran-
Fig. 11. Time course of chlorophyll a fluorescence from dark-adapted mesophyll and guard cell chloroplasts of Chlorophytum comosum intact leaf segments. The onset of actinic illumination occurred at zero time.

sients OPSMT, as reported previously (Govindjee and Papageorgiou, 1971). Guard cell chloroplasts showed a single transient that peaked at about the same time as P in mesophyll chloroplasts, and a subsequent gradual decay to a steady-state level, which resembled the MT transition.

We determined that the single fluorescence transient peak from guard cell chloroplasts responds like P in mesophyll chloroplasts both in terms of its time of appearance under actinic illumination and its restoration kinetics in darkness (not shown). Guard cell chloroplasts also exhibit the MT transition, as evidenced by the reversible slow fluorescence yield decrease occurring after 10 s of actinic illumination (Fig. 11). The SM transition, however, is either lacking in guard cell chloroplasts or, if present, it is of small amplitude and is thus masked by other features of the fluorescence changes.

Effect of CO₂ on the Fluorescence Transients from Guard Cell Chloroplasts. The fluorescence transients from guard cell chloroplasts exposed to bicarbonate or Ca(OH)₂ were markedly different from their control. Figure 12 shows the reversible effects of a Ca(OH)₂ and bicarbonate solution on the fluorescence kinetics of guard cell chloroplasts. The single fluorescence transient peak GC of these chloroplasts was largely unaffected by either treatment; however, the secondary fluorescence yield decrease corresponding to the MT transition was significantly accelerated by Ca(OH)₂ or prevented by bicarbonate, a mode of action identical to that in mesophyll chloroplasts.

In the preceding article (Zeiger et al., this Report), evidence was presented that guard cell chloroplasts operate a photosystem I- and II-dependent linear electron transport chain. The present work provides evidence that guard cell chloroplasts show the fluorescence transient OP91, thus increasing the likelihood that guard cell chloroplasts generate reducing equivalents in the form of NADPH as a direct result of the electron transport activity in these organelles.

The slow fluorescence decrease (MT transition) was also detected in guard cell chloroplast samples. In mesophyll chloroplasts, the MT transition has been correlated with the accumulation of high-energy compounds (ATP) and their intermediates (ΔpH, Δions) in the chloroplast stroma (Bennet et al., 1980; Briantais et al., 1979; Horton and Black, 1980; Krause, 1974). It appears, therefore, that guard cell chloroplasts have the capability of photophosphorylation. The generation of metabolic energy in the form of ATP and possibly NADPH by the guard cell chloroplasts is of particular importance for stomatal physiology and strengthens the notion that these organelles play a central role in stomatal movements.
Fig. 12. Effect of Ca(OH)\textsubscript{2} and bicarbonate on the slow chlorophyll fluorescence decrease of guard cell chloroplasts. The same sample was illuminated three times with a 6-min dark interval between the individual kinetic runs. The water surrounding the control sample was replaced by a 5 mM Ca(OH)\textsubscript{2} solution during the first dark interval. Similarly, the Ca(OH)\textsubscript{2} solution was replaced by a 50-mM NaHCO\textsubscript{3}, pH 6.7 solution during the second dark incubation. Note the reversibility of the effect.

The inhibition of the MT fluorescence transition by CO\textsubscript{2} provides the first evidence for a specific response of the guard cell chloroplasts to CO\textsubscript{2}. Such a response was unexpected because guard cell chloroplasts are reportedly unable to fix CO\textsubscript{2} photosynthetically (Raschke, 1979; Outlaw \textit{et al.}, 1979). By analogy to mesophyll chloroplasts, CO\textsubscript{2} in guard cells must trigger photosynthetic energy consumption by an as yet unidentified process which prevents the formation of the high-energy state and the concomitant MT fluorescence transition.

REFERENCES


EFFECTS OF DESiccATION ON THE
EXCITATION ENERGY DISTRIBUTION IN THE
RED ALGA Porphyra perforata,
THE LIVERWORT Porella navicularis, AND THE
ISOLATED LICHEN GREEN ALGA Trebouxia pyriformis

Gunnar Öquist and David C. Fork

We showed in Year Book 79 (193–197) that desiccation of the intertidal red alga Porphyra perforata caused a loss of photosystem II (PS II) fluorescence emission \( F \) at 77 K. It was concluded from emission and excitation spectra analyses that this was achieved by an increased energy transfer from the phycobilins to PS I. Desiccation of mosses such as Dendroalsia abietinia and Scleropodium tourettei, of liverworts such as Porella bolanderi and Porella navicularis, and of the lichen Cladonia implexa and the symbiotic green alga Trebouxia pyriformis, isolated from Cladonia also induced a preferential loss of PS II fluorescence emission at 77 K. This change is shown in Fig. 13 for Porella navicularis and Trebouxia pyriformis.

Light desiccation caused a stronger loss of PS II emission than did dark desiccation.

The reason for the preferential loss of PS II emission upon desiccation was investigated by following the kinetics of PS I and PS II emission during PS II trap closure at 77 K (Butler, 1978). Porphyra represents a species that can tolerate desiccation under sun exposure at low tides, whereas Porella navicularis is a species that undergoes desiccation in its very shaded habitat. Porella was collected from the base of a live oak tree trunk (Quercus agrifolia) in the redwood forested area of Woodside, California. Trebouxia was obtained from the culture collection of algae at the University of Texas at Austin, Texas (#1712). It was grown on agar slants containing the nutrient medium described elsewhere (Sigfridsson and Öquist, 1980). The microprocessor-based spectrofluorimeter described earlier (Year Book 78, 196–199) was used in these experiments.

It has been demonstrated that \( F_T = f(F_{II}) \) when the PS II traps are closed at 77 K (Kitajima and Butler, 1975). The function gives a straight line in an X-Y-plot, and it can be shown that the slope of the line

\[
F_T F_{II} = k_{T(II-I)} \cdot \phi F_{II} / F_{II}.
\]

where \( \alpha \), the fraction of light initially absorbed by PS I, is proportional to the intercept obtained when the straight line is extrapolated back to the Y-axis, which represents the PS I emission (see Fork, Öquist, and Powles, this Report).

Figures 14, 15, and 16 show such X-Y plots for the PS II and PS I emission of Porphyra (collected in September because spring-collected algae [Year Book 79, 193–197] showed in the dry state no PS II emission at all), Porella, and Trebouxia, respectively. \( F_o \) and \( F_m \) values are summarized in Table 5. Desiccation of Porella caused a very marked decrease of both \( F_o \) and \( F_m \) at 685 nm (PS II) and 730 nm (PS I). Trebouxia responded with only minor decreases whereas Porphyra was intermediate. In Porphyra, desiccation induced a small increase of the slope of the straight-line plot (10%) and \( \alpha \) increased by 47 units (Fig. 14). For reasons we do not fully understand, the plot of wet Porphyra always crossed the Y-axis below zero. It means, however, that PS II of wet Porphyra receives some excitation energy which does not transfer to PS I. This fraction should be proportional to the intercept on the PS II axis when the PS I emis-
Fig. 13. Low-temperature (77 K) fluorescence emission spectra with deconvoluted emission bands of wet, dark-dried, and light-dried *Porella navicularis* and *Trebouxia pyriformis*. Fluorescence was sensitized by chlorophyll *b* excitation (478 nm). The emission was corrected to equal absorbance at 478 nm, as indicated in the second footnote of Table 5. The spectra were matched to equal heights of the dominant peaks using the multiplication factor given. The fluorescence properties of *Porella* represent equal leaf areas and those of *Trebouxia* equal chlorophyll concentrations. The error curves show the differences between the recorded (dotted-curve) spectrum and the sum of the components.

<table>
<thead>
<tr>
<th>Wavelength, nm</th>
<th>Porella</th>
<th>Trebouxia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A wet</td>
<td>x1</td>
</tr>
<tr>
<td></td>
<td>B dark dried</td>
<td>x1.9</td>
</tr>
<tr>
<td></td>
<td>C light dried</td>
<td>x1.9</td>
</tr>
<tr>
<td></td>
<td>D wet</td>
<td>x1</td>
</tr>
<tr>
<td></td>
<td>E dark dried</td>
<td>x1.4</td>
</tr>
<tr>
<td></td>
<td>F light dried</td>
<td>x2.6</td>
</tr>
</tbody>
</table>

The possible effects on the slopes of the straight-line plots by desiccation-induced changes in $k_{I_{II}-I_{III}}$, $k_{F_{II}}$, and $\phi F_I$ were evaluated. The procedure for doing this is exemplified for *Porphyra*. The effect of desiccation on $\phi F_I$ was estimated from values of $F_{730}$ when exciting chlorophyll *a* at 433 nm (Table 5); $\phi F_I$ dry = 0.65 · $\phi F_I$ wet. The straight-line plot, dry (corr $\phi_I$), of Fig. 14 was obtained by dividing the $F_{730}$ values of dry *Porphyra* by 0.65 in order to correct for the desiccation-induced drop of $\phi F_I$. The estimation of possible desiccation-induced effects on $k_{F_{II}}$ is less straightforward, but it was done as follows: First, $F_{730}$ of dry *Por-
Porphyrac was corrected to what it would have been if desiccation had not affected $\phi F_I$. This correction increased $F_0730$ from 99 to 153 (Table 5). Second, the 1.6-fold increase in $F_0730$ upon drying seen after the above correction had been applied was concluded to be caused by an increase in $a$ and/or $k_{II-I_{-10}}$. If we assume therefore that $F_0695$ was decreased with the same factor that caused the increase in $F_0730$ (because increases in $a$ and $k_{II-I_{-10}}$ would lower $F_0695$), we can calculate that $F_0695$ would be lowered from 93 to 57 upon desiccation. As the calculated value of $F_0695$ is identical with the measured one (Table 5), we assume that desiccation did not affect $k_{P_l}$ and that the slopes of the straight lines (Fig. 14, wet and dry [corr $\phi F_I$]) would be proportional to $k_{II-I_{-10}}$, i.e., $k_{II-I_{-10}}$ increased 1.7 times and $a$ increased 55 units upon desiccation. A similar calculation performed on dark-dried Porella revealed that the preferential loss of PS II emission must be caused first of all by a strong decrease in $k_{P_l}$ ($k_{II-I_{-10}}$) and $a$ even decreased slightly on desiccation. In dark-dried Trebouxia, $k_{P_l}$ was not significantly affected, and the increase of the slope of the line dry (corr $\phi F_I$) must be the result of a 10–20% increase of $k_{II-I_{-10}}$. Whether or not $k_{P_l}$ (radiationless decay in PS II) was affected by desiccation cannot be evaluated. The more pronounced desiccation-induced decreases in $F_{m685}$ than in $F_{o685}$ in both Porella and Trebouxia must be the result of a specific quenching of $F_v$, possibly by some quenching by $P680^+Q^-$ which may accumulate to some extent in dry
samples exposed to light at 77 K. Light desiccations induced a stronger loss of PS II emission than did dark desiccation (Fig. 13). Relative two-point action spectra of *Porella* and *Trebouxia* (chlorophyll *a* and chlorophyll *b* excitation with 433 nm and 478 nm, respectively) showed that light merely accentuates the changes taking place in \( k_{II-1} \), \( k_{FII} \), and \( \alpha \) in dark-dried samples.

In conclusion, the preferential loss of PS II fluorescence emission at 77°C in *Porphyra* was mediated by increases in \( k_{II-1} \) and \( \alpha \), whereas in *Porella* it was caused preferentially by a decrease in \( k_{FII} \). The small loss of PS II emission upon desiccation of *Trebouxia* was the consequence of a small increase in \( k_{II-1} \), whereas \( k_{FII} \) and \( \alpha \) were unaffected.

Ecological implications can be suggested for the differences observed in energy distribution in the plants studied. Since *Porphyra* can be desiccated at low tide and be exposed to bright sunlight, a mechanism has been developed to transfer excess excitation energy to PS I where it can be transformed eventually into heat. Such a mechanism would protect PS II from photodynamic damage in the desiccated state (compare Sigfridsson and Öquist, 1980). *Porella*, on the other hand, is found in extremely shaded habitats and is not exposed to bright sun, either in the wet or dry state. Perhaps PS II of *Porella* can handle small amounts of excess excitation in the desiccated state without damage to PS II. *Porella* has therefore not developed a specific mechanism for coping with large amounts of excess excitation energy. It is interesting to note in this regard that the leaves of *Porella* roll together in the dry state and thus decrease their absorption of light.

**Acknowledgement**

The authors are grateful to Professor W. B. Schofield of the University of British Columbia, who provided identification of the mosses and liverworts used in this work.
**TABLE 5.** The $F_o$ and $F_m$ Values of Wet and Dry *Porphyra, Porella,* and *Trebouxia.*

<table>
<thead>
<tr>
<th>433 nm Excitation</th>
<th>554 nm Excitation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Porphyra, wet</strong></td>
<td><strong>Porphyra, dark-dried</strong></td>
</tr>
<tr>
<td>$F_{730}$</td>
<td>$F_{695}$</td>
</tr>
<tr>
<td>$88 \pm 4$</td>
<td>$93 \pm 5$</td>
</tr>
<tr>
<td>$57 \pm 2$</td>
<td>$57 \pm 9$</td>
</tr>
<tr>
<td>478 nm Excitation</td>
<td>554 nm Excitation</td>
</tr>
<tr>
<td><strong>Porella, wet</strong></td>
<td><strong>Porella, dark-dried</strong></td>
</tr>
<tr>
<td>$F_{685}$</td>
<td>$F_{730}$</td>
</tr>
<tr>
<td>$124 \pm 6$</td>
<td>$47 \pm 2$</td>
</tr>
<tr>
<td>$64 \pm 9$</td>
<td>$13 \pm 1$</td>
</tr>
<tr>
<td><strong>Trebouxia, wet</strong></td>
<td><strong>Trebouxia, dark-dried</strong></td>
</tr>
<tr>
<td>$F_{715}$</td>
<td>$F_{685}$</td>
</tr>
<tr>
<td>$87$</td>
<td>$63 \pm 6$</td>
</tr>
<tr>
<td>$73$</td>
<td>$58 \pm 5$</td>
</tr>
</tbody>
</table>

*In *Porphyra, $F_{695}$ (PS II) and $F_{730}$ (PS I) were sensitized by chlorophyll (433-nm) or phycoerythrin (554-nm) excitation. In *Porella* and *Trebouxia, $F_{685}$ (PS II) and $F_{730}$/$F_{715}$ (PS I) were sensitized by chlorophyll a (700-nm) or chlorophyll b (478-nm) excitation. Standard deviations for $n = 3-4$ are given.

$^t$To give the dry samples the same absorbance as the three-layered wet sample at 433 nm. The measured value was multiplied by 1.15. The $F_{715}$ ($Trebouxia$) and $F_{730}$ ($Porella$) values were corrected as if the dry and wet samples had equal absorbance at 700 nm. For this correction the measured values of dry *Porphyra* were multiplied by 1.0 and those of dry *Trebouxia* by 0.68.

$^s$In addition to the correction listed above ($^{tt}$), these fluorescence values have been corrected to what they would have been if $\phi F_1$ was unaffected by desiccation; for *Porphyra* $\phi F_1$ dry $= 0.65 \phi F_1$ wet, for *Porella* $\phi F_1$ dry $= 0.55 \phi F_1$ wet, and for *Trebouxia* $\phi F_1$ dry $= 0.84 \phi F_1$ wet (see text).

**REFERENCES**


We showed previously (Year Book 79, 193–197) that desiccation of the red alga *Porphyra perforata* (to 91% water loss) caused increased transfer of quanta absorbed by PS II to PS I. It was suggested that this effect may protect *Porphyra* from photodynamic damage to PS II when the plant is exposed to full sunlight and extreme desiccation during low-tide periods. The former study used measurements of fluorescence of PS I (730 nm) and PS II (685 nm and 697 nm) made at 77 K, and the model of Butler (1978), to suggest how desiccation affects energy distribution between the two photosystems of photosynthesis.

Since *Porphyra* is unusual in having a fluorescence emission band at room temperature that is sensitized by PS I (Fork, Öquist, and Hoch, this Report), we wanted to take advantage of this property to extend our investigation of how desiccation affects energy transfer between two photosystems of photosynthesis at physiological temperatures.

The fluorescence emission and excitation spectra were measured as described in another section of this Report (Öquist and Fork; Fork, Öquist, and Hoch). The kinetics of fluorescence were measured either with blue or green actinic light, the intensity of which was decreased until fluorescence could be measured with a two-pan chart recorder. *Porphyra*, which is only one cell layer in thickness, could be easily dried in darkness by passing an air stream over pieces of the thalli. Drying was also done in the presence of strong white light.

Excitation of *Porphyra* with blue light that excites PS I of photosynthesis predominantly gives rise to the emission spectrum shown in Fig. 17, curve A. As was discussed in another section of this Report (Fork, Öquist, and Hoch), PS I excitation in *Porphyra* produced a distinct long wavelength fluorescence emission band at room temperature. Figure 17, curve B, shows that desiccation in the light to 91%
water loss produced an increase in the intrinsic fluorescence yield of PS I (of 3.6 times) and a 10-nm shift of the 727-nm peak to longer wavelengths. When the Porphyra was dried in darkness, the fluorescence yield increased 4.3 times (Fig. 17, curve C).

Excitation of PS II in Porphyra with green light produced the spectrum shown in Fig. 18, curve A. The shoulder near 643 nm and the peak at 660 nm correspond to emissions from phycocyanin and allophycocyanin, respectively. Chlorophyll a of PS II produces the 687-nm peak and, as discussed previously, PS I produces the long wavelength shoulder at 727 nm. Curve A of Fig. 18 represents the spectrum for fluorescence in the F_o state, since Q, the primary electron acceptor of PS II, remained largely in the oxidized state in the relatively weak actinic light intensities used. If we treated Porphyra with DCMU, then Q became reduced as a result of the interruption of normal electron flow between the two photosystems. Under this condition (F_m) we saw increases of 3.6 times at both the 687-nm and 730-nm bands compared to the F_o spectrum (Fork, Öquist, and Hoch, this Report). DCMU, therefore, increased fluorescence yield but did not change the shape of the fluorescence emission spectrum. By contrast, desiccation produced a large change in the shape of the emission spectrum (Fig. 18, curve B). In this case, Porphyra dried in light showed a large increase of the far-red emission band, a loss of the 687-nm band, and an increase of the allophycocyanin band at 658 nm. As was seen by measurements of fluorescence kinetics in the light-dried alga, the reaction center of PS II (Q) does not become reoxidized in darkness.

Since Fig. 17 shows that the intrinsic fluorescence yield increased upon desiccation of Porphyra either in the light or the dark, we wanted to replot the results of the phycoerythrin-sensitized spectra so that this drying-induced intrinsic fluorescence yield increase of PS I would be removed. After doing this manipulation, it would be possible to see more clearly if drying produced effects on emission spectra other than the intrinsic fluorescence yield increases of PS I. In Fig. 19, the relative fluorescence emission spectra measured using green actinic light were corrected for equal intrinsic fluorescence yields of PS I for both dark-dried and light-dried Porphyra (Fig. 19, curves C and B) and were then compared to a wet sample (Fig. 19, curve A). These spectra show that desiccation produced dramatic increases in the relative fluorescence yields at 737 nm (PS I) and decreases of fluorescence at 687 nm (PS II). In Porphyra dried in strong light (Fig. 19, curve B), there was almost a complete disappearance.

![Fig. 18. Fluorescence emission spectra measured at 24°C in wet and dry Porphyra perforata using phycoerythrin excitation (643-nm interference filter, 9-nm half-bandwidth, Corning glass filter 4-96; Calflex C, 9 ,mol quanta m^-2 s^-1). (A) Wet, dark-adapted (three layers). (B) Dried in strong, white light (one layer).](image-url)
of the PS II 687-nm band. In *Porphyra* desiccated in darkness, only a small shoulder at 687 nm remained (Fig. 19, curve C). It is interesting to note that, except for the 658-nm allophycocyanin band, desiccation in strong light produced less fluorescence yield increase at 737 nm than did desiccation in the dark. The 737-nm and 687-nm fluorescence bands were decreased by about equal proportions upon desiccation in strong light, as compared to the dark-dried sample.

We measured the kinetics at 24°C of the rise of 730-nm fluorescence upon excitation of wet and desiccated *Porphyra* with green actinic light. Since *Porphyra* is photosynthetically active at 24°C, the value of $F'_o$ was the same as $F_m$ because Q did not accumulate in the reduced form under the relatively low light intensities used. Adding DCMU to the wet sample produced a fluorescence increase of 2.5 times at 730 and a similar increase at 687 nm (Fork, Öquist, and Hoch, this Report). Illumination of a sample dried in the dark with green light produced a 1.4-fold increase of fluorescence. This suggests that PS II traps could still be reduced to a substantial degree (56%) in the dry state. Light-dried *Porphyra*, on the other hand, did not have the ability to reduce PS II traps; both $F'_o$ and $F_m$ were the same, probably because all PS II traps were closed in the light-dried sample.

The $F_m$ value of fluorescence at 730 nm in dark-dried *Porphyra* was double that in wet *Porphyra* (no DCMU) and 1.4 times higher than that of light-dried *Porphyra*. This finding agrees quite well with emission spectra results presented in Fig. 19. The desiccation-induced increase of the fluorescence yield at 730 nm and the decrease at 687 nm seen in emission spectra and kinetic measurements may arise from an enhanced energy transfer from PS II to PS I as the result of drying. If this is the case, then the excitation spectrum of dry *Porphyra* would show an enhanced PS II (phycobilin) effectiveness to excite 730-nm fluorescence in the dry samples, as compared to the wet.

Figure 20 shows excitation spectra for wet and dry *Porphyra*. The relative effectiveness of green light (548, 570 nm) absorbed by the phycobilins to excite the far-red fluorescence band at 730 nm is higher in both the light-dried and dark-dried *Porphyra* than in the wet alga.

The changes of the fluorescence properties of *Porphyra* dried in the dark, such as the reduced efficiency for PS II trap closure, the almost complete loss of PS II emission at 687 nm with concomitant relative increase of PS I emission at 730 nm, and the increased phycoerythrin/chlorophyll efficiency ratio for sensitizing PS I emission, are quali-

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Fig. 19. Fluorescence emission spectra measured at 24°C in wet and dry *Porphyra perforata* using phycoerythrin excitation, as for Fig. 18. (A) Wet (three layers). (B) Dried in strong, white light and corrected for fluorescence yield increase of chlorophyll a upon drying, as explained in the text. (C) Dried in darkness and corrected as described in (B).
Fig. 20. Excitation spectra measured at room temperature for 730-nm fluorescence emission in wet Porphyra perforata and in Porphyra dried in strong light and in the dark.

Fig. 20. Excitation spectra measured at room temperature for 730-nm fluorescence emission in wet Porphyra perforata and in Porphyra dried in strong light and in the dark.

...tatively similar to the fluorescence changes observed at 77 K (Year Book 79, 193–197; Oquist and Fork, 1981). It can be concluded that Porphyra, even at room temperature, responds to desiccation by producing a strong increase in the distribution of excitation energy from phycoerythrin to PS I. Whether this shift is mediated by increases in $\alpha$ or spillover (see Butler, 1978) cannot be determined from the data presented, since there was almost a complete absence of the PS II emission at 685 nm in Porphyra desiccated at room temperature. However, the observation that the $F_o$ value of the dark-dried and the $F_m$ of the wet Porphyra (+ DCMU) were about the same favors the assumption that the desiccation-induced increase in energy transfer from phycoerythrin to PS I at room temperature is mediated by a substantial increase in $\alpha$. A similar interpretation was made for these algae studied at 77 K (Oquist and Fork, 1981). The loss of the capacity for trap closure by about one-half, induced by desiccation, can also be explained by an $\alpha$ increase. It is possible, however, that reaction center quenching may account for some of this loss in the dark-dried sample (see below).

Measurements of the kinetics of fluorescence of Porphyra dried in strong light showed that $F_v$ was not much affected, and part of $F_o$ was decreased in comparison with dark-dried algae. This $F_v$ loss was reflected in the emission spectrum shown in Fig. 19, curve B, where, as noted previously, the relative fluorescence yield was lower in the light-dried sample at both 687 and 737 nm. Both the 687-nm and 737-nm bands were decreased proportionately. Loss of $F_v$ but not $F_o$ suggests a mechanism of reaction center quenching (Butler, 1978). This type of quenching occurs under conditions where PS II reaction centers accumulate in the state where P680 is oxidized and Q is reduced (P680$^+$ Q$^-$. If the decreased fluorescence in the light-dried sample compared to the dark-dried sample is caused by an increase in the rate constant for nonradiative decay of fluorescence ($k_d$), then decreases at both $F_o$ and $F_m$ would have been seen. It is possible that the decreased capacity for trap closure of about 50% in the dark-dried Porphyra may have been caused to some degree by this type of reaction center quenching.

Our previous conclusion that desiccation gives rise to an increased energy transfer from PS II to PS I was based on studies made at liquid nitrogen temperatures (Year Book 79, 193–197). The present studies at physiological temperatures also favor the interpretation of increased energy transfer to PS I as a result of desiccation. As we noted previously, this mechanism may serve to protect the more light-sensitive reaction centers of PS II from photochemical damage when these desiccation-tol-
erant algae are exposed during periods of low tide to both extreme drying and high light intensities.

ROOM-TEMPERATURE PHOTOSYSTEM I
FLUORESCENCE EMISSION IN THE RED ALGA *Porphyra perforata*

David C. Fork, Gunnar Øquist, and George Hoch

At 77 K a fluorescence band at 725 nm can be seen clearly in the red and blue-green algae, but at room temperature this band is usually not seen (Goedheer, 1972). However, Murata and Takamiya (1967) saw a peak in this region in the room-temperature fluorescence emission spectrum that was sensitized by blue light in the red alga *Porphyra yezoensis*.

A room-temperature fluorescence emission band that can be shown clearly to originate in PS I would be useful, since it would permit measurements at physiological temperatures of photosynthetic reactions that can be followed using fluorescence techniques (Butler, 1978). We have seen a room-temperature fluorescence band at 730 nm in *Porphyra perforata* that is emitted from PS I.

*Porphyra perforata* used in these experiments was collected along the Pacific Coast north of Santa Cruz, California, and kept under illumination in open dishes of seawater held at 13°C. Fluorescence emission spectra were measured with the microprocessor-based system described previously (Year Book 79, 196-199). The alga, which is one cell layer thick, was held submerged in a Dewar at 24°C. Exciting and fluorescent light were directed to and collected by a quartz light guide positioned just on the surface of the algal thallus. Emission spectra were corrected by using a standard lamp.

Fluorescence excitation spectra were done with the same system except actinic light from a monochromator was directed on the sample with the light guide. The fluorescence was conducted by another light guide to a Hamamatsu TV R928 photomultiplier fitted with either a 730- or 685-nm interference filter (5-nm half-bandwidth). Excitation spectra were corrected by a technique that used Rhodamine B as a fluorescence standard.

Fluorescence kinetics were measured with a trifurcate fiber bundle. One branch served to illuminate the sample and the other two conducted fluorescence at two different wavelengths to two photomultipliers having 685- and 730-nm interference filters, respectively. The actinic light was kept low enough that the kinetics of fluorescence could be followed with a two-pen strip chart recorder.

*Porphyra* illuminated at room temperature with green actinic light had an emission spectrum (Fig. 21, curve A) with three peaks at 659 nm (allophycocyanin), 687 nm (Chl *a* of PS II), and 732 nm. As will be described, the 732-nm band is apparently emitted from Chl *a* of PS I. This spectrum represents the initial *F₀* fluorescence state, since it was measured in a physiologically active thallus under nonsaturating green actinic light. In the *F₀* state all the photochemical reaction centers are in the open (oxidized state). After addition of DCMU to this sample, the emission spectrum had maxima at 685 and 734 nm and a shoulder near 663 nm (Fig. 21, curve B). DCMU blocks electron transport between PS II and PS I, and reaction centers of PS II accumulate in
the reduced state. In this condition, fluorescence attains its maximum level ($F_m$), as all photochemical reaction centers are reduced. The difference between curves A and B of Fig. 21 (curve C) represents the fluorescence spectrum having a variable yield ($F_v$). This spectrum has peaks at 685 and 734 nm and no phycobilin peaks, indicating that DCMU did not interrupt energy transfer from the phycobilins to chlorophyll $a$ fluorescing at 685 nm. The increased fluorescence intensity at 734 nm after DCMU addition does not originate in PS I but comes from energy absorbed by PS II chlorophyll and transferred to PS I because of the closed PS II reaction centers (Butler, 1978).

Excitation of PS I of Porphyra by blue light gives a fluorescence emission spectrum with only one major peak at 732 nm (and a minor inflection around 687 nm) (Fig. 22, curve A). Adding DCMU increased both the 732- and 685-nm fluorescence bands (Fig. 22, curve B).

Excitation spectra (Fig. 23) show that blue light (440 nm) is more efficient in exciting fluorescence at 730 nm in Porphyra than is green actinic light. By contrast, green light at 553 and 568 nm is more efficient than blue light in exciting fluorescence at 685 nm. These measurements represent excitation spectra for $F_v$ fluorescence, since they were done at room temperature, in the absence of DCMU, and under relatively low light intensities. These excitation spectra, plus the fluorescence emission spectra described earlier, and the results of Murata (1967), demonstrate that the long-wavelength fluorescence band in Porphyra at room temperature originates from chlorophyll associated with PS I. These re-
Fluorescence yield changes have been suggested by Duysens and Sweers (1963) to reflect changes in the redox state of the primary electron acceptor \( Q \) of photosystem II of photosynthesis. The characteristic fluorescence yield changes seen upon reduction of \( Q \) can still be seen at the temperature of liquid nitrogen (77 K). Only primary photochemical reactions can function at 77 K; all other reactions are inhibited (Amesz, 1977).

We have seen a biphasic dark reoxidation of \( Q \) in bean leaves at 77 K that is not affected by DCMU. This result may be a reflection of the heterogeneous nature of photosystem II.

The bean leaves used in these experiments were grown in the greenhouse under natural illumination during late summer at 25°C day and 20°C night temperatures. The leaves were either used directly or vacuum infiltrated with DCMU.

Fluorescence induction at 695 nm was followed with the apparatus described by Fork and Öquist (this Report). The leaves were dark adapted and cooled to 77 K and then illuminated with actinic light until no further fluorescence increases took place. After dark periods of varying lengths the actinic light was again turned on and the fluorescence rise measured.
Figure 24 shows the kinetics of reoxidation of reduced Q at 77 K in untreated bean leaves and in leaves infiltrated with DCMU. It can be seen that DCMU did not have any effect on the rate of Q reoxidation at liquid nitrogen temperature. The curve of Fig. 24 could be deconvoluted into two first-order decay components, a fast component having a half-life of 0.8 min and a slow component having a half-life of 180 min. The fast component constitutes about 13% of the total fluorescence recovery seen at 77 K.

Murata et al. (1973) saw dark recovery of fluorescence induction at 77 K in isolated spinach chloroplasts that showed maximum recovery of the variable fluorescence after about 20 min of darkness. After that, fluorescence remained unchanged for 60 min of darkness.

Measurements by Melis and Homann (1975, 1976) of the fluorescence increase in chloroplasts treated with DCMU showed it to be biphasic. Two different kinetic components could be distinguished, a fast (α) component and a slow (β) component. These and other measurements (Melis and Duyssens, 1979; Rijgersberg et al., 1979) suggest that photosystem II of photosynthesis is heterogeneous with regard to its physical makeup and photochemical reactions, and it was suggested that the slow (β) component originates from the photosystem II complexes exposed in the stroma membranes whereas the fast (α) component originates from the photosystem II complexes in the appressed membranes (Melis and Thiel, 1980). The biphasic reoxidation kinetics of Q at 77 K shown in Fig. 24 may be a reflection of this physical heterogeneity of the photochemical reaction centers of PS II, so that the fast and slow reoxidation components of Q occur in the exposed stroma and the appressed thylakoid membrane regions, respectively.

The differences between these results and those of Murata et al. (1973) may be caused by the use of different experimental material—leaves vs. extracted chloroplasts. Leaves could be expected to have a more intact functioning photosystem II and thylakoid aggregation compared to extracted chloroplasts.

**References**


Heat inactivation of photosynthesis may be related to the breakage of short-range bonds of hydrophobic or hydrophilic character in photosystem II, as manifested in the functional disorganization of photosystem II at high temperatures (Schreiber and Armond, 1978). It was of interest therefore to investigate whether adaptation to high temperature in plants leads to an increased strength of the chlorophyll-protein interactions, as reflected in the heat stability of the sodium dodecyl sulfate (SDS) solubilized chlorophyll-protein complexes of photosystem I and II, CP-aI and CP-aII. We used the thermophilic blue-green alga Synechococcus lividus because it contains only CP-aI and CP-aII (no light-harvesting CP-a/b complex, which overlaps CP-aII) and also because considerable physiological data is already available on the photosynthetic properties of this alga (Fork et al., 1979).

Synechococcus lividus (strain SY-4 from Mercedes Edwards) was grown at 38° and 55°C, as described previously (Fork et al., 1979). Thylakoids were isolated by a lysozyme treatment (modified after the techniques reported by Ono and Murata [1979]), followed by French pressure cell treatment (77 kg cm⁻²), and harvesting of thylakoids by centrifugation at 48,000g for 40 min. The thylakoids were solubilized for 5 min in SDS at temperatures between 0° and 65°C, and SDS-polyacrylamide gel electrophoresis (PAGE) and molecular weight calibration were performed at 4°C, as described earlier (Öquist and Samuelsson, 1980). The proportion of chlorophyll in the different bands of the gel was calculated by planimetry. Low-temperature absorption and fluorescence emission spectra were measured and deconvoluted into components by RESOL.

Figure 25 shows gel scan profiles of extracts obtained at 20° and 55°C from thylakoids of Synechococcus grown at 55°C. When grown at this temperature, the alga has a lipid phase transition temperature near 43°C (Fork et al., 1979). Similar profiles were obtained from algae that were grown at 38°C (phase transition temperature near 37°C [Fork et al., 1979]). The profile obtained from thylakoids that were solubilized above the phase transition temperature had bands of CP-aI and CP-aII (apparent molecular weights of 85,000 and 43,500, respectively), whereas extracts made below the phase transition temperature showed only traces of CP-aI. Instead, a new band, CP-aI', appeared that had a slightly lower mobility (apparent molecular weight 90,000-98,000) than CP-aI'.

Figure 26 shows the relative distribution of CP-aI', CP-aI, CP-aII and SDS-Chl (solubilized chlorophyll) in the scan profiles obtained after SDS-PAGE of SDS-extracts made at temperatures between 0° and 65°C for Synechococcus grown at 38°C (Fig. 26A) and at 55°C (Fig. 26B). Clear bands of CP-aI were obtained from SDS-extracts made above 40°C, near the phase transition temperature of the 55°C-grown cells (Fig. 26B). There was a tendency for the CP-
a<sub>1</sub> to appear at a few degrees lower temperature in 38°C-grown algae (Fig. 26A). CP-a<sub>1</sub> of both algal cultures started to deteriorate when the solubilizing temperature ranged above 60°C. CP-a<sub>1</sub>', unlike CP-a<sub>1</sub>, was solubilized at temperatures between 0° and 40°C. Above this temperature it started to disappear. Thus, CP-a<sub>1</sub>' disappeared from the scan profile as CP-a<sub>1</sub> appeared. Although under optimal conditions both CP-a<sub>1</sub>' and CP-a<sub>1</sub> made up 40–50% of the chlorophyll on the gel, the chlorophyll content of CP-a<sub>1</sub> never
exceeded from one-third to one-half of the chlorophyll content in CP-a\textsubscript{I} (compare Fig. 25). The reason for this discrepancy between the presentations in Fig. 25 and Figs. 26A and 26B, is that much less chlorophyll entered the gel if the extracts were obtained below rather than above the phase transition temperature. Extracts obtained below the transition temperature always gave rise to considerable amounts of chlorophyll, which showed a tendency to enter and remain at the very top of the gel, whereas no (or only very little) residue was obtained with extracts solubilized above the phase transition temperature. When present, this residue had absorption and fluorescence emission properties typical for the antennae of photosystem I (data not shown).

CP-a\textsubscript{II}, unlike CP-a\textsubscript{I}, was solubilized at high yield even at 0°C, and it started to decrease in the scan profile above about 40°C. It could scarcely be found in extracts solubilized at 55–65°C. The relative proportion of SDS-Chl stayed at 15–25% as long as the solubilization was performed below about 40°C. With increasing solubilization temperature, it began an exponential rise as CP-a\textsubscript{II}, and later CP-a\textsubscript{I}, started decreasing in the scan profiles.

Low-temperature absorption and fluorescence emission spectra were typical for CP-a\textsubscript{I} and CP-a\textsubscript{II}, and no specific effects of the two growth temperatures on these properties were observed (data not shown). CP-a\textsubscript{I}' and CP-a\textsubscript{I} had identical absorption properties, and as they both had in addition a dominating far-red emission band we conclude that they are both derived from the reaction center antennae of photosystem I.

The phase transition temperature was considered to be critical for the solubilization of CP-a\textsubscript{I}, either because of the difficulties of SDS (especially as it forms micelles at low temperatures) in penetrating the solidified membrane lipids at temperatures below the phase transition or because the CP-a\textsubscript{I} monomers of the photosystem I antennae are so strongly bound to each other that they cannot be dissociated by SDS before thermal agitation has reached a certain level, achieved only above the phase transition temperature. Below this temperature, only a fraction of the photosystem I antennae (CP-a\textsubscript{I}') is accessible to SDS. Although CP-a\textsubscript{II} of Synechococcus could be solubilized at high yield even at 0°C, it was remarkably heat-stable in comparison with what is normal for mesophilic plants. We consider the difficulties in solubilizing CP-a\textsubscript{I} at sub-transition temperatures, and the heat stability of the two complexes, both as adaptations enabling Synechococcus to grow under extreme high temperature regimes.

REFERENCES
A FLUORESCENCE DECLINE AS AN INDICATOR OF PHOToinHIBITION IN INTACT Bryopsis CHLOROPLASTS UNDER ANAEROBIC CONDITIONS

Kazuhiko Satoh and David C. Fork

It is well known that under aerobic conditions chlorophyll fluorescence shows a typical induction phenomenon in leaves, algal cells, or intact chloroplasts (Kautsky and Appel, 1960; Govindjee and Papageorgiou, 1971; Katoh et al., 1975). However, under anaerobic conditions this typical induction pattern of chlorophyll fluorescence is replaced by another, simpler one. For example, with short incubation of photosynthetic organelles under anaerobic conditions, the PS1 decline is largely inhibited, and sometimes a slow fluorescence decay after the peak P is observed (Bannister and Rice, 1968; Schreiber and Vidaver, 1974).

Almost all work concerning the effects of anaerobiosis on the fluorescence time course has been done using complex systems such as leaves or algal cells. Moreover, little work on the slow fluorescence decline which replaces the PS1M1 transient has been done until now. Since oxygen was found to act as the main electron acceptor after photoactivation of electron transport had taken place (Satoh, this Report), it was also interesting to investigate what would happen if chloroplasts were illuminated in the absence of oxygen.

In this study, we used intact Bryopsis chloroplasts and observed the effects of anaerobic treatment on the time course of chlorophyll fluorescence and on the electron transport reactions. We found that the fluorescence decline was related to photoinhibition of the electron transport system.

Figure 27 shows effects of anaerobiosis on the time course of chlorophyll fluorescence at low and high light intensities. In order to obtain anaerobic conditions, 5 mM dithionite (Fig. 27, b and e) or glucose + glucose oxidase (Fig. 27, c and f) were added. Under aerobic conditions, the typical DPS1M1 transient of chlorophyll fluorescence was observed (Fig. 27, a). The fluorescence decline from the peak M1 was very slow even at the higher light intensities used (Fig. 27, d). However, under anaerobic conditions, this PS1M1 transient was replaced by a simpler decline from the peak P, and fluorescence reached much lower levels than those obtained under aerobic conditions.

Under iso-osmotic conditions (corresponding to 1.0 M sorbitol), electron carriers were not reduced by the addition of dithionite in intact Bryopsis chloroplasts (data not shown). Therefore, the effects of dithionite addition can be attributed to the consumption of oxygen by dithionite in the reaction mixture rather than to its effects on electron carriers directly. This fluorescence decline was also observed in chloroplasts incubated with glucose and glucose oxidase, although the rates were lower in this case (Fig. 27, c and f).

These results suggest that under anaerobic conditions the electron transport pathway of the chloroplasts was modified so that a simpler fluorescence decline replaced the PS1M1 transient.

Under conditions of low CO2 concentration, light is known to induce inhibition of photosynthesis (Powles and Osmond, 1979). This photoinhibition is attributed to the presence of excess light energy because there may be essentially no acceptor pools for electrons under low CO2 conditions. As mentioned previously, oxygen was shown to act as a principal electron acceptor after photoactivation of electron...
transport. If the above hypothesis is true, then in the absence of oxygen almost any light intensity may bring about photoinhibition. Therefore, we measured activities of photosystem I and II after illumination of the chloroplasts under anaerobic conditions. The activity of photosystem I (methyl viologen photoreduction in the presence of DCMU and DCIPH$_2$) decreased slowly and was about 87% of its initial activity after 45 s of illumination (Fig. 28, square symbols). On the other hand, the activity of photosystem II (DCIP-Hill reaction, Fig. 28, circles) decreased sharply and then continued to decrease slowly. The fluorescence time course under anaerobic conditions is also shown in Fig. 28. It also has two phases, a rapid and a slow phase. The time course of the initial rapid phase of the activity decrease of photosystem II was similar to that of the rapid phase of the fluorescence decline. Illumination of the chloroplasts under aerobic conditions had no effect on the photosystem I and II activities (data not shown). These results suggest that the rapid phase is directly related to photoinhibition of photosystem II, and although the rates are different, the slow phase is concerned with photoinhibition of both photosystems.

If we accept the hypothesis that oxygen is a main electron acceptor after the photoactivation has taken place, this photoinhibition must be induced by low intensities of light under anaerobic conditions. Table 6 shows the effect of various intensities of actinic light on the rate of the DCIP-Hill reaction. The illumination period was also
changed to observe the effect under conditions in which the same numbers of photons were absorbed by the chloroplasts. Light even at intensities as low as $7.7 \times 10^2 \text{erg cm}^{-2} \text{s}^{-1}$ induced photoinhibition of photosystem II. These results also show that this system may provide a good model to study the initial event of photoinhibition of photosynthesis.

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**PHOTOINHIBITION IN BEAN: A FLUORESCENCE ANALYSIS**

David C. Fork, Gunnar Öquist, and Stephen B. Powles

Photoinhibition of photosynthesis results from exposure of plants to excessive light energy. The first signs of such damage are a reduction of the quantum yield for photosynthetic CO₂ uptake accompanied by inhibition of PS II electron transport and loss of fluorescence emission from PS II (Powles et al., 1979; Powles and Critchley, 1980). These results suggest that photosynthesis...
to inhibition results as a consequence of over-energization of the photosynthetic reaction centers. In other studies, we have been examining how desiccation alone, and in combination with light, affects energy distribution in photosynthesis in marine algae, liverworts, and lichens (Oquist and Fork, Fork and Oquist, this Report; Year Book 79, 193-197). Even in these studies, a preferential loss of fluorescence emission from PS II was induced. In measurements with algae, liverworts, and lichens, the preferential loss of fluorescence emission from PS II could be explained by (1) changes in \( k_{III-1} \) (spillover of light energy from PS II to PS I), (2) changes in \( \alpha \) (the fraction of light distributed originally to PS I), (3) changes in \( k_{FI} \) (the rate constant for PS II fluorescence), or (4) by reaction center quenching produced by accumulation of inactive reaction centers (P680+ Q\(-\)) of PS II (see Butler, 1978).

In this study we wanted to determine whether the preferential loss of PS II emission under photoinhibition treatments could be explained by any of the above factors and if so by which one(s).

Bean plants (Phaseolus vulgaris C_v Hawkesbury Wonder) were grown under 25°/20°C temperatures during the late summer and autumn months when light intensities regularly reached quantum fluxes of 2000 \( \mu \text{mol quanta m}^{-2} \text{s}^{-1} \). Photoinhibition was accomplished by treating intact attached fully expanded bean leaflets for 3 h at 6°C under white light at 2000 \( \mu \text{mol quanta m}^{-2} \text{s}^{-1} \) (see Year Book 79, 157-160, for description of treatment procedures).

Measurements of fluorescence emission spectra were done at 77 K, as described previously (this Report). Fluorescence was measured simultaneously at 695 and 740 nm using a trifurcated fiber optic light guide. One branch served to illuminate the sample. The other two branches were fitted with interference filters (Infra Red Industries, 5-nm half-bandwidth) and attached to photomultipliers (EMI 9558B and Hamamatsu TV R928). The amplified signals were each fed to the inputs of a two- pen strip chart recorder.

A quartz light guide was attached to the optic fiber bundles and served to hold the sample submerged in a Dewar of liquid nitrogen while guiding actinic light to the leaf surface and collecting fluorescence from the same surface. The intensity of the actinic light was decreased until it became possible to follow the kinetics of fluorescence at 77 K.

Figure 29, curve A, shows the emission spectrum of a bean leaflet that had been kept at 6°C for 3 h in the dark (in normal air). In Fig. 29, curve B, the intact bean leaflet was given a 3-h exposure to 6°C (normal air) at a photon fluence rate of 2000 \( \mu \text{mol quanta m}^{-2} \text{s}^{-1} \).

![Figure 29](image-url)

Fig. 29. Low-temperature (77 K) fluorescence emission spectra for an intact bean leaflet that had been kept in the dark for 3 h at 6°C (A) and for a leaf kept at 6°C in white light having a photon fluence rate of 2000 \( \mu \text{mol quanta m}^{-2} \text{s}^{-1} \) (B). The fluorescence was sensitized by chlorophyll \( b \) excitation (6 \( \mu \text{mol quanta m}^{-2} \text{s}^{-1} \), obtained with a Balzers interference filter 478 nm, half-bandwidth 9 nm, plus Corning 5-60 and Calflex C filters. The slits of the monochromator were set to pass a half-bandwidth of 1.6 nm.
These spectra show that exposure of a bean leaflet to a high light intensity while at a chilling temperature results in a preferential decrease of the PS II fluorescence peaks at 687 and 695 nm. The ratio of fluorescence peak height at 740 (PS I) to that at 695 nm was 3.6 for the control and 5.4 for the photoinhibited leaflet. The ratios of 740/685 fluorescence were 4.2 for the control and 7.0 for the photoinhibited leaflet.

The preferential loss of PS II activity in the photoinhibited leaflet was not seen when the fluorescence emission spectrum of the underside of the same leaflet was measured (data not shown). Since the upper surface of the treated leaflet was maintained perpendicular to the light during the photoinhibition treatment, the chloroplasts in cells toward the lower leaflet surface were shielded from the light energy impinging upon the plastids in the upper layers of the leaflet. There is, therefore, probably a gradient of damage to the plastids through the leaflet. Therefore, damage exhibited by chloroplasts close to the upper exposed leaf surface will be underestimated.

This is exemplified in Fig. 30, which shows emission spectra for chloroplasts isolated from leaf material photoinhibited in this way will contain plastids from throughout the leaf. Therefore, damage exhibited by chloroplasts close to the upper exposed leaf surface will be underestimated.

It has been demonstrated that $F_I = f(F_{II})$ when the PS II traps are all closed (reduced) at 77 K (Kitajima and Butler, 1975). This function gives a straight line in an X-Y plot. It can be shown that the slope of the line

$$\frac{F_I}{F_{II}} = k_{T_{II}} \cdot \phi F_I / k_{F_{II}}.$$  

The intercept obtained when this straight line is extrapolated back to the Y-axis is proportional to $\alpha$, the fraction of light energy absorbed by PS I directly and independently of the state of the PS II traps (Butler, 1978).

Figure 31 gives the plots of PS I (at 740 nm) and PS II emission (at 695 nm) during trap closure of a control and of a photoinhibited bean leaflet. Figure 31 shows results obtained by measuring the upper surface and the under surface of the photoinhibited leaflet. The upper side of the photoinhibited leaflet had a strong decrease of $F_m$ particularly at 695 nm since $F_m$ decreased from about 150 to 60, but $F_o$ of the photoinhibited leaflet did not change in comparison to the control leaf. Photoinhibition, in addition, produced about a 45% increase.
in the slope of the line and an \( \alpha \) decrease from 120 to 113 units.

The observation that the PS II fluorescence was quenched specifically in \( F_v \) and not in \( F_o \) indicates that chloroplasts in the bean leaflets exposed to photoinhibitory treatment exhibited reaction center quenching produced by accumulation of \( P680^+Q^- \). It is characteristic of this type of quenching that it affects only \( F_v \) but not \( F_o \), a conclusion indicated by studies of UV effects (Malkin and Butler, 1968; Yamashita and Butler, 1968) and ferricyanide effects on photosynthesis (Okayama and Butler, 1972; Butler, 1978). The partial loss of \( F_v \), 740 can also be explained by \( P680^+Q^- \) quenching, which would decrease the amount of excitation energy spilled over to PS I. It has been convincingly shown before that the variable portion of PS I fluorescence at 77 K is a function of the state of the traps of PS II (Butler, 1978).

The increased slope of the line produced by photoinhibition (curve A, Fig. 31) may be a sign of increased spillover, \( k_{II-I} \), but it could also be caused by an increase in the quantum yield of PS I fluorescence (\( \phi F_I \)), which was seen to increase by about 15% in a test experiment (excitation at 700 nm). The function \( k_{II-I} \) probably does not change, since \( F_o \) was unaffected. However, more studies will be needed to determine if the slope change induced by photoinhibition is produced by increases in \( k_{II-I} \) or \( \phi F_I \) or both. It can be seen in Fig. 31, curve B, that the under surface of the leaflet responded much less than did the upper surface to the photoinhibitory treatment. These results confirm the emission-spectra findings and demonstrate clearly that chloroplasts in cells of the upper surface of the leaflet became preferentially photoinhibited.

Effects similar to those described for Fig. 31, curve A, were seen in chloroplasts isolated from photoinhibited leaflets except that they were much less pronounced. This result, like the result obtained for emission spectra shown in Fig. 30, would be caused by the chloroplast preparation containing a mixture of photoinhibited and non-photoinhibited thylakoids.

Measurements of \( F_I \) and \( F_{II} \) fluorescence at 77 K from water-stressed and sun-exposed or water-stressed and shaded Nerium oleander leaves also induced a specific quenching of \( F_v \). This reaction center quenching was stronger in water-stressed, light-exposed leaves than in water-stressed, shaded leaves (this Report), and it was correlated directly to the loss of the capacity for electron transport in PS II (Year Book 79, 150-157).

It would appear from these and other studies that photoinhibition of photosynthesis can occur after photosynthesis is inhibited either directly (e.g., by removal of \( CO_2 \)) or by environmental stress (e.g., temperature and/or water stress). As demonstrated above, photo-
inhibition effects can be observed from X-Y plots of PS I vs. PS II fluorescence, as had been done in the desiccation studies of algae (Oquist and Fork, 1981). In order to test further the hypothesis that photoinhibition can occur as soon as photosynthesis is inhibited, we measured X-Y plots of PS I vs. PS II fluorescence in leaf discs that were infiltrated with DCMU and floated on water in the presence or absence of light. Figure 32, curve A, shows that the \( F_I \) vs. \( F_{II} \) plots of dark control leaf discs \( \pm \) DCMU-infiltration were identical; both lines had the same slopes, the same \( F_m \) values, and about the same \( F_o \) values. By contrast, a 5-min exposure of the DCMU-infiltrated leaflet to white light produced a marked quenching of \( F_u \) (Fig. 32, curve B) analogous to the effects of the high light, chilling-induced effects on \( F_u \) in bean leaflets (compare Fig. 31, curve A). However, photoinhibition in the presence of DCMU produced no slope or \( \alpha \) changes but caused an increased \( F_o \). The increase in \( F_o \) may have been produced by a fraction of the reaction centers that did not become reoxidized during the 1-5 min dark periods which followed the photoinhibitory treatment before freezing to 77 K.

The preferential loss of PS II emission observed in photoinhibition studies at room temperature (Critchley and Smillie, 1981), at 77 K (Powles et al., 1979), and in this study can be ascribed to some type of damage to PS II. Photoinhibition effects could not be alleviated by adding to PS II electron donors that can bypass the water-splitting complex (Critchley, 1981). This result suggests that the site of damage lies at or very close to the PS II reaction center. The present results suggest that the primary damage done by photoinhibition must be in the immediate environment of P680 or in the protein, carotenoids, or neighboring lipids with which P680 is associated (see Satoh and Fork, this Report).

The loss of \( F_u \) in photoinhibited leaves may be caused by the creation of a more oxidizing environment that allows accumulation of oxidized P680. Such a condition is conducive to an interaction between excitation energy and oxygen, resulting in the formation of singlet oxygen. This species of oxygen is thought to be very active in hydrophobic environments such as PS II (Fridovich, 1976), where it can initiate potentially destructive oxidative reactions.

**REFERENCES**


Last year we reported on the effect of water stress on the photosynthetic characteristics of *Nerium oleander* (Year Book 79, 150–157). It was found that next to stomatal responses, photosynthetic electron transport and photophosphorylation are the component processes most sensitive to water stress.

Our preliminary observations indicated that high irradiance levels may aggravate the detrimental effects of water stress. During the past year we have therefore studied the interactions between irradiance level and leaf water status on photosynthetic reactions in *N. oleander* plants.

For this purpose, plants were grown in soil (18-l containers) in the Department's experimental garden during spring and summer of 1980. In late summer, water stress was imposed on some of these well-established plants by withholding the water supply to the soil. The leaf water potential ($\psi_w$), monitored several times weekly, was allowed to fall from about $-1.0$ to $-6.0$ MPa over a one-month period. Plants serving as controls were supplied with ample water throughout the experiment. One set of stressed plants and control plants received full daylight irradiance levels while another set was placed in the shade, receiving 5–10% of full daylight irradiance. All leaves used for experiments were kept horizontal throughout the treatment period. Leaf water potential, stomatal conductance, chlorophyll content, coupled and uncoupled electron transport, and the fluorescence kinetics at liquid nitrogen temperature were followed periodically on stressed and control plants during the treatment period. The methods used here have been previously described (Year Book 79, 150–157; Fork, Öquist, and Powles, this Report).

Some of the results of these studies are summarized in Tables 7 and 8. The values for whole-chain electron transport by isolated chloroplasts and variable fluorescence ($F_v$) at 695 nm from stressed leaves are expressed as percentages of those obtained from concurrent measurements on control plants.

It is evident from Table 7, that in plants exposed to the full daylight levels under which the leaves had developed, the rate of uncoupled whole-chain electron transport showed a progressive decline as the leaf water potential decreased. The control values, as well as the chlorophyll content of both control and stressed plants, did not show any marked variation during the treatment period. Photosystem I–driven electron transport was much less affected by low leaf water potential than whole-chain electron trans-
TABLE 7. Effect of Leaf Water Potential ($\psi_w$) on the Uncoupled Rate of Whole-Chain Electron Transport (ET) of Isolated Chloroplast Membranes of *Nerium oleander*, Maintained in the Sun and in the Shade

<table>
<thead>
<tr>
<th>$\psi_w$ MPa</th>
<th>Whole-Chain ET, % of Watered Control</th>
<th>Photosystem I ET, % of Watered Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sun</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-3.9</td>
<td>79</td>
<td>99</td>
</tr>
<tr>
<td>-4.2</td>
<td>63</td>
<td>83</td>
</tr>
<tr>
<td>-6.1</td>
<td>13</td>
<td>42</td>
</tr>
<tr>
<td>Shade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-3.9</td>
<td>92</td>
<td>88</td>
</tr>
<tr>
<td>-4.5</td>
<td>93</td>
<td>96</td>
</tr>
<tr>
<td>-6.0</td>
<td>66</td>
<td>83</td>
</tr>
</tbody>
</table>

*For watered control plants, $\psi_w$ ranged from -0.5 to -1.2 MPa and the rate of electron transport (H$_2$ O - MV) for these plants was 107 ±6 (sun) and 79 ± 17 (shade) mmol O$_2$ s$^{-1}$ Chl s$^{-1}$ at 25°C.

TABLE 8. Effect of Leaf Water Potential ($\psi_w$) on Variable Fluorescence ($F_v$) from the Upper Surface of Leaves of *Nerium oleander*, Maintained in the Sun and in the Shade

<table>
<thead>
<tr>
<th>$\psi_w$ MPa</th>
<th>$F_v$, % of Watered Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sun</td>
<td></td>
</tr>
<tr>
<td>-1.9</td>
<td>73</td>
</tr>
<tr>
<td>-2.7</td>
<td>20</td>
</tr>
<tr>
<td>-6.0</td>
<td>13</td>
</tr>
<tr>
<td>Shade</td>
<td></td>
</tr>
<tr>
<td>-3.4</td>
<td>119</td>
</tr>
<tr>
<td>-5.8</td>
<td>111</td>
</tr>
<tr>
<td>-6.3</td>
<td>72</td>
</tr>
</tbody>
</table>

*Fluorescence emission at 695 nm was measured at liquid nitrogen temperature, as described by Fork, Oquist, and Powles (this Report). For the present purpose we define variable fluorescence as $F_v = \left( F_m - F_o \right) / F_m$, where $F_m$ and $F_o$ denote the maximum and the initial fluorescence yields, respectively. The $F_v$ values for the control plants were 2.4 ± 0.2 (sun) and 4.7 ± 0.4 (shade). It should be noted that because of light gradients in the leaf, high-light-induced inactivation is likely to have a greater effect on the fluorescence characteristics of the upper leaf surface than on the rate of electron transport of a population of chloroplasts isolated from exposed leaves.

Port. Also, there was no marked change in the ratio between the rates in the presence and in the absence of added uncoupler, either for whole-chain or photosystem I-driven electron transport (data not shown). As shown in the lower part of Table 7, the effect of $\psi_w$ on whole-chain electron transport was much smaller when the leaves were kept in the shade than when they were exposed to full daylight. Much lower water potentials were required to produce a significant inhibition of electron transport, and at any given water potential this inhibition was much less pronounced in the shade than in full daylight.

As shown in Table 8, plants exposed to full daylight at low leaf water potential exhibited a progressive decline in the variable fluorescence ($F_v$) emitted from photosystem II of leaves at 695 nm. This decline in $F_v$ was primarily caused by a decrease in the $F_m$ level; there was no pronounced or consistent change in the $F_o$ levels with changes in $\psi_w$. The fact that water-stressed leaves exposed to high irradiance had a specific quenching of $F_v$ of photosystem II and not $F_o$ indicates that this treatment led to an inactivation of photosystem II reaction centers as a result of a type of reaction center quenching that is discussed in another section of this Report (Fork, Oquist, and Powles).

As shown in the lower part of Table 8, water stress had only a small effect on $F_v$ over a very wide range of $\psi_w$ when the leaves were kept in the shade during the stress treatment period. These results are in close agreement with those obtained in the electron transport measurements on isolated chloroplasts.

The results of this study demonstrate that there is a close interaction between irradiance level and leaf water status on inactivation of photosynthetic reactions. The inactivation of photosystem II activity and the quenching of the photosystem II reaction centers were much more pronounced...
when water stress was combined with a high irradiance level than when it was combined with a low irradiance level. However, studies now in progress show that leaves of *N. oleander* grown under low or moderate irradiance levels suffer substantial photoinhibition when exposed to full daylight irradiance levels, even when the leaf water potentials remain as high as $-0.5 \text{ MPa}$. These photoinhibitory effects very closely resemble those resulting when field-grown *N. oleander* plants are subjected to water stress.

These results indicate that water stress, by some yet unknown mechanism, increases the susceptibility to photoinhibition of *N. oleander* leaves. One may speculate that this is the main agent by which water stress causes damage to the photosynthetic apparatus of plants under natural conditions.

**LEAF MOVEMENT IN THE SHADE SPECIES Oxalis oregana. I. RESPONSE TO LIGHT LEVEL AND LIGHT QUALITY**

*Oxalis oregana* Nutt. (redwood sorrel) is an ubiquitous and dominant component of the evergreen vegetation of the densely shaded floor of the redwood (*Sequoia sempervirens*) forests of northern California. Although this *Oxalis* species predominates in the most densely shaded sites occupied by higher plants (about 0.5% of full sunlight), it extends to the borders of forest clearings. In more open sites, the plants may be subjected to intense sunflecks; in sites adjacent to clearings the quantum fluence rate may reach that of full sunlight and last for periods up to one or two hours. Under these conditions, changes in irradiance of about 200-fold may occur within a few seconds, thus imposing a drastic shift in the radiation environment of the leaf.

Like many other species, *Oxalis oregana* is capable of changing the orientation of its leaves, enabling it to maximize light interception as the direction of the light changes. Such maximization is advantageous in light-limited habitats. In *Oxalis oregana* and many other members of the Oxalidaceae, the leaf consists of three leaflets, each of which is attached to a single vertical petiole by its own pulvinus. These organs, serving as swivel joints, permit each leaflet to change its angle (to the horizontal plane) over a range of $\pm 90^\circ$.

Our field observations show that for most of the day, the three leaflets of *Oxalis oregana* are oriented in essentially the same plane, facing the brightest part of the forest canopy (Fig. 33, left). However, when the leaves are struck by direct solar radiation, they respond by folding downward toward a position in which the leaflets are parallel to the sun's rays, thus minimizing light interception (Fig. 33, right; this is also the nighttime position of the leaflets). This folding response is remarkably rapid. As shown in Fig. 34, the change in leaf angle, following a change in incident quantum fluence rate from approximately 4 to 1600 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$, is $20^\circ$ per min. The lag is only about 10 s and the approximately $90^\circ$ change in leaf angle (equivalent to a change in light interception from over 90% to less than 10%) is complete in approximately 6 min (Fig. 34; compare Fig. 33, right). The return of the leaves to an essentially horizontal position after departure of the direct sunfleck is a considerably slower process. In the example given in Fig. 34, there was a lag of
Fig. 33. Photographs showing the position of Oxalis oregana leaves immediately before (left) and 6 min after (right) the plant was struck by an intense sunfleck. San Mateo Memorial Redwood Park, San Mateo County, California, April 13, 1981 (a clear day), Experimental Site #2.

about 10 min, followed by a change in leaf angle at a rate of about $3^\circ$ per min. The total time required for the leaves to return to the original position after departure of the sunfleck was 30–35 min.

The effect of such changes in leaf angle on the radiation environment of the leaves, photosynthetic performance, and the role of leaf folding in minimizing light-induced injury to the photosynthetic system are considered in the following article.

Subsequent studies both in the native redwood sites and in the laboratory were undertaken to determine the relationship between quantum fluence rate and leaf folding, the action spectrum for the folding response, and the sensory mechanism involved. A brief summary of some of the results are given below.

The threshold light level for triggering the rapid leaf-folding response in young leaves is the range of 300–400 $\mu$mol quanta $m^{-2} s^{-1}$. Similar results were obtained with natural sunlight, and with xenon arc and Metal arc lamps as the light sources. Older leaves required somewhat higher light levels for triggering the folding reaction and they tended to respond more slowly than young leaves. Laboratory measurements on Oxalis plants, grown under natural shade in the garden at Stanford, showed that a minimum quantum fluence rate of 600 $\mu$mol quanta $m^{-2} s^{-1}$ was required to trigger the rapid folding reaction in 50% of the leaves of varying age for a population of 20 plants. This value may be somewhat higher than that for plants growing in their native habitat, perhaps because the average quantum fluency rate under which the leaves developed was several times higher in the shade garden than in a typical native site.

Increases in the quantum fluence rate above the threshold level decreases the lag time, increases the rate of leaf folding, and also increases the final change in leaf angle. For example, at a quantum fluence rate of 800 $\mu$mol m$^{-2}$ s$^{-1}$, the lag time was 1 min, the rate of change in leaf angle was $10^\circ$ per min, and the final change in leaf angle was $69^\circ$ for a given leaf. Corresponding values for the same leaf at a quantum fluence rate of 1500 $\mu$mol m$^{-2}$ s$^{-1}$ were 20 s, $19^\circ$ per min, and $89^\circ$.

The primary sensory organ involved
in the rapid folding reaction of *Oxalis oregana* leaves is the pulvinus itself. No folding response was obtained when the pulvinus was shaded, even when the remainder of the leaf was illuminated with bright light (2000 µmol quanta m$^{-2}$ s$^{-1}$). Conversely, full response was obtained when the pulvinus alone was illuminated and the remainder of the leaf was shaded. Since illumination of one pulvinus while shading the other two pulvini elicited a response only in the leaflet attached to the illuminated pulvinus, it appears that the responses of the three leaflets are largely independent of one another.

Although a leaf-folding reaction can be induced by mechanical agitation of the leaf or by rapidly changing the water relations of the leaf (e.g., by excision of the petiole), the primary agent in eliciting the folding reaction is light, more specifically, blue light. When wavelengths shorter than 500 nm are removed from sunlight (by applying appropriate filters), the folding reaction is totally abolished, even when the total quantum fluence rate in the visible region is kept as high as 2200 µmol m$^{-2}$ s$^{-1}$. Irradiation with wavelengths in the waveband 700–2400 nm also did not trigger a folding reaction even at intensities considerably in excess of full sunlight infrared radiation, indicating that heating effects are not responsible for the folding response. Illumination of the pulvinus, or of whole leaves, with light in the waveband 400–500 nm at a quantum fluence rate equivalent to that present in full sunlight (in the same waveband) was as effective in causing leaf folding as unfiltered sunlight, indicating that this portion of the spectrum is solely responsible for the leaf-folding response.

Finally, determinations of the effect of monochromatic light were also made in the range of 375–740 nm at approximately 25-nm intervals. (Shorter intervals were used at the wavelengths in the range 300–540 nm.) As expected, no action could be detected at wave-
Fig. 35. Action spectrum for the rapid folding response of *Oxalis oregana* leaves. Prior to each determination, the petiole of each leaf was excised under water and placed in a small vial in the laboratory under illumination with white light at a quantum fluence rate of 50 μmol m\(^{-2}\) s\(^{-1}\) for at least 30 min. After the leaflets had assumed an essentially horizontal position, a beam of monochromatic light (half-bandwidth = 12–15 nm) at a quantum fluence rate of 150 μmol m\(^{-2}\) s\(^{-1}\) was focused on a 4-cm\(^2\) area centered on the junction of the three pulvini, and the change in leaflet angle (α) was determined for the next 10 min. The change in cosine α during the first 4 min was taken as a measure of the action. If no change in leaf angle could be detected in 10 min, the quantum fluence rate was raised to 300 μmol m\(^{-2}\) s\(^{-1}\), and if there was still no change in leaf angle the action was considered to be zero. Different leaf samples were used for each determination, and at least three replicates were used for determination of the action at each wavelength.

An action spectrum for the folding response in *Oxalis* generally resembles those of action spectra for a variety of phenomena involving blue-light-induced movements in algae, fungi, and higher plants. (Senger, 1980; Haupt and Feinleib, 1970). Although the action spectrum shown in Fig. 35 is not inconsistent with the notion that a flavoprotein serves as the photoreceptor, no direct evidence concerning the identity of the photoreceptor is yet available.

Implications of leaf folding in *Oxalis* leaves exposed to direct sunlight on photosynthetic function are discussed in the following article.

**REFERENCES**


As is evident from the preceding report, the shade species *Oxalis oregana* (Nutt.) responds by folding down its leaves when suddenly exposed to intense light. In this article we will consider how this response affects the radiation environment of the leaves and the role it may serve in protecting the leaves from damage by excessive radiation.

To provide answers to these questions, measurements of incident radiation, rate and extent of leaf movement, and the effect of such movement on photosynthetic performance and photoinhibition of *Oxalis oregana* leaves were conducted at different sites on the floor of a redwood forest. Complementary studies were also made in the laboratory at Stanford. The field studies were made during the period from March 30 to April 20, 1981, in the Wurr Flat area within the San Mateo Memorial Park, San Mateo County, California. We thank Mr. William Lawrence, Park Supervisor, for his interest and help in this project. The study period, characterized by clear skies, was preceded by heavy rainfall, ensuring excellent soil-water relations during our studies.

Our mobile laboratory, used in these studies, in addition to its usual equipment for continuous gas-exchange analysis, was equipped with instrumentation for micrometeorological and fluorescence kinetic measurements. The latter instrument is similar in design to that described elsewhere in this Report (Fork, Öquist, and Powles), except that it is field-portable and housed in a special enclosure, thereby permitting us to maintain the leaf samples in total darkness for a period before and during the addition of liquid nitrogen to the leaf sample in the optical system. Fluorescence emission was measured at 690 nm; excitation was at 470 nm with a quantum fluence rate of 0.4 μmol m\(^{-2}\) s\(^{-1}\).

For measurements of photosynthetic gas exchange in situ, a single leaf (three leaflets) with its petiole was enclosed in a specially designed chamber with a transparent, hemispherical top. This arrangement permits illumination of the leaf with a natural light field and allows free movement of the leaflets. Wire supports were used to maintain the leaf in a horizontal position when desired.

Studies were obtained from plants of *Oxalis oregana* in three native sites described below. Not shown are additional studies of the daily carbon balance obtained from *Oxalis* at three sites.

Of the study sites, Site #1, in the deep shade, was never reached by sufficiently bright light to trigger a leaf-folding response or to supersaturate photosynthesis. The total daily quantum fluence rate at Site #1 was 0.73 mol m\(^{-2}\) day\(^{-1}\) and the peak fluence rate (averaged over 10 min) was 153 μmol m\(^{-2}\) s\(^{-1}\). The corresponding values for Site #2, situated on the edge of an opening in the forest, were 2.3 mol quanta m\(^{-2}\) day\(^{-1}\) and 1590 μmol quanta m\(^{-2}\) s\(^{-1}\). As much as 71% of the total daily quanta received at this site was contributed by two sunflecks, one just before and the other just after noon, the duration of the first and most intense sunfleck being about 20 min. Site #3, situated on the edge of a very large clear-cutting in the forest and facing southwest, permitted full insolation (about 1700 μmol m\(^{-2}\) s\(^{-1}\)) for periods of up to two hours. The total quanta received at Site #3 were 8.14 mol m\(^{-2}\) day\(^{-1}\); the major sunfleck, which arrived at this site at noon, contributed...
as much as 83% of the total. The Oxalis plants growing at Site #3 had a stunted appearance, and their chlorophyll content per unit leaf area was lower than in plants growing at Sites #1 and #2. Site #3 is atypical for Oxalis oregana habitats and probably represents the extreme of radiation with which this species can cope.

Figure 36 (upper) shows the effect of leaf folding on leaf inclination relative to a plane perpendicular to the sun’s rays for the three leaflets of an Oxalis oregana plant growing at Site #2. The bottom part of Fig. 36 shows the actual quantum fluence rates incident on the leaflets. In this example, leaflet #3 had essentially the same azimuth as the sun (i.e., the axis from the pulvinus through the mid-rib of the leaflet was pointing south). Leaflets #1 and #2 each had an azimuth of approximately 120° in relation to leaflet #3 (and the sun). At the instant the sunfleck struck the leaf (time = 0), the three leaflets were oriented in the same plane with an angle (β) of 14° in relation to a plane perpendicular to the sun’s rays. The solar radiation incident on the leaf was 97% (β = 14°; cosine β = 0.97) of the maximum possible (β = 0, cosine β = 1.0). Within 20 s after the arrival of the sunfleck, the leaves started to fold down, and after approximately 4 min, leaflets #1 and #2 received only 18% (cosine β = 0.18) of maximum fluence rate while leaflet #3 was still receiving about 56% (cosine β = 0.56). After about 6 min, leaflets #1 and #2 received no direct radiation (although they still received weak diffuse light) while leaflet #3 still received 30% direct light. Similar results were obtained with other Oxalis plants growing in Sites #2 and #3, as well as in our laboratory studies.

The leaf-folding response has a significant influence on the photosynthetic performance of Oxalis plants exposed to bright sunflecks in the field. In experiments conducted at Site #2, the rate of photosynthetic CO₂ uptake was measured in situ at a constant quantum fluence rate of 38 µmol m⁻² s⁻¹ (using artificial light) before and after exposure to a natural sunfleck, having an
average quantum fluence rate of 1500 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) and a duration of 18 min. (The leaf temperature was prevented from rising during the exposure.) When the leaflets were held in their original (essentially horizontal) position throughout the experiment, the rate at 38 \( \mu \text{mol quanta m}^{-2} \text{s}^{-1} \) measured after the departure of the sunfleck was 30\% lower than before the arrival of the sunfleck. (The rate returned to the presunfleck value over a 2-hour period.) In a similar experiment on the same plant, where the leaflets were permitted to fold down naturally, no such inhibition could be detected. The high-light-induced inhibition of photosynthetic rate was not caused by partial stomatal closure, since the stomatal conductance and the intercellular CO\(_2\) pressure, after the exposure to the sunfleck, were higher than before the exposure. Thus, the decline in photosynthetic rate must reflect an inhibition of intrinsic photosynthetic reactions.

It is noteworthy that the photosynthetic rate during the sunfleck was as high when the leaf was permitted to fold down as when it was held in a horizontal position. This result is understandable in view of the fact that light saturation of photosynthesis is reached at quite a low quantum fluence rate in this shade species. Our measurements show that for the *Oxalis oregana* plants at the three experimental sites the quantum fluence rates required to reach 50\% and 90\% of the light-saturated photosynthetic rate (3.0 \( \mu \text{mol CO}_2 \text{ m}^{-2} \text{s}^{-1} \)) were about 30 and 100 \( \mu \text{mol quanta m}^{-2} \text{s}^{-1} \), respectively. The light intercepted by the leaves during the sunfleck must obviously have exceeded 100 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) even when they were in the folded position. The results reported in the preceding article indicate that at incident quantum fluence rates in the range of approximately from 300 to 1200 \( \mu \text{mol m}^{-2} \text{s}^{-1} \), the leaf-folding response is not a simple "on/off" switch. Rather it may serve to adjust the leaf angle so that the light intercepted by the leaves is maintained at sufficiently high levels to permit photosynthesis to operate at light saturation while at the same time avoiding excessive light levels that would cause injury to the photosynthetic system.

Measurements of fluorescence at liquid nitrogen temperatures show that a substantial decline in the variable component, \( F_v \), takes place when *Oxalis* leaves are exposed to bright light (Tables 9 and 10). As discussed elsewhere in this Report (Fork, Oquist, and Powles; Björkman, Powles, Fork, and Oquist), such specific quenching of \( F_v \) of photosystem II with little or no ef-

<table>
<thead>
<tr>
<th>Table 9. Reduction in Variable Fluorescence After Exposure of <em>Oxalis oregana</em> Leaves to Intense Sunflecks in Native Sites #2 and #3*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Site #</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>
| 2 | 35 | 35 | 24 | (13)
| 2 | 42 | 42 | 11 | 4
| 2 | 60 | 60 | 26 | 12
| 3 | 44 | 44 | 33 | (14)
| 3 | 54 | 54 | 31 | 11
| 3 | 47 | 47 | Mean ± S.D. | 47 ± 9 | 25 ± 9 | 0 ± 12 | 9 ± 5 |

*Variable fluorescence is defined as \( F_v = (F_m - F_o)/F_o \). The mean quantum fluence rate and duration of exposure for Site #2 were 1573 ± 158 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) and 20 min, respectively. Corresponding values for Site #3 were 1638 ± 59 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) and 50 min.†The initial value for \( F_v \) at the two sites was 5.06 ± 0.75.‡Values in parenthesis indicate apparent stimulation; these values are assumed to be zero in calculations of the average reduction in \( F_v \) for the whole area of the leaf.
TABLE 10. Effect of Exposure Time and Quantum Fluence Rate on Subsequent Reduction in Variable Fluorescence ($F_v$)* in Oxalis oregana Leaves

<table>
<thead>
<tr>
<th>Quantum Fluence Rate, µmol m$^{-2}$ s$^{-1}$</th>
<th>Exposure Time, min</th>
<th>Leaves Free to Move</th>
<th>Reduction in $F_v$, % of Initial*</th>
</tr>
</thead>
<tbody>
<tr>
<td>640</td>
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<tr>
<td>1750</td>
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*($F_v$ before exp. - $F_v$ after exp.)/$F_v$ before exp.; mean value of $F_v$ before exposure was 6.20 ± 0.49 (n = 14).

†The light beam, from a Metal-arc lamp, was vertical in these laboratory experiments.

fect on $F_v$ may be attributed to photoinhibitory inactivation of the photosystem II reaction centers.

As shown in Table 9, exposure of Oxalis leaves growing at Sites #2 and #3 to intense sunflecks while preventing the leaves from folding down, resulted in 47% average reduction of $F_v$. The reduction was much smaller in experiments where the leaves were free to move. Significant reduction could only be detected in that portion of the leaf area having an azimuth similar to that of the sun; thus it was exposed to moderately bright light in spite of the folding response. In leaves permitted to fold, the average reduction in $F_v$ over the entire area of the leaf was about 9%.

Table 10 shows the time course for the reduction in $F_v$ in Oxalis oregana leaves exposed to two different quantum fluence rates in the laboratory. In this experiment, the light beam was vertical and the leaves were held in a horizontal position. It is evident from these results that the extent of the decline in $F_v$ increases with both the duration and the intensity of the exposure. There was only a small effect on $F_v$ when the leaves were free to move under these conditions when the light beam was vertical.

It should be noted that high-light-induced reduction in $F_v$ tends to be more pronounced than the reduction in photosynthetic rate. This may be expected, since most of the fluorescence signal comes from those chloroplast layers in the leaf that receive the most intense light during the sunfleck. The photosynthetic rate represents the integrated activity of all chloroplasts in the leaf.

It is noteworthy that leaves of another shade species, Trillium ovatum, growing adjacent to Oxalis oregana at Site #3, suffered a reduction in $F_v$ following exposure to the intense sunfleck at this site. This reduction in $F_v$ in Trillium (which does not possess a leaf-folding response) was about 40%, comparable to that of Oxalis plants that were prevented from folding their leaves.

The leaf-folding response of Oxalis oregana thus provides an important mechanism for minimizing photoinhibitory injury to the photosynthetic system in this shade species. It is likely that the leaf-folding response has the additional advantage of improving the water and heat balance of the leaves during exposure to high radiation. One may expect this latter function to be particularly important later in the season when soil-water relations are less favorable, temperatures are higher, and the evaporative demand is greater.
THE RELATIONSHIP BETWEEN PHOTOSYNTHETIC PERFORMANCE AND THE LEVELS AND KINETIC PROPERTIES OF RuBP CARBOXYLASE-OXYGENASE FROM DESERT WINTER ANNUALS

Jeffrey R. Seemann, James M. Tepperman, and Joseph A. Berry

Last year (Year Book 79, 146-147), we reported a survey of rates of photosynthetic CO$_2$ fixation by species of winter annuals native to and growing in Death Valley, California. While considerable variation was found, the photosynthetic capacities of these C$_3$ species were in general much higher than those of other species having this pathway of CO$_2$ fixation, and were similar to those of the most productive C$_4$ species. The original report (Mooney et al., 1976) of a high photosynthetic rate (~60 µmol m$^{-2}$ s$^{-1}$) in an individual of the C$_3$ species Camissonia claviformis suggested that such capacity could be the result of a combination of high concentrations of the CO$_2$-fixing enzyme ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) and of low diffusional resistances to the movement of CO$_2$ through the stomates. We have examined some of the physiological and biochemical characteristics of some of the more common desert winter annuals in an attempt to account both for these high capacities for carbon fixation and for apparent species-specific differences observed in the photosynthetic capacity per unit of leaf-protein nitrogen. Our results indicate that these high capacities for photosynthesis are not the result of any apparent differences in the basic processes of C$_3$ photosynthesis, but rather can be at least partially explained by levels of leaf protein, particularly Rubisco, that are significantly higher than in many C$_3$ species from a wide range of other environments (Björkman, 1980). Furthermore, leaf internal CO$_2$ concentrations consistent with and necessary for this photosynthetic performance have been demonstrated. Most notably, however, differences in photosynthetic capacity between two of these desert winter annual species appears to be the result of differences in the kinetic properties of Rubisco.

MATERIALS AND METHODS

Determinations of photosynthetic rates and stomatal conductances were made on plants occurring naturally in Death Valley, California (for details, see Year Book 79, 146-147). Leaves used for these measurements were frozen in liquid nitrogen for later biochemical analysis in the laboratory. Plants for biochemical studies were also grown in controlled-environment chambers at Stanford. Rubisco concentrations were determined quantitatively and specifically by the radioimmuno-precipitation technique of Collatz et al. (Year Book 78, 171-175). Total protein concentrations were determined by Kjeldahl analysis. Soluble protein concentrations were determined by Coomassie Blue protein binding (Bradford, 1976) after calibration against known concentrations of bovine serum albumin (BSA), spinach soluble protein, and Rubisco. It should be noted that Rubisco displayed an approximately twofold higher reactivity per unit protein than did BSA in this assay.

Kinetic studies of Rubisco were performed essentially as described by Lorimer et al. (1977), either with crude leaf extracts or with purified enzyme prepared immediately prior to experiments.

RESULTS AND DISCUSSION

The levels and relative allocational patterns of total protein, soluble pro-
tein, and Rubisco for nine species of desert winter annuals are shown in Figs. 37 and 38. Despite large differences in absolute levels of proteins, the allocational patterns between protein pools for these species appears quite consistent. Furthermore, these protein ratios are quite typical of C₃ plants in general. The protein concentrations are usually quite high, with Rubisco concentrations as high as 500 µg cm⁻² in some field-grown individuals. Apparently, these annual species possess the capacity to accumulate large quantities of nitrogen from their native environment for the production of photosynthetic machinery, most notably Rubisco. Certainly other components of the photosynthetic apparatus in addition to Calvin cycle enzymes must be concomitantly increased (i.e., chlorophyll concentration, which is maintained in a ratio with Rubisco of ~1:6 [g/g] in these species, typical of C₃ species examined to date [Berry and Downton, 1981]), and we have seen no evidence suggesting mechanistic differences between the photosynthetic functioning of these species and that of other C₃ species.

Accompanying this enhanced carboxylation capacity is the necessity for the maintenance of a sufficient internal CO₂ concentration to make effective use of such high Rubisco concentrations. Our previous study (Year Book 79, 146–147) documented the existence of high stomatal conductances to CO₂ in these species. Calculation of the ratio of internal CO₂ concentration (Cᵢ) to ambient CO₂ (Cₒ), a measure of the extent of nonstomatal limitations on photosynthesis (Berry and Downton, 1981), reveals that the value of Cᵢ/Cₒ (0.73 ± 0.04) for these desert winter annuals is similar to values reported for
other C₃ species, and is independent of the rates of photosynthesis (Fig. 39). By comparison, the value of C_i/C_o for C₄ species is typically 0.2–0.4, a consequence of the CO₂-concentrating function of this pathway. Photosynthetic capacity of these C₃ species would be strongly reduced if stomatal limitation of CO₂ uptake were comparable to that of C₄ species. The conclusion to be drawn is that the very high photosynthetic capacities of these C₃ desert winter annuals, while comparable to rates in agriculturally important and native C₄ species, are obtained by quantitative increases in the capacity of steps of C₃ photosynthesis rather than by qualitative modification of carbon-fixation pathways.

It was also noted earlier (Year Book 79, 146–147) that two closely related species of desert winter annuals, *Camissonia claviformis* and *C. brevipes* (Onagraceae), possessed rates of photosynthesis that were, on average, significantly higher than those of all other species. This difference could not be accounted for simply on the basis of proportionately higher concentrations of Rubisco or associated proteins. Individuals of *Geraea canescens* (Asteraceae), another common desert annual, have been determined to contain Rubisco in concentrations equal to *Camissonia*, yet possess lower photosynthetic capacities. This observed difference in the apparent efficiency of Rubisco between these two genera in relation to photosynthetic capacity is shown in Table 11 (right column). For equal Rubisco concentrations, the whole-leaf CO₂ fixation capacity of *Camissonia* exceeds that of *Geraea* by ~1.8 X. This difference is uncorrected for differences in C_i between species. As is shown below, however, the existence of such a difference (values of C_i for *Camissonia* were ~1.07 X greater than those of *Geraea*) cannot account for the entire difference in the efficiency of Rubisco utilization.

To provide at least a partial explanation for this phenomenon, we have examined the in vitro kinetic properties of Rubisco from these two genera. Differences in the specific activity (µmol
TABLE 11. Values for the Kinetic Efficiency of Rubisco from Two Species of Desert Winter Annuals

<table>
<thead>
<tr>
<th>Species</th>
<th>Photosynthetic Efficiency</th>
<th>Kinetic constants in vitro at 25°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmol CO₂ min⁻¹ mg Rubisco⁻¹</td>
<td>V_max (μmol CO₂ min⁻¹ mg Rubisco⁻¹)</td>
</tr>
<tr>
<td>Camissonia sp.</td>
<td>0.77 ± 0.18</td>
<td>2.85 ± 0.26 (n = 15)</td>
</tr>
<tr>
<td></td>
<td>0.73 ± 0.19</td>
<td></td>
</tr>
<tr>
<td>Geraea canescens</td>
<td>0.51 ± 0.09</td>
<td>2.40 ± 0.41 (n = 14)</td>
</tr>
<tr>
<td></td>
<td>0.41 ± 0.04</td>
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</table>

*In vitro kinetic constants were determined on the basis of ¹⁴CO₂ incorporation into acid-stable products. Photosynthetic efficiency was determined on the basis of gas-exchange analysis of whole leaves. Data for *Camissonia brevipes* and *C. claviformis* is placed together as *Camissonia* sp. Predicted photosynthetic efficiency was calculated as described in the text using kinetic constants determined in vitro for Rubisco, measured stomatal conductances and a leaf temperature of 20°C. Values of V_max and Kₘ (CO₂) were calculated by the method of Wilkinson (1961).

¹⁴CO₂ fixed · min⁻¹ · mg Rubisco⁻¹) (measured at saturating HCO₃⁻ and RuBP concentrations) of the enzyme between *Camissonia* and *Geraea* were found to parallel the previously discussed differences in the apparent efficiency of Rubisco determined on the basis of whole-leaf photosynthesis. *Camissonia* Rubisco was found to have a specific activity ~1.2 X higher than that of *Geraea*. (See Table 11 for actual in vitro values.) We have found that values for Rubisco specific activity are reproducible only within a certain range, as the enzyme in our hands suffers a nonrecoverable loss of activity within the first hour after purification. Variability in the maximal state of activation of the enzyme, despite the presence of effectors such as 6-phosphogluconate, also contributes to a degree of noise in our specific activity determinations. Determination of the Michaelis constants for CO₂ (Kₘ(CO₂)) for Rubisco from these species has revealed no significant difference between genera, as is shown in Table 11. The values of ~ 310–331 μl/liter (~10 μM) are in agreement with previously reported values in the literature for the fully activated enzyme.

In order to evaluate our results in the light of present-day knowledge of the kinetic behavior of Rubisco in the photosynthetic process, we have used a biochemical model of C₃ photosynthesis based upon the Michaelis-Menten equations for enzyme catalysis in the presence of a competitive inhibitor. This allows us to predict the whole-leaf rate of photosynthesis given our measured values of Rubisco V_max, Kₘ(CO₂), CO₂ concentration, and stomatal conductance (in order to calculate the effective internal CO₂ concentration). For our purposes here, we have assumed that both the carboxylation and oxygenation reactions of Rubisco are RuBP-saturated. This assumption is probably valid for the light and temperature regimes used during the measurements of photosynthesis considered here. This model allows us to determine on the basis of Michaelis-Menten kinetics if our biochemical measurements can account for both the relative differences observed in photosynthetic performance of *Camissonia* and *Geraea* and the absolute magnitude of their photosynthetic rates. Figure 40A demonstrates that if the model is used to predict photosynthetic rates using the specific activity of spinach Rubisco as determined by Badger (1976) (2.2 μmol/ min/mg Rubisco at 25°C) for both Ca-
missonia and Geraea, there is not exact agreement with the observed rates of photosynthesis. However, if our measured in vitro values for these two species (Table 11) are substituted in the model, the agreement between observed and predicted rates of photosynthesis for both species is improved (Fig. 40B), particularly in the case of Camissonia. These data are also summarized in Table 11, where on the basis of predicted photosynthesis per unit of Rubisco, it can be seen that a difference of ~1.5X exists between Camissonia and Geraea (after adjustment for C₃ differences), in close agreement with the observed values (Table 11).

That we can account for the rates of CO₂ fixation observed under these conditions indicates that our techniques for extraction, quantification, and kinetic analysis of Rubisco at least predict sufficient biochemical capacity. Furthermore, these techniques provide a plausible explanation for apparent differences between Camissonia and Geraea at a physiological level based upon differences in the properties of Rubisco measured in vitro. We are confident that there is a significant difference in the $V_{\text{max}}$ of Rubisco of these two species. However, other factors not yet considered in our study may affect photosynthetic performance in vivo. Most critically, we know very little concerning the control of the activation state of Rubisco (in vivo), and we have not tested the assumption that RuBP levels were saturating for CO₂ uptake under the conditions used for photosynthetic measurements in vivo. It is also conceivable that the concentration of CO₂ at the site of its fixation in the chloroplast stroma may differ significantly from that in the intercellular air spaces. Further experiments are underway to examine these possibilities and to qualify more precisely the kinetic properties of Rubisco from these and other species. How widespread variation in Rubisco kinetic properties will turn out to be remains an unanswered question, as evi-
dence for variability of this enzyme is decidedly limited (for review, see Ogren and Hunt, 1978). In conclusion it should be emphasized that these studies have considered the relationship between biochemical characteristics and whole-leaf photosynthesis studied under a single condition chosen such that Rubisco activity would likely be rate-limiting for photosynthesis.

References

SOLAR TRACKING (PHOTOTROPISM) IN LEAVES OF
Lavatera cretica AND Malva parviflora

Dov Koller

Schwartz and Koller (1978) have shown that the photoreceptor for the vectorial phototropic response of Lavatera cretica and Malva parviflora leaves is located in the lamina, probably in association with the veins, not the mesophyll or pulvinus. They have suggested that the photoreceptor is excited by oblique illumination of a vein in its plane of symmetry. The nature of the response depends on the direction of the oblique beam. A tip-oriented beam causes the vein to incline (upward) by transmitting a signal that results in an increase in turgor in the segment of the pulvinar motor tissue associated with this vein, while a similar, but base-oriented beam causes the vein to decline (downward) by decreasing turgor in the same segment.

The exact location and biochemical nature of the photoreceptor are unknown, except that it is excited by the blue, not red, region of visible radiation. Its physical organization within the cell is fundamental to its mode of action. The photoreceptor molecules could all be similarly oriented in a unique geometrical relationship to the axis of the vein, or they could be attached in a nonspecific arrangement on the surface of some optically dispersive or opaque subcellular structure situated at right angles to the axis of the vein. If the molecules are uniquely oriented, one can expect the response to be strongly dependent on angle of incidence of the oblique beam on the vein. On the other hand, in the case of the alternative arrangement any oblique illumination of the linear array of subcellular structures should result in different fluences reaching the photoreceptor molecules on their opposite sides, with relatively small effect of angle of incidence (smaller than the critical). One way of discriminating between these alternative possibilities is to establish the quantitative relationship between angle of incidence and kinetic parameters of the response. This, however, is not a simple undertaking, since as far as we know the response to light is non-inductive. This means that if the oblique beam is fixed, its angle of incidence on the lamina would change progressively as the lamina reorients, until it becomes normal to the beam. Such a situation evidently does not occur during actual solar tracking, where the
solar azimuth is continuously changing. Therefore, a way had to be found to determine the kinetics of laminar response while maintaining a constant angle of incidence.

One approach was based on the possibility that the response might be studied in detached leaves, as was the case in leaves exhibiting photonastic and circadian movements (Hillman and Koukkari, 1967; Satter et al., 1974a, b), or even in some leaves exhibiting phototropic responses (Wien and Wallace, 1973). To do this, lamina of detached leaves were placed flat on a horizontal perforated partition, through which their petioles were threaded into a water-filled vessel with transparent walls. The lamina thus remained stationary, maintaining a constant angle of incidence with a fixed oblique beam directed at it. Phototropic response at the pulvinus could then only take place by movement of the suspended petiole, a process that could be continuously observed through the transparent walls using nonphototropic illumination if necessary. However, the water relations of the leaf proved very sensitive to excision (although carried out under water, with or without the node), and led to wilting in light unless the leaves were allowed to recover in darkness for a number of hours. By that time, the submerged petioles were exhibiting curvatures unrelated to phototropism. These undesirable effects were not remedied by controlling the pH, by employing a nutrient solution, or by supplying sucrose. This approach was therefore abandoned.

A different approach was to tilt intact potted plants so as to bring a specific leaf in each of them to the desired angle of incidence with a vertical beam, and keep on tilting as to correct for the laminar reorientation and keep constant the angle of incidence. The rate of tilting of each pot could be continuously monitored. As the plants used were in the rosette stage, it was hoped that geotropic responses of the short stem, if any, would not interfere with the phototropic response. However, it was found that the geotropic stimulus was either sensed by the petioles or transmitted to them, causing them a rapid negative geotropic curvature even in darkness. This approach was therefore also abandoned.

The limitations in the two previous approaches led to the present method, which employs intact, erect plants. By this method, the light source is continuously reoriented on the lamina so as to maintain the angle of incidence constant despite laminar movement. The modified photoelectric device for recording leaf movements, developed by Tibbitts et al. (1973), was initially used. However, it was designed for a type of leaf quite different from the malvaceous, and it would have required extensive structural modifications for this purpose. Hence, a manually operated apparatus was designed and constructed, in which the pulvinus of the treated leaf is maintained in a fixed position, and changes in the angle (inclination or declination) of a single vein around this axis can be continuously followed with a resolution of 0.5°, without touching the leaf. The low infrared light source (Sylvania Cool-Lux Spot Lamp 150 PAR 38/2 SP) is directed toward the laminar surface at the desired angle of incidence, and its movement is manually linked to that of the lamina. This lamp is equipped with a dichroic reflector and can provide fluence rates up to 1000 μmol m⁻² s⁻¹ between 400 and 700 nm uniformly on the leaf surface, without appreciably overheating the leaf over extended periods. The plants are kept adequately watered and are ventilated by means of a fan. On the basis of extensive testing and modification of the prototype, additional devices are under construction and nearing completion. These will enable replication and averaging of results of simultaneous and equal treatment, as well as simultaneous comparison of different treatments.
During its period of testing, the prototype has already yielded some significant observations. For instance, at a given angle of incidence, the lamina can sustain a reorientation at a constant angular velocity for considerable lengths of time (about 1 h), before changing the rate (usually to a slower one). A stationary phase of variable duration (and of unknown origin) may occur, most commonly in the transition to a new angular velocity. In suitable leaves, angular velocity may readily exceed that of the earth's rotation (i.e., the sun's azimuth), even though irradiance is only a fraction of the sun's. Maximal rates obtained were about 90° h⁻¹ (inclination or declination). Vectorial excitation of the lamina is transmissible to the petiole (which is shaded against direct phototropic excitation), causing it to bend toward the beam, thus enhancing the reorientation by the pulvinar response. A similar transmission was already reported from vectorially excited cotyledons to the darkened hypocotyl (Schwartz and Koller, 1980). The extent to which the petiole participates in the vectorial phototropic response depends on its mechanical resistance to bending and is thus affected by plant age, leaf age, and growing conditions. Whereas pulvinar bending results from differential, reversible changes in cell volume, bending of the petiole may or may not involve differential and irreversible extension growth. Observations on solar-tracking leaves of other species, which lack morphologically distinct pulvini, suggest that the entire petiole may be operating as a "diffuse" pulvinus.

It was also found that once laminar reorientation (declination) had proceeded for some time under constant base-oriented excitation, it could continue at virtually the same rate for a considerable period (1 h or longer) after the light had been turned off. Reexamination of previous data (obtained with a fixed beam) indicated that a similar phenomenon may take place also in the tip-oriented response. Continued reorientation in darkness following vectorial excitation could be a result of phototropic induction or of inertial translocation of solute and water that had started during vectorial excitation. This phenomenon merits further study, as well as verification for the tip-oriented response, using the leaf-tracking beam.

Previous studies showed that when a lamina which had inclined upwards in response to tip-oriented vectorial excitation is transferred to darkness, it starts to decline and comes to rest at approximately 90° to the petiole. This is the typical "night" position (until onset of the predawn reorientation to face sunrise). Base-oriented excitation of such inclined leaves causes the lamina to reorient in the same direction but at a faster rate. Furthermore, the response to a tip-oriented fixed beam increased linearly with log fluence rate (up to about 400 μmol m⁻² s⁻¹), while the response to a base-oriented fixed beam was saturated at a very low level (about 30 μmol m⁻² s⁻¹). These results suggest that (1) excitation by a tip-oriented beam may lead to active uptake of solutes into the motor cells, (2) a return to darkness stops this uptake, leading to passive dissipation of the osmotic potential gradient, and (3) excitation by a base-oriented beam accelerates the passive dissipation, possibly by increasing membrane permeability to the solutes. However, by using a base-oriented, leaf-tracking beam to elicit the declination response, it was possible to cause the lamina to decline almost vertically down, assuming quite acute angles (<90°) with the petiole, i.e., well beyond the 90° dark-equilibrium position. This suggested that the response to excitation by a base-oriented beam may result from active transport of solutes out of the motor cells.

These nearly round malvaceous leaves are equipped with seven major veins radiating from the pulvinus (ap-
approximately 40°, 85°, and 120° on either side of the midrib/central vein). It is therefore conceivable that a base-oriented beam for any single vein may simultaneously provide partial tip-oriented excitation for at least two other veins on the opposite side of the pulvinus. The active uptake on the opposite of the pulvinus could thus supplement the leakage caused by the base-oriented excitation, leading to the extension of reorientation beyond the equilibrium position.

This possibility was studied by using dissected leaves, possessing only the midvein and its immediate neighbor on either side, plus the connecting mesophyll, where the possibility of inadvertent tip-orientation is eliminated. The cut surfaces were immediately sealed with lanolin to prevent damage by desiccation. Such leaves exhibited the full response to both tip-oriented and base-oriented excitation over several cycles, suggesting that both responses may operate by means of active transport, though in opposite directions.

The photoreceptive tissues associated with the veins still await identification. Our attention was drawn to a peculiar strand of colorless collenchymatous tissue overlying the veins and protruding well above the leaf surface. In preliminary studies, surgical interruption of the strand's continuity failed to affect the vectorial response, ruling out any role of it as an optical fiber. Surgical removal of long segments (along the basal 1/2–2/3 of the major veins) did not have any apparent effect on the mechanical strength of the lamina, nor did it seem to have a significant effect on the capacity for the vectorial phototropic response, even when the intact portions were shaded by a charcoal-lanolin paste. Again, the mutilated portions of the leaf were protected against desiccation by lanolin. Although these results cast doubt on participation of this tissue in photoperception, they are very preliminary and deserve further study.

**Acknowledgements**

Dr. T. W. Tibbitts, Dept. of Horticulture, University of Wisconsin, Madison, provided loan of his apparatus; Dr. R. Stout, Dept. of Botany, University of Washington, Seattle, collaborated during a brief visit to this laboratory; Mr. R. Hart of this laboratory gave invaluable assistance in the design and construction of the leaf-tracking apparatus.

**References**

The major emphasis of the past year's research has been to determine if the general features of mRNA-coding DNA are similar to or different from total DNA. Two basic approaches have been taken: the first is a comparison of the pattern of repeat sequence interspersion in coding and noncoding regions of the pea genome. The second approach is to compare the chromatin organization of coding and noncoding DNA in pea and wheat.

Pea Coding DNA Sequence Organization. Numerous studies on repeat sequence interspersion have followed Britten and Davidson's (1969) hypothesis that interspersed repetitive elements might play a role in the coordinate regulation of transcription. Data in sea urchin and mouse suggest that most transcribed sequences are contiguous with repetitive elements (Davidson et al., 1975; Costantini et al., 1980; Kuroiwa and Natori, 1979), and fine-scale analysis of specific genes has often revealed repetitive sequences in close proximity to transcribed DNA (e.g., Adams et al., 1980; Coggins et al., 1980; Duncan et al., 1979). However, it is not yet entirely clear whether the occurrence of repeats adjacent to mRNA-coding sequences reflects a functional relationship or merely the random dispersal of repetitive elements.

The pea genome is considerably larger and characterized by more extensive short-period repeat sequence interspersion than any of the animal systems used in the above studies. From previous work, we know that 85% of 1300-nucleotide-long randomly sheared pea DNA fragments contain highly repetitive sequences (about 5000 copies per haploid genome) and essentially all of the rest contain repeats present in about 50 copies per haploid genome (Murray et al., 1978). It is also clear that the sequences transcribed into mRNA cannot represent more than 1–2% of the pea genome (Murray et al., 1981). Consequently, it was of interest to determine the extent to which the pattern of repeat sequence organization in the small fraction of the DNA that contains genes reflects that seen in the total genome (Murray and Thompson, in press).

To address this question, randomly sheared pea DNA fragments 1300 nucleotides in length were separated on the basis of whether or not they contain highly repetitive elements by means of DNA reassociation and hydroxylapatite fractionation. The relative concentration of sequences complementary to mRNA in the fractions of long fragments that did or did not also contain highly repetitive elements were then compared by following the reassociation of a cDNA tracer when driven by these two fractions as well as with total pea DNA. The mRNA-coding sequences were found to be enriched in the small fraction (about 10%) of 1300-nucleotide fragments that did or did not also contain highly repetitive elements were then compared by following the reassociation of a cDNA tracer when driven by these two fractions as well as with total pea DNA. The mRNA-coding sequences were found to be enriched in the small fraction (about 10%) of 1300-nucleotide fragments that did or did not also contain highly repetitive elements. They were found to be depleted in the fraction of long fragments bearing the high-frequency repeats.

The fraction of long fragments enriched for mRNA-coding sequences but depleted in high-frequency repeats was found to be composed primarily of low-frequency repeats and single-copy sequences. Analysis of the thermal stability of these low-frequency repeats indicated that they had undergone considerably less sequence divergence than the average pea repeat. The experimental approach did not allow us to conclude whether or not the mRNA-coding sequences were present on the same 1300-nucleotide-long fragments as the low-frequency repeats. The data
do however provide evidence that the genes occupy a significantly different domain of genome organization. Whereas most sequences in the pea genome are within 1300 nucleotides of a highly repetitive element, the genes themselves are not. In contrast, any repeats that may lie within a distance of 1300 nucleotides of an mRNA-coding sequence must belong to a distinctive subset of pea repeats characterized by a relatively low copy number and an unusually low degree of sequence divergence.

To facilitate a more precise analysis of sequence organization in gene-containing regions, considerable effort was devoted to the isolation of pea DNA long fragments which contain transcribed sequences. These experiments were based on the observation that RNA:DNA hybrids are more stable than DNA duplexes in concentrated trichloroacetate salt solutions (Chien and Davidson, 1978). The optimum condition for the formation of RNA:DNA duplexes was found to be 45°C in 3.0 M sodium trichloroacetate. Hybrid formation proceeded at a rate equivalent to that observed in 0.12 M sodium phosphate at 60°C with little evidence of nucleic acid degradation, at least for a period of about 48 h. Since the $T_m$ for duplex DNA in 3.0 M sodium trichloroacetate is about 32°C, DNA:DNA renaturation could be completely eliminated. By treating the hybridization mixture with 1.0 M glyoxal (see Kaback et al., 1979) in 3.0 M sodium trichloroacetate at room temperature, the nonhybridized DNA could be rendered nonrenaturable, thereby permitting further manipulations in conventional solvents.

Two approaches were used to isolate hybrid molecules. First, the RNA was mercurated prior to reassociation and the hybrids retained by sulfhydryl agarose affinity chromatography (Dale et al., 1975; Brown and Balmain, 1979). The second approach was to use poly-U sepharose to retain hybrid molecules involving polyadenylated RNA. While both approaches seemed feasible in model experiments, neither proved practical for the isolation of gene-containing fragments of total pea DNA. From 1 to 3% of starting 5000–10,000 base pair fragments were typically retained upon the first cycle of fractionation. While a fairly low fraction was expected, it soon became obvious that any contamination with nonhybrid DNA would pose serious problems. It did not prove practical to prepare sufficient quantities of gene-containing fragments through several cycles of fractionation in order to allow further characterization. The general approach might be more useful in a smaller genome such as the mung bean, where the fraction of coding DNA is expected to be much larger than in pea.

*Studies on Wheat and Pea Chromatin.* Experiments are now in progress to compare the chromatin organization of coding and noncoding DNA in both pea and wheat. A vast body of evidence in animal systems suggests that transcriptionally active sequences are in an altered chromatin conformation. One of the major findings leading to this conclusion is that potentially active genes are more sensitive to digestion by DNAses than are the bulk of the DNA in intact nuclei and chromatin. Very good correlations exist between the increased nuclease sensitivity and the preferential localization of a distinctive class of nonhistone chromosomal proteins called high-mobility-group proteins (reviewed in Mathis et al., 1980). To date, no comparable data exists for higher plants.

Experiments done in collaboration with Dr. Steven Spiker at Oregon State University have examined whether transcriptionally active chromatin in wheat is preferentially sensitive to DNAse I. Following approaches developed in animal systems (Mathis et al., 1980), we isolated wheat chromatin or nuclei from unimbibed wheat germ and treated these preparations with DNAse
such that from 5% to 20% of the DNA was rendered acid soluble. We then compared the relative concentration of mRNA-complementary sequences in the DNAs-resistant DNA to that in total DNA by following the reassociation of a cDNA tracer when driven with the various DNAs. cDNAs prepared from either dry embryo total poly-A⁺ RNA or 3-h-imibed polysomal poly-A⁺ mRNA have been used with similar results.

In initial experiments using wheat chromatin isolated according to Simon and Becker (1976), no preferential digestion of coding sequences was observed. However, this method involves the use of 50 mM ammonium sulfate and thus may have permitted some histone redistribution during chromatin isolation. When experiments were repeated using chromatin isolated under low ionic strength conditions, preferential digestion of mRNA-coding sequences was observed. Preferential digestion was also seen when intact nuclei isolated under low ionic strength were used. Conditions that lead to the preferential digestion of transcriptionally active chromatin also lead to the release of high-mobility-group proteins. The data in wheat are thus consistent with those in various animal systems. An interesting difference in the wheat studies is that whereas all previous work in animals had been performed on transcriptionally active tissue, the studies in wheat were performed with unimbibed and thus transcriptionally quiescent embryos. It has now been well established that de novo RNA synthesis is essential for the rapid resumption of growth following imbibition (Jendrisak, 1980). The fact that the mRNA populations in dry wheat germ and in 6-h imbibed embryos are qualitatively very similar (Caers et al., 1979) would be consistent with the view that the desiccated embryo is merely an arrested stage of embryogenesis. One might logically expect that aspects of chromatin structure involved in gene regulation would be maintained in the desiccated embryo.

Differential nuclease sensitivity is also being used to examine pea chromatin, but with a somewhat different approach. In various animal systems, specific sites hypersensitive to DNase I have been identified in the vicinity of transcribed sequences. These sites are preferentially nicked with DNase treatments that do not lead to any significant degradation of the DNA (e.g., Stalder et al., 1980). Experiments are under way to compare the relative susceptibility to nicking for a variety of pea sequences: a transcribed tandem array as represented by ribosomal genes, a nontranscribed tandem repeat as represented by a cloned repeat probe, and transcribed single-copy sequence.

In these experiments, intact nuclei are isolated and samples treated with varying DNase I concentrations to introduce nicks at a frequency ranging from 1 per 1000 base pairs to 1 per 25,000 base pairs. The resulting DNA size distributions are resolved on low-percentage alkaline agarose gels, and the DNA is transferred to nitrocellulose (Southern, 1975). The nitrocellulose blots are then hybridized with various 32P-labeled probes. The relative size of the complementary sequences represented by the probe can then be compared to that of the total DNA.

Initial experiments on the ribosomal genes suggest that these genes are somewhat less subject to nicking than is the bulk of pea DNA. Leber and Hemleben (1979) reported that the ribosomal genes in Brassica chromatin are less sensitive to digestion than bulk chromatin. While surprising, these results may not be totally unexpected, for a number of reasons. If only some of the 8000-odd copies of the ribosomal gene in pea (Ingle and Sinclair, 1972) are actually transcribed at any one time, then one might not expect the total population to show increased nuclease sensitivity. The lower sensitivity to nicking observed for
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ribosomal genes relative to total DNA might be explained if in pea, as has been shown in corn, the majority of the ribosomal genes are heterochromatic (Givens and Phillips, 1976). Additional work is in progress to compare the relative frequency of nicking in sequences transcribed into mRNA, as well as those in a cloned repeat showing a tandem pattern of organization.

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PHYTOCHROME CONTROL OF TRANSCRIPT ABUNDANCE IN DEVELOPING PEA LEAVES

Marylee Everett, Richard A. Jorgensen, and William F. Thompson

The pea leaf cDNA clones described previously (Year Book 79, 116–119) have been used to characterize changes in RNA populations extracted from apices of pea seedlings during greening and leaf development. Of particular interest are two of the cloned cDNAs, pEA5 and pEA28, which hybridize to RNAs that show large increases in abundance when RNAs from etiolated buds and light-grown leaves are compared. We have studied the time course of these changes and the role of phytochrome in the induction.

RNA was extracted using the method of Gilsen et al. (1974) from six-day-old etiolated pea buds and from similar pea buds greened by exposure to continuous white fluorescent light for periods ranging from 5 to 96 h. In addition, other batches of seedlings were exposed once a day during days 3, 4, and 5 of growth in darkness either to 3 min of red light or to 3 min of red light followed by 8 min of far-red light. On the sixth day, these plants were brought into white light, and RNA was extracted from their apices during greening. As an internal standard, a known amount of purified rabbit globin mRNA was added to these RNAs. They were then labeled with $^{32}$P in vitro by the polynucleotide kinase reaction and hybridized to recombinant plasmids contain-
ing the different cDNAs, including globin cDNA, immobilized in bands on nitrocellulose filters, as described previously (Year Book 79, 116–119). Autoradiographs of these filters were scanned to yield quantitative data. Further information was obtained by hybridizing \(^{32}P\) plasmid DNA to denatured RNA, which was separated by electrophoresis through agarose gels and then blotted onto nitrocellulose by the method of Thomas (1980).

RNAs homologous to pEA5 and pEA28 appear to be messages that are translated in vivo, since they are present on polysomes extracted from pea leaves. They are probably poly-adenylated because they are labeled by oligo-dT-primed reverse transcriptase in the presence of 8 mM pyrophosphate. Under these conditions, ribosomal RNA and chloroplast mRNA are labeled at least 500 times less strongly than they are with kinase. On blots of denatured RNA, pEA5 hybridizes to a single band with a size of about 700 nucleotides, and pEA28 hybridizes to a band of about 900 nucleotides and less strongly to a band of about 4400 nucleotides.

RNAs homologous to pEA5 and pEA28 are not detectable in etiolated buds above a background level of 0.01% of the total RNA population. They are slightly above this background level in RNA extracted from tissue greened for 20 h; by 96 h of growth in light they each represent about 0.02% of the total RNA. However, these amounts do not represent full induction, since in light-grown leaves they comprise 0.08% and 0.04% of total RNA, respectively. This slow development is also paralleled by increases in leaf fresh weight and total chlorophyll content, which do not reach the light-grown control values until after 120 h of growth in light.

Since phytochrome promotes leaf development (Parker et al., 1949) and speeds chlorophyll synthesis in white light (Raven and Shropshire, 1975), the effect of phytochrome on the levels of these two transcripts was determined. Buds of the red-light-treated seedlings do not possess detectable levels of either of the RNAs, but by 24 h of greening in white light each RNA is present at the level found in non-pre-treated seedlings greened for 72 h. Far-red light after each red light exposure reverses this effect. As such reversibility is characteristic of phytochrome action, the abundance of these two RNA transcripts is influenced either directly or indirectly by phytochrome action.

The two transcripts described here are relatively abundant in mature leaf tissue though their function is unknown. They could be involved in the photosynthetic process, since their abundance follows the same pattern as the accumulation of chlorophyll. We believe that other patterns of transcription in developing leaves will emerge when a recently constructed cDNA clone bank consisting of several hundred clones derived from etiolated, red-light-treated, and immature pea leaf RNA is characterized. Furthermore, investigation of a similar clone bank from mung bean leaves will provide an interesting comparison because mung bean leaves are better developed in the dark than pea leaves and green significantly faster. The methods described above will be used to investigate these new clone banks and to document further examples of light-induced alterations in transcription.

**References**

Last year (Year Book 79, 119–120), we reported preliminary observations of a set of cloned DNA fragments containing repetitive sequences from the pea genome. Among these clones were three, designated pPs 18, 42, and 84, which hybridized strongly to many of the same bands on Southern blots of pea DNA restriction fragments. This observation provided an initial indication that these three independent clones might contain representatives of the same repetitive sequence family. Further analysis in the past year has indicated that the different cloned fragments do indeed contain common repetitive elements. However, the clones are not homologous over their entire length, and the majority of sequences in each are quite distinct. Thus the cloned fragments do not represent identical elements of a simple repeating family, but instead are examples of repetitive sequences interspersed among other, unrelated, sequences in different regions of the genome.

Our first indication that the three cloned fragments contained different as well as similar sequences came from the Southern blot analyses mentioned above, since in addition to seeing bands hybridizing with all three probes, we saw bands which hybridized specifically to only one of the clones. Differences in the sizes of the cloned pieces (8.2, 2.7, and 2.2 Kb for pPs 18, 42, and 84, respectively) were also consistent with a different sequence composition. When detailed restriction maps were constructed for each clone, we could not discern any regions of homology from the spacing of restriction sites for seven different enzymes, leading us to conclude that the actual regions of interclone homology must be relatively short.

Confirmation of this hypothesis came from direct cross-hybridization experiments in which restriction fragments from one clone were hybridized to filter-bound fragments from a different clone. In this way, we could locate regions of interclone homology on the physical map of the cloned DNA. It is clear that these regions are mostly quite short; estimates range between 300 and 500 nucleotides.

It seems necessary to postulate some type of chromosomal rearrangement process to account for the presence of many short homologous sequences in a wide variety of different sequence environments. The importance of rearrangements is further suggested by the observation that the linear order and spacing of homologous regions is clearly different in at least two of our three clones.

Each of the three cloned fragments also contains a number of sequences which are repeated to various extents in the pea genome but which are not common to the cloned DNAs. These sequences have been characterized by various blot hybridization experiments involving the cloned DNA and total pea DNA. By hybridizing labeled pea DNA to filter-bound, restricted clone DNA, we can determine which regions of the clone contain sequences repeated in the pea genome. From the intensities of hybridization to different fragments, we can distinguish different repetition frequencies.

Each of these three clones contains much more repetitive DNA than is present in the common elements alone, and, in fact, there may not be any truly single-copy sequences represented. However, the repetition frequency of different elements within each clone does vary over a wide range, indicating that repetitive sequences of high and
low copy number are closely interspersed in these regions. We have previously concluded that extensive inter­
spersion of short repeats with other repeats is the characteristic pattern of organization throughout more than
90% of the entire pea genome (Murray et al., 1978, 1981).

It is noteworthy that in spite of the interspersed pattern of organization evident in the clones, each fragment hybridizes predominantly to a set of discrete bands on Southern blots of restricted pea DNA. Partial digestion experiments show that many of these bands result from tandemly repeated ar­
rays. Thermal dissociation profiles constructed by “melting” the hybrids off the pea DNA blots indicate that the hybrids formed in the band regions display a range of thermal stabilities but are generally less stable than hybrids formed in self-reactions with filter-bound clone DNA. Together with our previous analysis of the clones them­
selves, this evidence indicates that the tandemly repeated DNA sequences are related but not identical to the repeats in the clones. In other words, the clones we have studied contain representa­
tives of one or more repeat families containing both interspersed and tan­
demly repeated members.

Precisely this situation was envi­sioned on the basis of previous studies indicating considerable heterogeneity in pairing precision within repetitive sequence families of the pea genome (Preisler and Thompson, 1981; Year Book 79, 114–116). Thus, our studies with cloned DNA confirm in detail for a small sample of sequences a pattern of organization deduced from studies of the entire DNA complement. Both sets of data are consistent with a view of plant genome evolution as largely a stochastic process of amplification, di­
vergence, rearrangement, and second­
ary amplification, a process recently elaborated by ourselves (Murray et al., 1981) and others (Flavell, 1980). It is noteworthy that the use of both cloning and whole-genome approaches lends a combination of scope and precision to those conclusions that would be impos­
sible to achieve with either approach alone.

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Murray, M. G., D. L. Peters, and W. F. Thomp­

EVOLUTIONARY STABILITY OF THE HIGHER­
PLANT CHLOROPLAST GENOME

Jeffrey D. Palmer and William F. Thompson

We have used clone banks of mung bean and pea chloroplast DNA in the construction of detailed restriction maps of these two genomes and in a comparison of the linear organization of homologous sequences in these and several other higher-plant chloroplast genomes. A detailed description of part of this research is in press (Palmer and Thompson, 1981a).

A combination of double and triple digestsions of individual cloned frag­ments of mung bean and pea chloro­plast DNA (Year Book 79, 120–123; Palmer and Thompson, 1981b), as well as hybridization of the cloned frag­ments to nitrocellulose filter-bound, total chloroplast DNA restriction frag­ments, has enabled us to map all the cleavage sites for the restriction en-
donucleases Bst EII, Kpn I, Pst I, Pvu II, Sac I, Sal I, Sma I, and Xho I on the circular chloroplast chromosomes from mung bean and pea. In addition, we have located the positions of the chloroplast ribosomal RNA genes on these maps by hybridizing 16S and 23S chloroplast ribosomal RNA to filters containing chloroplast DNA restriction fragments.

The mung bean chloroplast genome measures 150 kilobases in length; it includes two identical sequences of 23 kilobases which contain the ribosomal genes and which are arranged as an inverted repeat separated by single-copy regions of 21 and 83 kilobases. The pea chloroplast genome is only 120 kilobases long, has only one set of ribosomal genes, and does not possess any detectable repeated sequences. The mung bean inverted repeat structure is common to all other nonleguminous higher-plant chloroplast genomes so far studied, while the pea structure has been found only in the closely related legume—broad bean (Koller and De- lius, 1980). We conclude from these data that loss of one copy of the inverted repeat sequence has occurred only rarely during the evolution of the Angiosperms and, in the case of the legumes, only after the divergence of the mung bean line from the pea—broad bean line.

We have compared the order of homologous sequences in the mung bean and pea chloroplast genomes by using each of 14 different cloned mung bean fragments, which together represent 99% of the mung bean genome, as hybridization probes against filter-bound pea chloroplast DNA restriction fragments. A summary of these experiments (Fig. 41) indicates that a number of rearrangements (deletions, insertions, transpositions, inversions) have occurred which have scrambled the order of sequences common to the mung bean and pea chloroplast genomes. In similar sets of experiments we find that sequences shared

Fig. 41. Arrangement of homologous sequences in the mung bean and pea chloroplast genomes. Fourteen nonoverlapping, cloned mung bean restriction fragments representing 99% of the genome were each hybridized to replica nitrocellulose filters containing both pea and mung bean Pst I, Sal I, and Sma I fragments separated on a 0.7% agarose gel. The extent of the mung bean fragments used as probes is indicated by the two lines which converge above the fragments, while the size of the fragments in kb is given below. The pea fragments to which the mung bean probes hybridize are indicated by the lines leading from the mung bean fragments to the pea fragments. Wherever two different mung bean fragments hybridized to the same pea fragment it was usually possible to differentiate which portion of the pea fragment hybridized to a given probe by determining which adjacent pea fragment(s) hybridized to the same probe. Restriction sites are represented by triangles (Pst I), squares (Sal I), and circles (Sma I).
by the mung bean and broad bean chloroplast genomes, as well as by the pea and broad bean chloroplast genomes, also show extensive rearrangement. When we compare the organization of the mung bean chloroplast genome with those of several plants outside the legume family, we find, quite surprisingly, that these plants share a strikingly similar pattern of chloroplast DNA sequence organization. For example, we can detect only a single rearrangement (a large inversion of approximately 50 kilobases of DNA within the large single-copy region) between mung bean and spinach chloroplast DNAs (Fig. 42). The petunia and cucumber chloroplast genomes are identical to that of spinach in gross organization, i.e., they also possess a large inversion within the single-copy region and in all other respects have the same organization as mung bean. The corn chloroplast genome also features the large inversion and, in addition, has one other rearrangement—an apparent transposition of several kilobases of DNA, again within the large single-copy region—that distinguishes it from the above four chloroplast genomes.

At first glance, this situation may seem paradoxical: the linear order of shared sequences is far more conserved between mung bean and four species of plants outside the legume family than between mung bean and two other legumes—pea and broad bean. Note, however, that mung bean, spinach (Herrmann and Possingham, 1980), petunia (Bovenberg et al., 1981), cucumber (unpublished data), and corn (Bedbrook and Bogorad, 1976), whose chloroplast genomes are very similar in sequence arrangement, all possess the inverted repeat sequence of 20–25 kilobases containing the ribosomal RNA genes. In contrast, extensive sequence rearrangements are found only in comparisons involving two plants, pea and broad bean, which have lost the inverted repeat. In light of this correlation, we hypothesize that the inverted repeat is a strong stabilizing factor in chloroplast genome evolution, such that those chloroplast genomes possessing the inverted repeat are very intolerant to gross sequence rearrangements, while those genomes which have lost the inverted repeat sustain rearrangements at a much higher frequency.

Fig. 42. Arrangement of homologous sequences in the mung bean and spinach chloroplast genomes. Experimental conditions and figure nomenclature are the same as in Fig. 41, except that hybridization of mung bean clones was to spinach rather than pea Pst I, Sal I, and Sma I restriction fragments.
A SURVEY OF THE RECIPROCITY RELATIONSHIPS FOR RESPONSES TO END-OF-DAY IRRADIATIONS IN FOUR PLANTS

Holly L. Gorton and Winslow R. Briggs

For phytochrome-mediated responses, tests of the Bunson-Roscoe reciprocity law have yielded basic information about the kind of photobiological control involved. Classical low-fluence, reversible, phytochrome-mediated responses in etiolated seedlings generally obey the reciprocity law. Light exposures of equal fluence elicit equal responses whether they are delivered as short intense or long dim irradiations. The interpretation is that these low-fluence responses must be limited by a single photoproduct, presumably Pfr. High-irradiance phytochrome-mediated responses generally show reciprocity failure such that long dim exposures are more effective than short intense exposures of the same total fluence (Mancinelli and Rabino, 1978). This pattern occurs because the light must be on for the high-irradiance responses to proceed. When the light is switched off, the response stops. Together with other evidence, this reciprocity failure suggests a requirement for the presence of a low level of Pfr over a long time.

We have tested the reciprocity law for stimulation of coleoptile and mesocotyl elongation and inhibition of anthocyanin accumulation in the coleoptiles of light-grown corn in response to end-of-day, far-red (FR) irradiation. These responses are easily reversible and require only low fluences, so one would expect reciprocity to hold, as it does for the classical responses in etiolated seedlings. However, all three responses showed rapid and nearly complete reciprocity failure such that short intense irradiations were more effective than long dim ones of the same fluence (Year Book 79, 128–131). This is opposite from the direction of reciprocity failure for the high-irradiance responses and hence is termed “reverse reciprocity failure.” Red (R) reversal of these three end-of-day effects in corn also showed rapid reciprocity failure, and again, short intense irradiations were more effective than long dim ones of the same fluence.

The rapid, reverse reciprocity failure for FR and R is difficult to explain in terms of Pfr action and the current phytochrome dogma. Thus, it was of interest to determine whether rapid, reverse reciprocity failure is a common occurrence for responses to end-of-day irradiations in light-grown plants or whether it is an idiosyncrasy restricted to corn. We have examined the reciprocity relationships of the end-of-day FR effects on oat coleoptile elongation, sunflower and mung bean hypocotyl elongation, and anthocyanin synthesis in mung bean hypocotyls. We have also examined one additional response to end-of-day FR in corn—phytochrome-mediated stimulation of its own accumulation. We have tested reciprocity for R reversal of the FR effects for all these responses except for hypocotyl elongation in sunflower.
TABLE 12. Photobiological Data for End-of-Day FR Induction and R Reversal of Phytochrome-Mediated Responses in Light-Grown Seedlings*

<table>
<thead>
<tr>
<th>Organ</th>
<th>Response to end-of-day FR</th>
<th>FR</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Threshold/ Saturation</td>
<td>Does Escape</td>
</tr>
<tr>
<td></td>
<td></td>
<td>log nmol cm⁻² (approximate)</td>
<td>Reciprocity Begins</td>
</tr>
<tr>
<td>Corn</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mesocotyl + elongation</td>
<td>1.3/2.5</td>
<td>reverse</td>
<td>1h</td>
</tr>
<tr>
<td>coleoptile + elongation</td>
<td>1.3/2.5</td>
<td>reverse</td>
<td>1h</td>
</tr>
<tr>
<td>coleoptile − anthocyanin accumulation</td>
<td>1.3/2.5</td>
<td>reverse</td>
<td>1h</td>
</tr>
<tr>
<td>leaf + phytochrome accumulation</td>
<td>1.3/3.0</td>
<td>reverse</td>
<td>&gt; 1h</td>
</tr>
<tr>
<td>Oat</td>
<td>coleoptile + elongation</td>
<td>1.3/2.6</td>
<td>reverse</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sunflower hypocotyl + elongation</td>
<td>1.3/3.0</td>
<td>reverse</td>
<td>100 s</td>
</tr>
<tr>
<td>Mung Bean hypocotyl + elongation</td>
<td>1.3/3.0</td>
<td>little or no reciprocity failure or escape for at least 2.5 h</td>
<td>-0.5/2.0</td>
</tr>
<tr>
<td></td>
<td>hypocotyl − anthocyanin accumulation</td>
<td>1.3/3.0</td>
<td>little or no reciprocity failure or escape for at least 2.5 h</td>
</tr>
</tbody>
</table>

*Threshold and saturation values were estimated from fluence-response curves obtained by varying time at a set fluence rate as follows. For FR: corn mesocotyl, coleoptile, and anthocyanin responses, 6.4 nmol cm⁻² s⁻¹; corn phytochrome accumulation response, 5.2 nmol cm⁻² s⁻¹; oat coleoptile response, 4.4 nmol cm⁻² s⁻¹; sunflower and mung bean responses, 4.8 nmol cm⁻² s⁻¹. For R: corn phytochrome accumulation response, 0.10 nmol cm⁻² s⁻¹; all other responses, 0.09 nmol cm⁻² s⁻¹. N.T. = not tested.
sue sections (coleoptile plus leaves). The amount of red light that emerged from the cut end of the tissue section was measured with a photomultiplier. All tissue sections were gently bent to prevent direct illumination of the photomultiplier. The log of the axially transmitted light expressed as a function of tissue length was the same for both mesocotyl and leaf tissues \( y = -0.135x + 1.86, r^2 = 0.97 \). These organs can transmit laterally applied light over at least 25 mm, a distance greater than that expected if light were randomly scattered through the tissues. The presence of the coleoptilar node or tip attenuated the axially transmitted light relative to the amount transmitted through tissue sections devoid of these anatomical structures. Tissue damage also significantly decreased the amount of axially transmitted light.

If we assumed there was a discrete site of photoperception, our quantitative data on light piping by etiolated tissues enabled us to localize more precisely the site(s) of photoperception. First, we did a fluence-response curve at the 2-mm region, where 100% response for the VLF and LF was obtained. Second, we solved the light-piping equation for tissue lengths (i.e., values of \( x \)) from 0 to 19 mm distant from the hypothetical site of perception. This told us how much light was reaching the photosensitive site, i.e., what the effective fluence was when we had irradiated different 2-mm regions of the intact seedlings. Third, we determined what the response of the seedling would have been had that fluence been given directly to the 2-mm region that gave the maximum response (above), simply by reading the expected response from the fluence-response curve done with a 2-mm irradiation field. The net result of these steps was a theoretical response pattern for 2-mm irradiations for which we knew precisely the location of the most sensitive site of perception (where \( x = 0 \)). This pattern could now be matched with our empirically obtained response pattern to 2-mm irradiation fields to determine the site of photoperception in the intact, etiolated seedlings. Light attenuation by coleoptilar node and tip was accounted for in the final analysis.

In conclusion, when the fiber optic properties of the seedling are known, the mesocotyl response can be explained using one site of photoperception within the mesocotyl itself. The coleoptile, however, apparently depends on two discrete sites of photoperception, one below the node (i.e., in mesocotyl tissue) and one above the node (i.e., in coleoptile or leaf tissue). It is interesting to note that neither site of photoperception is correlated with the node or tip regions where phytochrome, as detected spectrophotometrically (Briggs and Siegelman, 1965; Pjon and Furuya, 1968) or immunologically (Pratt and Coleman, 1974), is most concentrated. Hence, the total amount of phytochrome conversion is not the limiting parameter in the determination of the photomorphogenic response. Although phytochrome is found throughout the etiolated oat seedling (Briggs and Siegelman, 1965; Pratt and Coleman, 1974), the state of most of the phytochrome is apparently immaterial to these two photomorphogenic responses; only phytochrome conversion from about 4.5 to 6.5 mm below the node and from 1.5 to 2.5 mm above the node determines the photomorphogenic response of the plants.

The potential ecological significance of this light-piping property of etiolated oats is also addressed (Mandoli and Briggs, 1981b). A biophysical study which deals directly with axial light transmission within these tissues is under way.

References

The general experimental plan has been described (Gorton and Briggs, 1980). To allow phytochrome measurement in the light-grown corn plants, seeds were imbibed and seedlings grown in the presence of the chlorosis-inducing herbicide Sandoz 9789 (0.2 mM) (Gorton and Briggs, 1980). For all other measurements, the seeds were imbibed and grown with water. Details of the growth and irradiation protocols varied for the different species, but all received end-of-day light treatments on at least three consecutive days. Oat coleoptiles and hypocotyls for mung bean and sunflower seedlings were harvested for length measurement after 16 h of darkness, following the final end-of-day light treatment. Mung bean hypocotyls were then lyophilized and extracted for anthocyanin measurement (Gorton and Briggs, 1980).

Com leaves were harvested 42 h after the final end-of-day treatment for phytochrome measurement. One-gram leaf samples were frozen in liquid nitrogen and kept frozen up to one week. They were then ground to a fine powder and mixed with 400 mg CaCO₃ and 400 µl buffer (1 M tris HCl, pH 7.5 with 300 mM sodium ascorbate, 15 mM dithiothreitol, and 2.8 mM sodium diethyldithiocarbamate). The final sample was a thick paste. Phytochrome was measured as Δ(ΔA) between 660 and 730 nm. There was no loss of spectrophotometrically detectable phytochrome during the storage period. Possible scattering differences between samples were eliminated by the grinding and by the addition of CaCO₃.

Fluence-response curves for FR induction of these responses and for R reversal of a saturating FR exposure were obtained with a constant fluence rate for each experiment and varied duration of irradiation. Threshold and saturation values for FR and R fluence-response curves are summarized in Table 12 along with analogous data for the mesocotyl, coleoptile, and anthocyanin responses in corn. Rapid, reverse reciprocity failure for the mesocotyl, coleoptile, and anthocyanin responses in corn causes the fluence-response curves to be shifted to higher energy when low-fluence rates are used to obtain them, but a 25-fold difference in fluence rate is necessary to get a shift in the threshold of about one-half log unit (Gorton and Briggs, 1982). It is therefore unlikely that the small differences in fluence rates used would in themselves cause any significant shift in the fluence-response curves even if rapid, reverse reciprocity failure occurred. Also, since the responses are related to the log of the fluence, most of the differences in threshold and saturation between species and between responses are considered minor.

Reciprocity was tested for responses to end-of-day FR by varying fluence rate and duration of irradiation inversely, but maintaining the same sub-saturating fluence. Reciprocity for R reversal was similarly tested for all responses except hypocotyl elongation in sunflower. Where reciprocity failed, it was necessary to determine if the failure could be explained by escape from photoreversibility (Year Book 79, 128-131; Gorton and Briggs, 1982). For FR escape tests, plants were left in the dark for varying amounts of time between the end of the daily white-light period and the beginning of a short, high-fluence-rate FR irradiation of the same fluence used in the FR reciprocity experiments. Similar tests were done for escape from R reversibility by leaving the plants in the dark for varying amounts of time between saturating FR and the beginning of a short, high-fluence-rate R irradiation of the same fluence as that used in the R reciprocity tests. These results have been summarized along with the results from the corn coleoptile, mesocotyl, and anthocyanin responses to the end-of-day FR (Year Book 79, 128-131; Gorton and Briggs, 1982) in Table 12.

It is clear from Table 12 that rapid, reverse reciprocity failure not ex-
plained by escape is not an ubiquitous phenomenon. For FR, reverse reciprocity failure occurred in all responses examined in corn and oats, but for none of the responses tested in mung beans or sunflower. For R, reverse reciprocity failure was only observed for the mesocotyl, coleoptile, and anthocyanin responses in corn (Year Book 79, 128–131; Gorton and Briggs, 1982). The cause of the rapid, reverse reciprocity failure is unknown, but FR and R reverse reciprocity failure must stem from different causes, since they are separable in the cases of the coleoptile elongation response in oats and the phytochrome accumulation response in corn.

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LOCALIZATION OF THE REGION(S) OF PHOTOSENSITIVITY IN THE ETIOLATED Avena SEEDLING

Dina F. Mandoli and Winslow R. Briggs

Two photoresponses in both the mesocotyl and coleoptile of Avena sativa (cv Lodi) have been quantitatively described (Mandoli and Briggs, 1981a). Although both the very-low- and the low-fluence response (VLF and LF, respectively) involve changes in elongation rates of these organs in response to phytochrome conversion, they can be distinguished on the basis of differences in threshold and saturation energies of red light and percentage of Pfr required, far-red reversibility, and time at which red reciprocity fails. This report localizes the site(s) of photoperception for both the mesocotyl and coleoptile when fluences for red light sufficient to saturate either VLF or LF responses are given to small areas of each organ.

A masking technique was developed which allowed irradiation of just 2-mm regions of intact etiolated seedlings. Manipulations of the plants in darkness resulted in only about a 5% response in each tissue. Groups of plants for which different 2-mm regions were irradiated were allowed to grow in the dark for an additional 24 h until harvest. The percentage response of mesocotyl and coleoptile was graphed as a function of the position of the irradiation on the intact seedling. It was clear that a saturating illumination resulted in either a 100% VLF or LF response only if an area just below the node received light.

If a discrete site(s) of photoperception were present, we expected to obtain zero response when a nonsensitive region was irradiated and 100% response when a sensitive region was irradiated. Instead, we found that irradiation of any 2-mm region resulted in a response such that both the coleoptile and mesocotyl responses declined only gradually with distance from the most sensitive region for both VLF- and LF-saturating fluences. Either variation in length of the organs in the populations of seedlings irradiated in tissue light piping or scatter within the seedlings could reconcile this broad response pattern to discrete illumination with the idea of a discrete site of photoperception. However, since statistical analysis showed that the mesocotyl and coleoptile lengths were uniform in all seedlings used, this first explanation of the data was discarded. Red light was applied at 90° to the long axis of either mesocotyl or leaf tis-
and saturation fluences as growth inhibition (Fig. 44; compare Fig. 43). The relative insensitivity of the apical cm of the mesocotyl may be related to the presence of meristematic cells. It is fortuitous that the fluence-response curves for glucan synthetase and for growth have the same slope. Growth measured at the end of 12 h following irradiation (or dark control) is the integral of growth rate (constant in dark-grown plants, changing in irradiated plants; see Vanderhoef and Briggs, 1979; Year Book 76, 283-286), while glucan synthetase measurements represent the level of activity only at the 12-h time point.

Far-red light alone significantly inhibits both growth and glucan synthetase (Table 13). Far-red light given after red light can reverse both effects of red light back to the level of far-red alone (Table 13). Green safelight causes partial inhibition both of growth and of glucan synthetase level. A similar inhibition of mesocotyl growth by green light has been reported for oat (Mandoli and Briggs, 1981). In another experiment (not shown) a fluence of 126 nmol/cm² of far-red light alone inhibited growth and glucan synthetase by 43% and 39%, respectively. A comparison of these results with the data in Table 13 suggests that a far-red fluence of about 100 nmol/cm² is saturating. This result agrees well with the sensitivity of oat mesocotyl inhibition by far-red light.

In threshold fluence (Figs. 43 and 44) and timing (data not shown), the response of glucan synthetase activity corresponds closely with "first phase" inhibition of maize mesocotyl growth (Vanderhoef and Briggs, 1979; Year Book 76, 283-286). However, there are two discrepancies between the previously obtained results (Vanderhoef et al., 1979) and those reported here. First, the magnitude of the inhibition of mesocotyl growth by red light is higher in our results than in the previous report. Second, the far-red-inducible mesocotyl growth inhibition is not seen by Vanderhoef et al. (1979). The fact that green safelights were employed in the previous work could account for these two discrepancies. Green-light-induced mesocotyl growth inhibition (Table 13) may totally account for the increment of mesocotyl inhibition found here but not previously reported. This green light fluence also may have saturated the far-red-inducible mesocotyl inhibition. In addition, the red-light-induced mesocotyl inhibition reported by Vanderhoef et al. (1979) was measured after 24 h, rather than after 12 h as reported here. Since in the present experiments growth rate inhibition by red light shows recovery within 24 h (data not shown), the apparent magnitude of the inhibition induced by red light could be significantly reduced by measuring growth after this period of time following irradiation.
RED LIGHT INHIBITION OF GROWTH AND 
GOLGI-LOCALIZED GLUCAN SYNTHETASE 
ACTIVITY IN THE MAIZE MESOCOTYL

Jonathan D. Walton,* James R. Shinkle, and Winslow R. Briggs

During the study of red-light-induced changes in auxin binding in maize mesocotyls, it was discovered that Golgi-localized glucan synthetase activity (Ray et al., 1969), but not any of several other membrane marker activities, was lowered by red light irradiation of the seedling before extraction. We have done fluence-response curves and far-red reversibility experiments to study the relationship between the light-induced inhibition of growth (Vanderhoef and Briggs, 1979; Year Book 76, 283–286) and the light-induced reduction in glucan synthetase activity, and to test for phytochrome mediation of both responses.

Unlike the response reported for the inhibition of mesocotyl growth in oat (Mandoli and Briggs, 1981), the maize variety used in our experiments (WxWx X Bear Hybrid, CFS Research, 2761 N. Main, Decatur, IL 62526) lacks the very-low-fluence response (VLFR) which is saturated at a fluence of 3 × 10⁻³ nmol/cm². A fluence of 10⁻³ nmol/cm² has no effect on growth (Fig. 43). Other experiments indicate that the fluence-response curve in Fig. 43 is saturated at or near a red light fluence of 10 nmol/cm².

Golgi-localized glucan synthetase activity has been studied in several plants. The enzyme utilizes μM concentrations of UDP-glucose as substrate, and requires Mg. In vitro, the enzyme produces β-1,4-linked glucan. In vivo, it probably makes hemicellulosic mixed-linkage glucan that is transported via the Golgi vesicles to the extracellular space. The activity of this enzyme in pea epicotyl tissue is controlled by auxin (Ray, 1973).

The reduction of glucan synthetase activity by red light in the second cm below the node, but not in the apical cm, responds with the same threshold

*Department of Biological Sciences, Stanford University, Stanford, CA 94305.
Our results indicate that the effects of red light on inhibition of mesocotyl growth and on reduction of glucan synthetase activity are mediated at least partially by phytochrome (Table 13). In comparing the glucan synthetase inhibition and growth inhibition of the maize mesocotyl with the growth inhibition of the oat mesocotyl (Mandoli and Briggs, 1981), the threshold of the response is similar to the low-fluence response (LFR), but the far-red- and green-light-induced inhibition is characteristic of the very-low-fluence response (VLFR). Mandoli and Briggs (1981) explain the effect of far-red light alone by the absorption of far-red light by $P_r$, generating significant (1-3%) levels of $P_{fr}$. Vanderhoef et al. (1979) reported a two-phase fluence-response curve, but their second phase is a high-irradiance response (HIR) which requires a high fluence and a long exposure time to potentiate the response.

The physiological significance of the existence of multiple "phases" having different threshold requirements, and which are apparently induced by differing levels of $P_{fr}$, is unclear. These phases may be found to differ considerably between varieties, as does mesocotyl growth (Inge and Loomis, 1937; Avery et al., 1937). The evidence from the experiments reported here suggests that such phases of mesocotyl inhibition may be fused into a single, continuous fluence-response curve. This result implies that, at least in the maize variety studied here, there is a continuous sensitivity of the mesocotyl inhibition to increasing levels of $P_{fr}$, and that there may be a single mode of action of phytochrome in potentiating both the far-red reversible and far-red inducible portions of the fluence-response curve.

The red light inhibition of mesocotyl growth is preventable by supplying auxin to irradiated shoots floated in solution (Vanderhoef and Briggs, 1978). Our experiments (data not shown) indicate that the effect of red light on glucan synthetase can be prevented by floating irradiated intact plants in solution containing auxin. Thus, it appears that both glucan synthetase and growth are controlled, at least in part, by a phytochrome-modulated supply of auxin.

Glucan synthetase activity in the mesocotyl at a particular time would be expected to correlate with growth rate and with levels of physiologically active auxin at that time. Therefore, the evidence presented here supports the suggestion that the activity of this en-

### TABLE 13. Partial Reversibility by Far-Red Light of the Red-Light-Induced Inhibition of Growth and Glucan Synthetase*

<table>
<thead>
<tr>
<th>Light Treatment</th>
<th>Growth (mm/12 h)</th>
<th>% of Dark Control</th>
<th>Glucan Synthetase (cpm/mg Protein)</th>
<th>% of Dark Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dark</td>
<td>28.1 ± 1.1</td>
<td>100</td>
<td>60,017</td>
<td>100</td>
</tr>
<tr>
<td>Green Safelight</td>
<td>24.5 ± 1.2</td>
<td>87</td>
<td>59,000</td>
<td>87</td>
</tr>
<tr>
<td>Red</td>
<td>10.7 ± 1.0</td>
<td>38</td>
<td>26,475</td>
<td>44</td>
</tr>
<tr>
<td>Far-red</td>
<td>17.5 ± 1.5</td>
<td>62</td>
<td>38,302</td>
<td>64</td>
</tr>
<tr>
<td>Red/Far-red</td>
<td>17.7 ± 0.9</td>
<td>63</td>
<td>36,352</td>
<td>61</td>
</tr>
</tbody>
</table>

*The plants were grown in vermiculite for 78 h before irradiation and then returned to darkness for 12 h before harvest. Plants were handled under green safelight, which itself caused some inhibition. Average mesocotyl length at time of irradiation was 35.8 ± 1.3 mm. Light sources were as described by Gorton and Briggs (1980); total red light fluence = 9.0 nmol/cm²; total far-red fluence = 846 nmol/cm². Glucan synthetase was assayed in the subapical (1-2 cm below the node) cm of the mesocotyl. Average total particulate protein was 37.7 ± 1.5 μg/segment.
zyme may be a useful indicator of the auxin status of a tissue.

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FURTHER CHARACTERIZATION OF A BLUE-LIGHT-SENSITIVE CYTOCHROME-FLAVIN COMPLEX FROM CORN COLEOPTILE MEMBRANES

Ta-Yan Leong and Winslow R. Briggs

We have previously reported the purification and solubilization of a blue-light-sensitive cytochrome-flavin complex from corn coleoptile membranes by differential centrifugation, sucrose density gradient centrifugation, and Renografin density gradient centrifugation (Year Book 79, 131-134; Year Book 79, 134-155; Leong and Briggs, 1981). A specific b-type cytochrome in the membrane preparation is reduced by blue light, and the reduction is probably mediated by a flavin moiety (Briggs, 1980).

The photoactive membrane fraction is shown to have Mg++-dependent ATPase activity which is not stimulated by K+ and which is not inhibited by oligomycin, indicating that this membrane fraction is probably associated with the plasma membrane.

The presence of flavin in the purified membrane fraction is indicated by the lumiflavin assay as well as by a fluorescence emission maximum at 525 nm, obtained upon excitation at 450 nm.

The redox potential of the Triton-solubilized b cytochrome in the purified membrane fraction was measured in the presence of pyocyanine and potassium ferricyanide under anaerobic conditions. The redox states of both the pyocyanine and the b cytochrome were monitored simultaneously using a spectrophotometer interfaced to a computer. With increasing amount of dithionite, the pyocyanine peak between 600 and 800 nm decreases in height, while the Soret difference peak of the b cytochrome at 427 nm increases in height. The ratios of log [ox]/[red] as a function of the volume of dithionite added (which itself serves as a rough function of the potential) are then plotted for the pyocyanine and b cytochrome, as shown in Fig. 45. From the equation $E_h = E_m + RT/nF \log [ox]/[red]$, with $-34$ mV as the midpoint potential of pyocyanine at pH 7.0 (Dutton and Wilson, 1974) and $n = 2$ for pyocyanine, the midpoint potential of the b cytochrome is calculated to be $-65$ mV.

The kinetics of the blue-light-induced reduction and dark reoxidation of the b cytochrome suggest that the midpoint potential of the b cytochrome is not affected by Triton X-100 solubilization, since the rates of these reactions were not significantly changed by Triton.

From the above characteristics of the flavin-cytochrome complex, it is postulated that the complex is localized in the plasma membrane. When the flavin is excited by blue light, it acquires an electron (Massey, 1979), and on return to ground state, it passes the electron onto the b cytochrome. The midpoint potential of the b cytochrome will help to determine its position in this elec-
EFFECT OF DIPHENYL ETHERS ON THE BLUE-LIGHT-INDUCED ABSORBANCE CHANGE, PHOTOTROPISM, AND GEOTROPISM IN ETIOLATED CORN AND OAT SEEDLINGS

Ta-Yan Leong and Winslow R. Briggs

Blue-light-induced phototropism in coleoptiles of grass seedlings has been extensively studied over the years (Thimmann and Curry, 1960). Although a possible blue light photoreceptor system involving a flavin-cytochrome complex has been purified and characterized from corn coleoptiles (this Report), the physiological significance of this blue-light-sensitive membrane fraction with respect to phototropism is still unknown.

Diphenyl ethers are herbicides known to inhibit electron transport at the plastoquinone-cytochrome f level in chloroplasts (Bugg et al., 1980), and it is known that light is required for them to be effective. Apparently, light energy for activation is absorbed by xanthophylls. Some toxic reaction(s) occurs that leads to a rapid increase in cell membrane permeability and subsequent death of the tissues (Matsunaka, 1976).

The effects of one of those diphenyl ethers, acifluorfen (Blazer), on the blue-

Fig. 45. Titration of pyocyanine and b cytochrome against dithionite. The redox state of pyocyanine is estimated by the height of 600–800 nm peak; that of b cytochrome by the height of 427-nm Soret peak.

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light-induced absorbance change (reduction of b cytochrome) in corn membrane preparations and on phototropism and geotropism in etiolated oat seedlings, were investigated in this study.

The presence of acifluorfen in the 21,000 × g pellet from etiolated corn coleoptiles (see Goldsmith et al., 1980) leads to a stimulation in the amount of light-induced absorbance change in Δ(A_{428} - A_{410}). Within the range of concentrations tested (from 1 × 10^{-6} M to 1 × 10^{-4} M), the stimulation increases log-linearly, as shown in Fig. 46. The increase in magnitude of the light-induced absorbance change is caused by inhibition of the dark cytochrome rather than by any effect of the herbicide on the light reaction.

The effect of acifluorfen on the blue-light-induced phototropic curvature in etiolated oat seedlings indicates that the phototropism response is sensitized by acifluorfen. In the presence of acifluorfen, the fluence-response curve (see Zimmerman and Briggs, 1963) is shifted in the direction of lower fluences by about 0.6 log unit. The degrees of curvature obtained in the control experiment at 1 × 10^{-5} and 1 × 10^{-4} M concentrations of acifluorfen after a 10 s exposure of 1 × 10^{-13} mol cm^{-2} s^{-1} blue light are compared in Table 14. Stimulations of 31% and 24% in the amount of curvature are obtained at 1 × 10^{-5} and 1 × 10^{-4} M acifluorfen, respectively.

Acifluorfen, at a concentration of 1 × 10^{-5} M, has no effect on the geotropic response in etiolated oat seedlings, indicating that acifluorfen is only active

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**TABLE 14. Effect on Acifluorfen on the Blue-Light-Induced Phototropism in Etiolated Oat Seedlings**

<table>
<thead>
<tr>
<th>Acifluorfen</th>
<th>Control</th>
<th>Acifluorfen-Treated</th>
<th>Acifluorfen-Treated/Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.5</td>
<td>13.5</td>
<td>1.17</td>
</tr>
<tr>
<td>1 × 10^{-5} M</td>
<td>14.3</td>
<td>15.9</td>
<td>1.11</td>
</tr>
<tr>
<td>Acifluorfen</td>
<td>14.8</td>
<td>21.5</td>
<td>1.46</td>
</tr>
<tr>
<td>1 × 10^{-4} M</td>
<td>15.0</td>
<td>20.8</td>
<td>1.39</td>
</tr>
<tr>
<td>Acifluorfen</td>
<td>12.7</td>
<td>20.0</td>
<td>1.57</td>
</tr>
<tr>
<td>1 × 10^{-4} M</td>
<td>12.0</td>
<td>14.1</td>
<td>1.18</td>
</tr>
<tr>
<td>Ave:</td>
<td>13.4</td>
<td>17.6</td>
<td>1.31 ± 0.18</td>
</tr>
</tbody>
</table>

*Phototropism experiments on four-day-old oat seedlings were performed as described by Zimmerman and Briggs (1963). Acifluorfen, when present, was applied in the agar medium onto which 30-hour-old seedlings were transplanted. Data were from 10 s × 10^{-13} mol cm^{-2} s^{-1} exposures.*
in the presence of light. The above results suggest that the herbicide may be acting directly at the photoreceptor site.

The good correlation between the effect of acifluorfen at $1 \times 10^{-5}$ M on the in vitro blue light reaction (Fig 46, 40% stimulation) and the in vivo phototropism response (Table 14, 31% stimulation) may indicate that the blue-light-induced absorbance change obtained with isolated membranes could be closely related to the phototropism observed in intact seedlings, and could thus be of physiological significance. The higher concentration ($10^{-4}$ M) could be inhibiting other processes as well.

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INVESTIGATIONS OF THE SHIBATA SHIFT IN ETIOLATED PRIMARY LEAVES OF CORN AND OAT SEEDLINGS

Siegrid Schoch, Holly L. Gorton, and Winslow R. Briggs

When protochlorophyll(ide) is phototransformed to chlorophyll(ide), the initial product shows an absorption maximum between 680 and 684 nm. During the next 10–40 min, this maximum shifts to about 670 nm. This spectral change was first described by (and named after) Shibata (1957). The present study addresses three questions concerning the Shibata shift.

First, do carotenoids play a role either in determining the position of the absorption maximum immediately after phototransformation or on the Shibata shift itself? For this study (on corn), we used the herbicide Sandoz 9789, a strong inhibitor of carotenoid synthesis in chloroplasts (Barbel and Deitzer, 1978) but one that has little effect on protochlorophyll(ide) formation or phototransformation (Jabben and Deitzer, 1978). Preliminary evidence suggesting that carotenoids could not play any major role in the Shibata shift appeared previously (Gorton and Briggs, *Year Book* 78, 138–139). The current study presents more detailed analysis.

Second, does the reduction of the geranylgeraniol ester of chlorophyll play any role in the shift? For this study (on oats), we used the technique of Schoch et al. (1980) for preventing reduction of the ester to phytol by anaerobiosis without significantly affecting the esterification process itself.

Finally, does proteolytic cleavage of the chlorophyll(ide) holochrome account for the shift, as suggested by Stummann (1979)? For this study, we used the inhibitor of serine proteases, phenylmethylsulfonyl fluoride (PMSF) Fahrney and Gold, 1963), already known to be an effective inhibitor of proteolytic activity in oat extracts (Pike and Briggs, 1972). Since Butler and Briggs (1966) had shown that the Shibata shift occurs, in glycerol-buffer homogenates of etiolated leaves, we used such preparations for the protease studies. The techniques for growing the seedlings, applying the inhibitors, and
preparing the glycerol-buffer extracts are all described elsewhere, as are light sources and spectral methods (Schoch et al., 1981).

The top two curves in Fig 47 illustrate room-temperature difference spectra obtained by subtracting the in vivo absorption spectrum for protochlorophyll(ide) in etiolated corn leaves from the chlorophyll(ide) spectrum obtained immediately following phototransformation. The two curves have not been normalized and both represent approximately equal amounts of tissue. It is clear that inhibiting carotenoid synthesis to less than 1% of control amounts does not affect either the amount of protochlorophyll(ide) synthesized, the absorption spectrum of the protochlorophyll, or the absorption spectrum of the immediate product of phototransformation. The lower difference spectra of Fig. 47 similarly illustrate that anaerobic treatment known to prevent reduction of the geranylgeraniol ester to phytol in oats likewise has no effect, either on protochlorophyll synthesis or on the spectral properties of both the protochlorophyll(ide) and the immediately formed chlorophyll(ide).

Figure 48 illustrates room-temperature difference spectra for the same two preparations shown in Fig. 47, but obtained by subtracting the chlorophyll(ide) curves obtained immediately after phototransformation from those obtained 20 min later, after the Shibata shift had taken place. It is clear that neither carotenoids nor geranlygera-

![Fig. 47. In vivo difference spectra from leaves from four-day-old etiolated corn (upper two curves) and from eight-day-old etiolated oats (lower two curves). Corn spectra from plants grown in the absence (above) or presence (below) of 0.2 mM Sandoz 9789. Oat spectra from plants kept in air (above) or under nitrogen (below) for 16 h prior to harvesting. Samples measured at room temperature. Difference spectra obtained by subtracting spectrum obtained just before actinic irradiation from that obtained just after.](image1)

![Fig. 48. In vivo spectra as in Fig. 47, except obtained by subtracting spectrum obtained immediately after actinic irradiation from that taken after the Shibata shift had been permitted to occur for 20 min.](image2)
TABLE 15. Relative Amounts of Four Major Spectral Components of Chlorophyll in Control and Nitrogen-Treated Plants Before and After the Shibata Shift

<table>
<thead>
<tr>
<th>Treatment</th>
<th>S.E.*</th>
<th>Error¹</th>
<th>662nm</th>
<th>669nm</th>
<th>677nm</th>
<th>684nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, 1 min L*</td>
<td>0.30</td>
<td>11.05</td>
<td>12 (11)</td>
<td>27 (12)</td>
<td>40 (12)</td>
<td>22 (13)</td>
</tr>
<tr>
<td>Control, 1 min L, 10 min D*</td>
<td>0.33</td>
<td>12.32</td>
<td>14 (12)</td>
<td>36 (12)</td>
<td>36 (12)</td>
<td>13 (13)</td>
</tr>
<tr>
<td>Nitrogen, 1 min L</td>
<td>0.39</td>
<td>9.52</td>
<td>12 (11)</td>
<td>26 (12)</td>
<td>38 (13)</td>
<td>24 (13)</td>
</tr>
<tr>
<td>Nitrogen, 1 min L, 10 min D</td>
<td>0.32</td>
<td>10.82</td>
<td>17 (12)</td>
<td>36 (12)</td>
<td>32 (12)</td>
<td>13 (13)</td>
</tr>
</tbody>
</table>

*Abbreviations: D, dark; L, light; S.E., standard error.
¹Error: Factor by which difference between original curve and sum of components must be multiplied to give the error-difference curve produced by the RESOL program.

(ii) Figures in parentheses denote half-bandwidths.

niol esterification plays any role in the Shibata shift.

For the study of a possible role of protease activity, buffer-glycerol homogenates of oat leaves were used, and spectra were measured at liquid nitrogen temperature. PMSF at a concentration of $10^{-3}$ M, known to inhibit the activity of a neutral protease in oat extracts by about 80%, had no effect on the Shibata shift (data not shown). These experiments are not as conclusive as those with Sandoz 9789 or anaerobiosis, since it can be argued that the surviving 20% of protease activity that is insensitive to PMSF (Pike and Briggs, 1972) is that which is involved in the Shibata shift.

Spectra similar to the lower curves in Fig. 48 were obtained for oat glycerol-buffer extracts at liquid nitrogen temperature and subjected to curve analysis by the RESOL program (French et al., 1972). We wanted first to determine whether the Shibata shift in vitro could be modeled with the same four spectral components used by Virgin and French (1973) for their in vivo study of the Shibata shift and, second, if it could, whether this sensitive analytical technique could detect differences between spectra obtained from control plants and from those plants subjected to anaerobiosis, differences undetectable by straight difference spectroscopy. The quantitative aspects of these analyses are shown in Table 15. It is clear that the Shibata shift can be largely accounted for by a loss of the 684-nm component and its almost quantitative replacement by the 669-nm component, as found by Virgin and French (1973) in their in vivo studies. It is also clear that the results are the same whether the plants were incubated in air or nitrogen prior to harvest. The results strongly support the conclusions that reduction of the geranylgeraniol ester of newly formed protochlorophyllide has nothing to do with the Shibata shift.

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8To May 4, 1981
9To September 30, 1980
10To January 31, 1981
11To May 31, 1981