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INTRODUCTION

The Department of Plant Biology has continued its good fortune in attracting unusually gifted predoctoral and postdoctoral students and senior investigators. The strength of the current group of fellows and co-workers is measured not just by the articles that follow, but also by the Department's unusual record of outside publication. It merits note that even though two faculty members were on sabbatical leave a fair part of the year, the science continued unabated.

In past years it has been possible to organize this Introduction into relatively neat packages around the various subdisciplines in the Department—e.g., photosynthesis, molecular biology, development, physiological ecology. This year, however, the lines have become seriously blurred, as interdisciplinary activities have increased and molecular techniques have been applied to problems in other areas. But while the disciplinary edges have been blurred, the caliber of research has remained high, and the year's efforts have produced a number of significant findings. The following paragraphs represent an attempt to highlight them.

Two years ago, Thompson and his associates reported that DNA of genes undergoing transcription is more susceptible to nuclease digestion than is DNA that is not being transcribed. This year, Flavell and Thompson obtained evidence from wheat suggesting that active groups of ribosomal genes are less methylated than nonactive groups. Although it is still too early to conclude that this correlation holds at the level of the individual gene, the experiments provide a promising lead to our understanding of gene regulation.

In several elegant studies on the role of light in the regulation of development, Kaufman has successfully merged the photomorphogenic interests of the Briggs laboratory with the molecular approaches of the Thompson laboratory. Mandoli had shown previously that completely-dark-grown plants had at least two growth responses to red light, both probably mediated by the pigment phytochrome. One of these was potentiated by extremely small light fluences (the very low fluence response, VLF), while the other required four or more orders of magnitude more light (the low fluence response, LF). This year, in studies of the light regulation of the abundance of specific mRNAs, Kaufman has resolved these responses. In peas grown in total darkness, he finds that the abundance of messenger RNA used to synthesize one of the chlorophyll a/b-binding proteins (an important structural component of the light-gathering machinery of photosynthesis) is increased by light fluences in the VLF range, and then further increased by light treatments in the LF range. By contrast, the messenger abundance for the small subunit of RuBP carboxylase, the CO₂-fixing enzyme, is unaffected by VLF treatments, and increases only in response to light doses in the LF range. (Both of these mRNAs are coded by nuclear DNA.)

This year, plant mitochondrial DNA is mentioned for the first time. Stern and Palmer report the surprising finding that significant regions of plant mitochondrial DNA are homologous to chloroplast DNA. In corn, for example, the gene sequence for the large subunit of RuBP carboxylase (chloroplast DNA-coded) is represented in mitochondrial DNA sequences. The results raise the very real possibility that DNA has been exchanged between these two organelles during their evolutionary histories. In another study, Palmer and Shields obtained evidence that the mitochondrial DNA of the genus *Brassica* occurs in three circular DNA molecules, and that the two smaller circles are simply the conse-
quence of intramolecular recombination within the larger circle.

Polans and Thompson report steady progress in a study of the variation in nucleotide sequences in several phytochrome-controlled genes among different pea accessions. The wealth of phenotypic variation makes this system a particularly favorable tool for studying the multilocus phytochrome system. Watson, Palmer, and Thompson also report for the first time that higher-plant chloroplasts contain a gene for the elongation factor \textit{eufA} required for chloroplast protein synthesis. Finally, Palmer and Thompson present a model for chloroplast genome evolution based on their extensive studies (many with various collaborators) of the structure and variability of chloroplast DNA in various plant taxa.

Graduate students and fellows have been very active in photomorphogenesis studies. Using a variety of inhibitors, auxin analogs, antiauxins, and ionophores, Lomax has studied the uptake of the plant auxin indole-3-acetic acid by pH-tight vesicles from zucchini squash hypocotyls. She has shown that the uptake does not represent simple diffusion of the neutral (protonated) form of the acid through the vesicle membranes, but is, rather, highly specific for this active growth hormone. Using electron paramagnetic spin resonance techniques in collaboration with Mehlhorn at Berkeley, she has been able both to characterize the pH gradient from outside to inside of these vesicles and to calculate the accumulation ratio (an impressive thirtyfold) for the hormone.

In a continuing study of auxin-red light interactions, Shinkle has shown that physiologically effective concentrations of auxin can sensitize sections from etiolated oat coleoptiles to red light by four orders of magnitude. The auxin treatment converts the elongation reaction to red light from an LF to a VLF response. Somewhat lower auxin concentrations yield a red light fluence-response curve showing two distinct steps. The response is very similar to those obtained earlier by Mandoli with intact plants. It will be interesting to determine whether the hormone treatment has any effect on light-induced changes in mRNA transcript abundance. Here is another potential interface between the molecular and the developmental approaches.

In her continuing studies of light piping in plants, Mandoli, in collaboration with Boyer from the University of Illinois, demonstrated that there is an exponential decline in light-piping capacity with water loss in soybean hypocotyls, as well as an increase in the acceptance angle for maximum transmission of obliquely incident light. These optical properties may provide important tools for the nondestructive assessment of plant water status.

Since 1934, it has been assumed that phototropic curvatures of higher plants are mediated exclusively by blue light photoreceptor molecules. This year \textit{Iino} and Schäfer discovered and characterized phototropic responses of corn mesocotyls to red light. These responses are almost certainly phytochrome-mediated. \textit{Iino} has also shown by careful growth measurements that blue light-induced phototropic curvature of coleoptiles, at least in the most-sensitive range, is mediated by differential growth: a decrease of growth on the illuminated side is quantitatively matched by an increase on the shaded side. He also found that the growth differential migrated down the coleoptile at rates consistent with those for auxin transport. Taken together, \textit{Iino}'s results strongly affirm the hypothesis that this particular coleotilar phototropic response is mediated by light-induced lateral transport of auxin to create an auxin differential, which is then transported down the coleoptile.

Brown continues her long-standing interest in the nature of photosynthetic pigments and pigment complexes in vivo. She reports significant progress in fractionating spinach chloroplasts into three major complexes with little alteration of spectral properties. Curve analysis by the
RESOL program indicates that all higher-plant chlorophyll a can not be represented by four spectral components, as was generally believed. She also documents the sometimes very subtle and misleading effects that certain detergents such as sodium dodecylsulfate and Triton can have on the components of absorption spectra. Various technical advances, as well as an understanding of the pitfalls involved in attempts to solubilize pigment-protein complexes, have led to real progress in the isolation and characterization of absorption spectral components from primitive green algae and the notoriously refractory flagellate Euglena. Brown's initial studies of a group of primitive phytoplankton species represented by Mantoniella, which contains a protochlorophyll- or chlorophyll c-like porphyrin in addition to chlorophylls a and b and unusual xanthophylls, are leading to new insights both in pigment formation and in the evolution of eukaryotic algae.

Brown has also modified the RESOL program so that different half-bandwidths can be applied to the left and right sides of Gaussian or Lorentzian components in the analysis of complex spectra. An analysis of the red absorption band of chlorophyll a (in acetone) from Anacystis nidulans indicated that this spectrum cannot be matched completely by a single Gaussian-Lorentzian component, skewed or not. Obviously there is still much to learn about the basis of chlorophyll spectra.

Fork and his associates have continued to unravel the multitude of mechanisms whereby the intertidal red alga Porphyra perforata copes with unfavorable conditions. These mechanisms range from a simple imbalance in the amount of light reaching photosystems I and II to the crisis of extreme desiccation in high-intertidal plants at low tide on a hot day. It appears that Porphyra has at least four ways of coping with light imbalance. Besides the ability to redistribute energy between the two photosystems through the state I to state II transitions (studied previously by Fork and his collaborators), the alga has three other mechanisms to prevent overload of system II reaction centers by absorption of excessive system II light. The first of these involves some change in the system II pigment bed leading to dissipation of the absorbed energy by a mechanism other than transfer to system I. Satoh and Fork refer to this change as a state II to state III transition. Evidently, light-driven proton translocation across the thylakoid membranes plays some role in this transition. System I light can drive the transition back from state III to state II. Next, Porphyra seems to be able to cope with excess system II light by harmlessly cycling electrons around system II, preventing the formation of excessive amounts of oxidants or reductants. Using fluorescence techniques, Satoh and Fork readily distinguished between this mechanism and the others. Finally, Mohanty and Fork showed the presence of a fourth mechanism: When there is too much system II light, the alga can dissipate some of the energy through a long-wavelength fluorescence associated with system II. (Long-wavelength fluorescence is normally associated almost exclusively with system I.)

Smith, Satoh, and Fork find that Porphyra has yet another bag of tricks. Under conditions of severe desiccation, this facile alga is able to inhibit electron flow on the water side of system II, inhibit photoactivation of electron flow on the reducing side of system I, and inhibit energy transfer between pigment molecules. Inhibition of electron flow at these three different sites seems necessary to prevent permanent damage by photoinhibition. Evidently electron flow with only part of the system blocked can lead to photoinhibitory damage.

Brand, Mohanty, and Fork have shown that normal functioning of photosystem II in the blue-green alga Anacystis nidulans requires the divalent cation Ca²⁺. It is likely that this cation is needed for the stabilization of charges separated at the reaction center of photosystem II. In
addition, Mohanty, Fork, and Brand showed that Ca\(^{2+}\) plays a role in the regulation of energy distribution between the two photosystems by a mechanism associated with the state I–state II transitions and, further, that a long-term Ca\(^{2+}\) depletion leads to disruption of energy transfer from phycobilin pigments to chlorophyll \(a\).

Hoshina and Fork report progress in the preparation of three chlorophyll-protein complexes from Anacystis by a combination of detergent treatment and sucrose density gradient centrifugation. This method produces large amounts of purified chlorophyll-protein complexes that are photochemically active. Finally, Hoshina used the RESOL program to investigate the spectral consequences of temperatures above or below the phase transition for chlorophyll \(a\) in liposomes of phosphatidylcholine. The results suggest that chlorophyll \(a\) 662 is specifically converted to chlorophyll \(a\) 670 when the lipids pass from the liquid crystalline to the gel state, and that this change may reflect an alteration in the aggregation state of chlorophyll \(a\).

Three reports this year deal with the influence of various kinds of stress on higher plants. Using simple techniques, Levitt has shown that damage occurring during moderate wilting of cabbage leaves can be repaired during water reabsorption even if the process takes several days. Thus, conditions during the rehydration period are perhaps as important as the extent of dehydration in determining the recovery of a wilted plant. In the second study, Vallejos and Björkman report that a single night of low-temperature treatment (5°C) significantly reduces the subsequent photosynthetic performance of chilling-sensitive tomato plants, even without damage to photosystem II. Thus, photoinhibition is not the only way whereby low but not freezing temperatures can damage chilling-sensitive plants.

In the third study, Ludlow and Björkman have investigated the influence of paraheliotropic leaf movement (turning edgewise to the light) in preventing drought damage to the photosynthetic reactions of a Mexican legume, siratro (Macroptilium atropurpureum). When this plant is water stressed, its leaves move from a position of maximum to one of minimum interception of incident light. If the leaves are physically constrained, they show clear evidence of photoinhibition, particularly on the upper leaf surfaces. Fluorescence studies show that these leaf movements protect the leaves from photoinhibition, from high-temperature damage, and from interactive effects of high temperature and light. Siratro clearly provides an excellent model system for study of important protective responses found in many other species, including some (e.g., soybean) of economic importance.

Von Caemmerer, Coleman, and Berry used the unicellular green flagellate Chlamydomonas reinhardtii to probe the role of ribulose bisphosphate in regulating the rate of photosynthesis. If Chlamydomonas is grown under low CO\(_2\) conditions, it develops the capacity to accumulate carbon (from bicarbonate) to provide its ribulose bisphosphate carboxylase with sufficient substrate for effective photosynthesis. Quantitative comparison of photosynthetic behavior and ribulose bisphosphate concentration between high- and low-CO\(_2\)-grown cells clearly support the hypothesis that CO\(_2\) concentrations inside low-CO\(_2\)-grown cells are higher (at the same external CO\(_2\) concentration) than they are in high-CO\(_2\)-grown cells. These studies also show that ribulose bisphosphate itself plays an important role in the regulation of the carboxylation reaction. There was excellent agreement between responses measured in vivo and those predicted by in vitro studies of the carboxylase and from theoretical models published earlier by Farquhar, Berry, and von Caemmerer.

In a re-enactment of an historic experiment (see Year Books 68, 69, and 70), Nobs successfully repeated a cross between a C\(_3\) and a C\(_4\) species of Atriplex. Because the ribulose bisphosphate carboxylases from these two species have
clearly distinguishable kinetic properties, Berry, with Nobs, Osorio, Palmer, Tepperman, and Thompson, addressed the question as to which subunit of this enzyme—the small one (coded for by the nucleus) or the large one (coded for by the chloroplast)—determines these properties. The hybrid yielded a carboxylase with the kinetic properties of the C4 (maternal) parent. Restriction analysis of the chloroplast DNAs from both parents and three hybrids indicated a maternal inheritance for the chloroplasts, whereas the nuclear DNAs were clearly biparentally inherited. Since it is well known that chloroplast DNA codes for the large subunit of the carboxylase, it follows that the large subunit determines the kinetic properties of these enzymes. The study did not define a function for the small subunit, but it is still conceivable that some other genetically based differences in kinetics might be related to it. All the same, future efforts at kinetic modification of the carboxylase to improve photosynthetic yield should focus on the chloroplast genome, rather than on the far-more-complicated nuclear genome.

Several years ago, Berry and his colleagues reported the carbon-accumulation phenomenon mentioned above in Chlamydomonas reinhardtii, on transfer to low CO₂. In a recent, discipline-bridging study, Coleman, Berry, and Grossman, in collaboration with Togasaki from Indiana University, have shown that the induction of CO₂-concentrating capacity is accompanied by the de novo appearance of a polypeptide (molecular weight 37,000) identified as the enzyme carbonic anhydrase. The enzyme is secreted into the space outside the plasma membrane but inside the cell wall, where it may play an important role in maintaining bicarbonate at equilibrium levels, permitting more-rapid solubilization of gaseous CO₂ and providing inorganic carbon for the transport system. Coleman and Grossman then showed that the protein is synthesized on cytoplasmic ribosomes and that it fails to be excreted in the presence of an inhibitor of glycosylation. Finally, they noted how transferring plants from high to low inorganic carbon affects the synthesis of several additional proteins, including both subunits of the ribulose bisphosphate carboxylase (which may be affected differently).

Grossman, Talbott, and Egelhoff have conducted detailed investigations to determine the location of the DNA coding for the different proteins that form pigmented granules called phycobilisomes. Phycobilisomes contain the light-harvesting accessory pigments in red and blue-green algae. In the eukaryotic algae, where the phycobilisomes are contained within chloroplasts (or, in Cyanophora, within a specialized chloroplast-like organelle called a cyanelle), the pigment-binding proteins are synthesized within the organelle itself, as is the protein which anchors the phycobilisome to the photosynthetic membrane. The relationships among the pigmented polypeptides suggest that a gene family encoding both the pigment-binding and anchor proteins is located in the chloroplast. On the other hand, the so-called linker proteins, which bind the pigmented proteins to the anchor protein, are clearly synthesized in the cytoplasm. Thus phycobilisomes, like RuBP carboxylase, represent a division of labor between different genomes in the construction of a single functional unit.

Grossman and Brand report a new method for the rapid isolation of intact phycobilisomes from certain algae, and Grossman reports a technique for obtaining macromolecular aggregates of phycobiliproteins on denaturing gels. Both methods should be of considerable help in continuing efforts to elucidate the molecular architecture of phycobilisomes.

This brief summary fails to do justice to the reports that follow, nor does it give full sense of the depth and breadth of the research. The following articles, and more importantly the published papers that have or will arise from them, provide both the details and the perspective needed to measure the Department's accomplishments.

Winslow R. Briggs
The genes for ribosomal RNAs constitute a prominent multigene family in all eukaryotic organisms (reviewed by Long and Dawid, 1980). These genes occur in tandemly repeating units, usually at or near the sites of nucleolus organizers (NOR). Although the nucleotide sequences coding for ribosomal RNA are highly conserved during evolution, the copy number or repetition frequency of the genes can vary quite rapidly within a species from one genome to another. For example, among varieties of hexaploid wheat, ribosomal gene number varies between about 3,000 and 15,000 per 2C nucleus. Such large variations in gene number among otherwise very similar organisms indicate that many, or even most, of the genes are not essential for survival. Indeed, it is likely that many of the ribosomal genes are transcriptionally inactive (e.g., Phillips, 1978). Our interest centers on how the cell selects which genes should be active and which ones should remain (or become) inactive.

It seems likely that the nucleotide sequences of active and inactive genes are identical in most cases, so we predict that the selection process probably involves modifications of the DNA or chromatin structure at levels above that of the primary nucleotide sequence.

One type of DNA modification that has received much attention in recent years is the methylation of cytosine residues. Changes in the degree of cytosine methylation have been correlated with changes in expression of various genes in a number of animal systems (reviewed by Razin and Friedman, 1981). Although there are many apparent exceptions, the most frequent observation is that active (or potentially active) genes are less methylated at one or more sites than their inactive counterparts. Most observations have been made on protein-coding genes transcribed by RNA polymerase II rather than on ribosomal genes, which are transcribed by RNA polymerase I. However, there are indications that a similar correlation may hold for ribosomal genes in at least some animal systems (e.g., Bird et al., 1981a,b).

In wheat, the relation between methylation and rDNA activity has been examined in a series of chromosome substitution and addition lines containing widely varying numbers of ribosomal genes. The number of genes on each of the four pairs of NOR chromosomes was previously determined by hybridizing rRNA to DNA from a series of aneuploids in which the dosage of each of the 21 chromosomes was varied, and from an additional set of intervarietal substitution lines based on the original aneuploids. Using these lines and a cytological assay for nucleolar activity, Flavell and O'Dell (1979) were able to establish that the activity of a given nucleolar organizer depends in large measure on the fraction of the cell's potentially active rRNA genes which are contained in that organizer. Either the rate of transcription or the number of active genes in the organizer thus appears to be modulated by changes in total rDNA dosage. Data from Scheer et al. (1976) indicate that more genes are transcribed as rRNA synthesis increases in Triturus.

The methylation state of rDNA in wheat lines with different numbers of rRNA genes has been determined using the Hpa II/Msp I technique of Waalwijk and Flavell (1978). Both Hpa II and Msp I cleave the sequence CCGG. However, Hpa II will not do so if the internal cytosine residue is methylated. The inability of Hpa II to cut at sites cleaved by Msp I is therefore an index of the degree
of methylation at CG dinucleotides in CCGG sequences. From mapping experiments with cloned rDNA, and from Msp I digests of genomic rDNA, we know that there are many (often about fourteen) CCGG sites in each wheat rDNA repeating unit. When all these sites are cut the rDNA is reduced to fragments of low molecular weight.

Figure 1 shows an example of an experiment where wheat DNA was digested with Eco RI and Hpa II. Eco RI cuts once in the rDNA repeating unit, producing monomer fragments of about 9 kb if there is no further cutting by Hpa II. A very high degree of methylation is indicated by the large amount of rDNA remaining at the 9-kb position in the gel. The many faint bands below 9 kb result from Hpa II digestion at a few unmethylated sites in some of the molecules. Partial-digestion bands are particularly prominent at about 6.2 and at about 2.7 + 2.9 kb, indicating especially frequent undermethylation at a particular site 6.2 kb from the left-hand Eco RI site, near the 5' end of the nontranscribed spacer, and the two smaller bands at 2.7 and 2.9 reflect spacer-length heterogeneity within the rDNA complement.

The fraction of rDNA with at least one Hpa II site unmethylated was measured by quantitating the decrease in signal in the 9-kb Eco RI monomer upon digestion with Hpa II. Figure 2 shows that the number of genes cut at least once by Hpa II increases with the total dosage of ribosomal genes until the total dosage reaches about 9,000 and then levels off. The percentage of genes cut steadily decreases from about 80% to 40% with increasing gene number, so that the number of genes fully methylated is increasing more rapidly than the number of undermethylated genes even before the “saturation” dose of 9,000 is reached. This effect of gene number on methylation is similar to the effect of gene number on cytologically detectable nucleolar activity, in that both the average nucleolar activity and the fraction of undermethylated rDNA are highest when the total number of rRNA genes is low, and both decrease with increasing gene dosage. Thus there is a correlation between undermethylation (at least at the frequently unmethylated Hpa II site) and gene activity. If we assume that the control of nucleolar activity is exerted through modulation of the fraction of genes subject to transcription, this correlation would suggest that undermethylation and transcription might be causally related.

In Fig. 2, genes are scored as undermethylated if they are cut at least once by Hpa II. Since we know that undermethylation occurs preferentially at a single site, a question arises as to whether or not the level of methylation at other Hpa II sites also varies with gene dosage. The degree of overall methylation was estimated from the ratio of intensity of the 2.7 + 2.9-kb bands to the 6.2-kb band. Since the 6.2-kb fragment has many
more Hpa II sites than the smaller fragments, a similar degree of random undermethylation will have a much greater effect on the 6.2-kb band. Increases in the ratio \((2.7 + 2.9)/6.2\) thus indicate decreases in the overall degree of methylation. The results show that increases in this ratio correlate well with increases in the percentage of genes cut at least once, which we interpret as an indication that changes in methylation occur in parallel at many sites, although the percentage of molecules methylated at any given site may vary.

A further correlation between rDNA methylation and gene activity can be deduced from experiments with wheat plants containing chromosome 1 from *Aegilops umbellulata* in addition to the normal set of wheat chromosomes. Cytological observations by Martini, O'Dell, and Flavell (1982) showed that the nucleolus organizers on the *A. umbellulata* chromosome formed large nucleoli, while those on the wheat chromosomes were less active than in control plants, forming only micronucleoli. In a recent series of experiments it has also been shown that the *A. umbellulata* rDNA, which is distinguishable from that of wheat by virtue of its longer nontranscribed spacer region, is specifically undermethylated in these interspecific hybrids. In addition and perhaps even more significantly, the wheat rDNA is heavily methylated. Thus the suppression of wheat nucleolar organizer activity by the *A. umbellulata* chromosome is accompanied by an increase in the level of methylation of the wheat ribosomal genes.

The data obtained so far indicate that increases in the average level of activity of the ribosomal genes in a given plant or at a given nucleolar organizer site are associated with reductions in the average level of rDNA methylation. One interpretation of the data might be that active genes are less methylated than inactive ones. However, we do not yet have any way of knowing that the relationships we see in populations of ribosomal genes still hold at the level of an individual gene. To conclude that active genes are undermethylated, we would have to measure the methylation status of genes somehow fractionated according to transcriptional activity.

Since it may be difficult to fractionate ribosomal genes in this way, we have chosen first to examine the methylation...
status of genes showing different degrees of sensitivity to digestion when isolated nuclei are treated with an endonuclease such as DNase I. Sensitivity to nuclease digestion is a well-known property of active, or potentially active, genes in animal cells, and is believed to reflect a more-open or extended conformation of the chromosomal regions containing active genes (reviewed by Weisbrod, 1982). We have recently shown that the same general phenomenon also occurs in plant chromosomes (Year Book 80, 76–79, and Spiker et al., 1983). As in the case of methylation, the best-studied examples of DNase sensitivity involve protein-coding genes transcribed by RNA polymerase II, but there are some data showing similar sensitivity/activity correlations in ribosomal DNA (e.g., Bird et al., 1981a).

Work is currently in progress to see whether the fraction of DNase-sensitive ribosomal genes varies in parallel with the fraction showing undermethylation of Hpa II sites, and whether the rDNA from Aegilops umbellulata is more sensitive than wheat rDNA in the chromosome addition line where the Aegilops nucleolar organizer expression is dominant over that of the wheat organizers. Preliminary experiments indicate that useful data should be obtainable in these systems. Regardless of the outcome with respect to methylation, studies at the level of chromosome structure seem essential to an ultimate understanding of ribosomal gene expression.

REFERENCES


PHYTOCHROME CONTROL OF SPECIFIC mRNA LEVELS IN DEVELOPING PEA BUDS: THE PRESENCE OF LOW AND VERY LOW FLUENCE RESPONSES

Lon S. Kaufman, William F. Thompson, and Winslow R. Briggs

Previous Annual Reports and a subsequent publication by Thompson et al. (1983) describe a group of pea transcripts characterized by changing abundances during light-regulated bud development. The steady-state level for several of these transcripts is under the control of phytochrome. The protocols leading to these initial observations required three irradiations, one on each of three successive days. We have since attempted to find a single day or the fewest number of days on which single irradiations would result in red light fluence–response curves exhibiting low fluence responses and possibly very low fluence responses.

Initially, we assayed for chlorophyll and fresh weight of the bud. Six-day-old dark-grown peas were irradiated with a single pulse of red light. Seedlings were returned to the dark for an additional 24 h before receiving 24 h of white light. Red
light fluence–response curves measuring mg chlorophyll/bud, mg fresh weight/bud, and mg chlorophyll/mg fresh weight bud exhibit both low fluence responses and very low fluence responses (Mandoli and Briggs, 1981). The red light fluence curve for mg fresh weight/bud is shown in Fig. 3A. The very low fluence response has its threshold between 10^{-5} and 10^{-4} nmol cm^{-2} red light and becomes saturated between 10^{-2} and 10^{-3} nmol cm^{-2} red light. The low fluence response has its threshold at 10^{-1} nmol cm^{-2} red light and does not become saturated by 10^{3} nmol cm^{-2} red light. These results are in excellent agreement with those observed for oat coleoptile stimulation and mesocotyl suppression (Mandoli and Briggs, 1981).

Prior to initiating a similar set of experiments measuring transcript abundance, it was necessary to address the question of how to quantitate the RNA data. To this end, slot blots have replaced dot blots. The relative density of each slot may be easily and reproducibly obtained with a densitometer. The area under the resulting trace may then be determined by a computerized digitizer. Construction of standard curves from a DNA dilution series present on each slot set allows us to assign a relative abundance to each density.

Six-day-old dark-grown peas were irradiated with a single pulse of red light. Seedlings were returned to the dark for 24 h, after which the buds were harvested and total RNA extracted. Red light fluence–response curves were measured using hybridization techniques and the previously mentioned means of quantitation. Transcripts assayed thus far fall into one of three categories: those exhibiting a low fluence response only, those exhibiting both a low and a very low fluence response, and those exhibiting neither response. cDNA probes specific to the mRNA for the small subunit of RuBP carboxylase show a low fluence response only. cDNA probes specific to the mRNA for the chlorophyll a/b binding protein show both a low fluence and a very low fluence response. The fluence response curves for these probes are shown in Fig. 3B. The very low fluence response has its threshold at 10^{-4} nmol cm^{-2} and becomes saturated between 10^{-2} and 10^{-3} nmol cm^{-2}. The low fluence response has its threshold between 10^{-1} and 10^{0} nmol cm^{-2} red light and does not become saturated by 10^{3} nmol cm^{-2} red light. These values are in excellent agreement with those observed for mg chlorophyll/bud, mg fresh weight/bud, and mg chlorophyll/mg fresh weight. Far red light will reverse the low fluence response measured for either fresh weight or transcript abundance. Rever-
sal is saturated at $7.5 \times 10^{-2}$ J cm$^{-2}$ far red light.

In summary, peas irradiated with single pulses of red light exhibit both very low and low fluence responses for a variety of physiological parameters. Light-regulated transcripts may show low fluence responses only (i.e., the small subunit of RuBP carboxylase), both low and very low fluence responses (i.e., the chlorophyll $a/b$ binding protein), or neither response.

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WIDESPREAD PRESENCE OF CHLOROPLAST DNA SEQUENCES IN PLANT MITOCHONDRIAL GENOMES

David B. Stern and Jeffrey D. Palmer

Plant mitochondrial DNAs (mtDNA) are quite large in comparison to their fungal and mammalian counterparts and are also highly variable in size, ranging from 215 kilobase pairs (kb) in the genus *Brassica* to about 2,400 kb in muskmelon. Despite their large size, plant mtDNAs appear to encode only a few more mitochondrially synthesized polypeptides than do the smallest known mtDNAs, those of metazoan animals, which are approximately 16 kb in length. It seems likely, therefore, that higher plant mtDNA consists largely of non-coding sequences.

An unusual aspect of plant mitochondrial genome structure is the presence of sequences which have homology to chloroplast DNA (ctDNA). Corn mtDNA, for example, has been demonstrated to contain a 12-kb portion of the corn ctDNA inverted repeat (Stern and Lonsdale, 1982). We have since extended these earlier results, and have shown that numerous sequence homologies exist between cloned mung bean and spinach ctDNA restriction fragments and mtDNAs from corn, mung bean, spinach, and pea (Stern et al., 1983; Stern and Palmer, 1983). Our experimental approach has been to use $^{32}$P-labeled ctDNA clones spanning most of the mung bean chloroplast genome (Palmer and Thompson, 1981) as hybridization probes against Southern blots of restriction endonuclease digestions of the mitochondrial and chloroplast DNAs from each species. The ctDNA is included in a lane next to mtDNA prepared from the same plant as a control, since cross-contamination between organellar DNAs is an inevitable artifact of their isolation. In interpreting hybridization results, then, one can distinguish between mtDNA fragments with homology to ctDNA and ctDNA fragments contaminating the mtDNA preparation.

The major finding from these studies is that the presence of ctDNA sequences in the mitochondrion is a pervasive phenomenon in plants. All the ctDNA clones tested hybridized to at least one mtDNA restriction fragment (Fig. 4), although the strongest cross-homologies are between clones derived from the ctDNA inverted repeat and mtDNA from corn and pea. We have also found that the chloroplast gene for the beta subunit of the chloroplast ATPase has strong homology to a segment of mung bean mtDNA, and that the gene sequence (rbcL) for the large subunit of RuBP carboxylase is contained in corn mtDNA.

The presence of ctDNA sequences is a novel feature of plant mitochondrial genomes. One cannot rule out, however, that some of these sequences actually originated in the mitochondrion or its progenitor and were then transferred to the chloroplast. This is unlikely in the case
Fig. 4. Top: Map of the mung bean chloroplast genome adapted from Palmer and Thompson (1981). Pst I sites (arrows) and Sal I sites (boxes) are indicated. The clones used in this study are shown on the map; for example, MB 18.8 is a clone of an 18.8-kb Pst I fragment. The positions of the genes are according to Palmer and Thompson (1981), Palmer et al. (1982; unpublished data). The heavy lines just beneath the ctDNA map indicate the inverted repeats. Bottom: Schematic representation of the hybridization strength of the 32P-labeled ctDNA clones to mtDNA. Hybridizations were classified as very weak (dotted lines), weak (thin solid lines), strong (medium lines), or very strong (heavy lines). Clones not tested were MB 12.8, MB 17.2, and MB 20.5, whose sequences are largely contained within the inverted repeat and were mostly represented by MB 18.8 and MB 16.2, and MB 1.2. Hybridizations are shown directly under the cloned mung bean ctDNA fragment used as a probe.

of sequences that are clearly necessary for chloroplast survival, such as the transfer and ribosomal RNA (rRNA) genes, some of which are contained within the ctDNA inverted repeat, and rbcL.

Two types of mechanisms could account for random and widespread sequence transfer between cytoplasmic organelles. One type would require direct physical contact. Outer membrane continuities between chloroplast and mitochondrion have been observed in thin sections of the fern *Pteris vittata* (Crotty and Ledbetter, 1973). Also, enclosure of several mitochondria by a single chloroplast was observed in the grass *Panicum schenckii* (Brown et al., 1983). Membrane continuities might facilitate intermolecular recombination, whereas enclosure of mitochondria by a chloroplast suggests transformation of the mitochondrion as a likely mechanism for ctDNA uptake. On the other hand, exchange of DNA sequences between organelles may not require direct physical contact. DNA released into the cytoplasm from broken or lysed chloroplasts may be taken up by transformation of the mitochondria. Alternatively, there may exist in the cytoplasm specific vector molecules capable of transferring sequences between organelles. If such a vector could be isolated, it could prove invaluable for introducing genetic material simultaneously into chloroplasts and mitochondria of the same plant. Such a system, however, might exhibit sequence-specific selectivity. This could account for the apparently higher frequency of transfer of certain ctDNA sequences, such as parts of the inverted repeat.

We feel it unlikely that these integrated ctDNA sequences play any biological role in the mitochondrion. Significantly, the strongest cross-homologies we have observed are to ctDNA sequences unlikely to have a function in the mitochondrion: rbcL (corn) and the chloroplast rRNA genes (corn and pea). Rather, we feel that the widespread presence of ctDNA sequences in plant mitochondrial DNAs is best regarded as a dramatic demonstration of the dynamic nature of interactions between the chloroplast and the mitochondrion, similar to the ongoing process of interorganellar DNA transfer already documented between the mitochondrion and the nucleus in fungi and animals (Van den Boogaart et al., 1982; Wright and Cummings, 1983; Gellissen et al., 1983; Farrelly and Butow, 1983; Jacobs et al., 1983).
TRIPARTITE ORGANIZATION OF THE Brassica MITCHONDRIAL GENOME

Jeffrey D. Palmer and Clark R. Shields*

By far the largest mitochondrial DNAs known are those of higher plants, which range in size and sequence complexity from about 200 kilobase pairs (kb) to over 2,000 kb (Leaver and Gray, 1982). At the present time, the physical organization of higher-plant mitochondrial DNA is unclear, although various models have been proposed which postulate a complex organization of the genome into a collection of circles differing in size and sequence information (Leaver and Gray, 1982).

We have chosen to study the structure and function of one of the smallest known plant mitochondrial DNAs, that of various species in the genus Brassica (cabbage, turnip, mustard, rapeseed oil). Restriction fragment analysis of the Brassica mitochondrial genome indicates a size of just over 200 kb (Lebacq and Vedel, 1981; Palmer et al., 1983). We have already described an unusual, linear extrachromosomal plasmid found in the mitochondria of certain fertile and male-sterile Brassica cytoplasms (Palmer et al., 1983) and will not discuss this molecule any further in this report. Instead, we shall briefly summarize our recent studies on the physical organization of the main mitochondrial genome in Brassica.

Digestion of mitochondrial DNA from Brassica campestris (Chinese cabbage, turnip) with Pst I and Sal I produced a series of 29 and 26 restriction fragments, respectively. For each enzyme, all fragments have the same stoichiometry except for one pair of fragments which is at approximately one-third the stoichiometry of the rest and one pair which is at approximately two-thirds stoichiometry. The Va and 2fa stoichiometry pairs sum to the same molecular weight for each enzyme (26 kb for Pst I and 36 kb for Sal I), and subtraction of one pair from each total fragment summation (243 kb and 251 kb) gives a corrected genome size estimate of 217 kb (Pst I) and 215 kb (Sal I). Restriction maps of all four substoichiometric Pst I fragments reveal that they share a repeated sequence of approximately 3 kb, as defined by common Bam HI, Bgl I, and Pvu II sites (Fig. 5). Furthermore, restriction mapping and filter hybridizations indicate that each Pst I fragment is homologous on one side of the repeat to one of the other three fragments and, on the

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Fig. 5. Restriction maps of the four substoichiometric Pst I fragments of *B. campestris* mitochondrial DNA. Fractions ½ and ¾ at left indicate fragment stoichiometry. The 3-kb repeat sequence (efl) is flanked by four paired combinations of four unique sequences (klegmfjie, lflmlgeeifgk, khhe, ik). Solid bar indicates the minimum extent of the repeat sequence; open extensions of the bar indicate its maximum extent.

other side of the repeat, is homologous to a second one of the other three (Fig. 5). Thus, the ½ stoichiometry pair (P21.1 and P4.8) is equivalent in sequence complexity to the ¾ pair (P15.7 and P10.1), and the two pairs are interconvertible by a single reciprocal crossover within the 3-kb repeat.

The relative order of these four substoichiometric fragments and of the remaining fragments, of normal stoichiometry, was determined by hybridizing each Pst I or Sal I fragment (cloned into the plasmid vector pUCS) to a replica nitrocellulose filter containing total mitochondrial DNA digested with Pst I, Pst I-Sal I, and Sal I. Together with double digestions of the individual clones with Pst I and Sal I, these hybridizations enable construction of a physical map which accounts for all of the observed restriction fragments (Fig. 6). The simplest model consistent with both the linkage relationship data and the homologies among the four substoichiometric Pst I fragments (Fig. 5) is that the 215-kb *B. campestris* mitochondrial genome consists of three circular chromosomes (Fig. 6). One of the circles (215 kb) contains the entire genome, including two copies of the 3-kb element present as direct repeats separated from one another by 80 kb. We postulate that reciprocal recombination occurs between intramolecu-

Fig. 6. Three circular linkage groups of the *B. campestris* mitochondrial genome. Arrows denote positions and relative orientations of the 3-kb repeat sequence (Fig. 5). The three molecules are drawn to scale at their outside circumferences.
larly paired repeat sequences (Fig. 7) to
generate two smaller circles of 80 kb and
135 kb (Fig. 6). Based on the stoichi­
ometry of the four repeat-containing Pst
I fragments, it is estimated that the two
small circles are present in roughly equal
amounts and at twice the molarity of the
215-kb circle.

The process of recombination between
repeated sequences to give alternate
states of Brassica mitochondrial genome
organization—either two small circles or
one large cointegrate circle—is analo­
gous to the process of lambda phage in­
tegration-excision into the E. coli
chromosome (Nash, 1981) and to that of
bacterial transposable-element cointe­
grate formation-resolution (Kostriken et
al. 1981). By analogy to these and
mechanistically related specialized site­
specific recombination systems (Simon et
al., 1980; Broach, 1982; van der Putte et
al., 1980), we hypothesize the existence
of a specialized recombination system,
possibly encoded within the Brassica
mitochondrial genome and physically linked
to the 3-kb repeat sequence, which me­
diates recombination between specific
recombination sites embedded within the
3-kb repeats. Further analogy to pro­
cesses of low-frequency recombination
between lambda DNA and related sec­
ondary sites in the E. coli chromosome
(Nash, 1981) lead to the prediction that
recombination may also occur between
the strong recombination sites located in
the Brassica 3-kb repeats and the weaker
recombination sites located elsewhere in
the genome. This possibility will be stud­
ied in Brassica relative to the high levels
of small, circular mitochondrial DNAs
observed in suspension cultures of other
plants (Sparks and Dale, 1980; Dale et
al., 1981; Dale, 1981; Brennicke and Blanz,
1982).

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Fundamental to an understanding of the multilocus associations that characterize complex genetic systems is the existence of appropriate marker loci to serve as experimental tools. A potential source of such loci is the collection of cDNA clones previously described in connection with phytochrome regulation of pea transcript levels (Year Book 81, 101–105). Several of these clones represent nuclear genes that encode messenger RNAs whose abundance is regulated by light acting through the phytochrome system (Thompson et al., 1983); as such, these clones may present a unique opportunity to investigate the relationships among a group of genes expressing regulatory and possibly functional associations.

Historically, genetic studies have used marker loci encoding morphological, quantitative, and, more recently, biochemical traits. In particular, protein electrophoresis has been widely used in such investigations because of the ease with which unambiguous genetic assignment can be made (e.g., see Marshall and Allard, 1969). An analogous method is to analyze DNA restriction fragment length polymorphisms (see Botstein et al., 1980), which defines presumptively neutral, codominant marker loci but which is not restricted to genes encoding soluble enzymes. DNA “alleles” are not only useful as classical Mendelian segregates but can extend analyses to variations occurring in different classes of DNA, including noncoding DNA or sequences flanking a gene of interest.

To examine the potential of using restriction fragment length polymorphisms in the study of phytochrome-regulated pea loci, we carried out digestion of DNAs from various Pisum accessions and pea relatives with an appropriate restriction endonuclease (usually Eco RI, Hind III, or Bam HI) and fractionated them according to fragment size on agarose gels. After transferring these DNA fragments to reusable membranes (Southern, 1975), specific sequences were localized by hybridization with a radioactive pea cDNA clone belonging to the previously described collection.

In place of more conventional nitrocellulose filters, rehybridizable transfer membranes are used to maximize the efficiency and comparability of the screening process. In this way, the same set of DNAs, located on a single blot, can be hybridized perhaps as many as a dozen times, with different probes recognizing different DNA sequences. Total-leaf DNA can be prepared in a rapid, small-scale procedure (Jorgensen, unpublished modification of procedure in Murray and Thompson, 1980). If appropriate, one or two cycles of CsCl banding may be added.

Preliminary results indicate substantial restriction fragment length polymorphism across a range of Pisum accessions including several wild annual species in addition to commercial varieties, experimental lines, subspecies, land races, and primitive cultivars of Pisum sativum. Although DNAs from different sources did occasionally display similar band-pattern phenotypes, the general result was widespread variation among lines, varieties, and populations. In fact, where interlineal similarity did occur, it was usually restricted to a single probe. Members of the same line, variety, or...
Fig. 8. Genomic blot of experimental pea line DNAs hybridized with pea cDNA clone, pAB96. DNAs extracted from the leaves of single pea plants and banded through CsCl were digested with Eco RI, fractionated on agarose gels, and bound to Gene-Screen membranes. These were then probed with pAB96, a pea cDNA clone corresponding to a portion of the αβ light-harvesting complex. Lanes 1 and 2 (from left to right), which contain DNAs from different plants belonging to the same line, show identical band patterns. The remaining DNAs are all derived from different lines. Lanes 5 and 10, and lanes 7 and 11, also show what may be identical band patterns. Lanes 5 and 7, and lanes 10 and 11, display similar but not identical patterns. Lane 6 is blank. Seed material was obtained through the courtesy of G. A. Marx. R. A. Jorgensen kindly provided the particular DNA samples presented. From left to right, the lanes contain DNA from the following accessions (G. A. Marx, NYS Agr. Exp. Sta.): B77-288(1), B77-288(2), B77-289, B77-290, B77-296, blank lane, B77-279, B77-279, B77-279, B77-245, B77-246, B77-246, B77-249.

population, however, displayed identical restriction fragment banding patterns—a result consistent with the highly selfing nature of pea.

An example of the variation observed in pea restriction fragments is presented in Fig. 8. The DNAs pictured were extracted from individual plants belonging to a series of experimental lines, digested with Eco RI, and bound to Gene Screen rehybridizable membranes (New England Nuclear). They were then probed with pAB96 (a cDNA clone obtained from N.-H. Chua), which corresponds to polypeptide 15 of the chlorophyll αβ light-harvesting complex (Broglie et al., 1981). Although both similar and identical patterns are evident in the DNA samples (see Fig. 8 legend), the complexity of the patterns and the high level of polymorphism displayed is representative of each combination of DNA, restriction endonuclease, and probe examined to date.
The preliminary evidence, then, indicates a wealth of phenotypic variation for pea restriction endonuclease fragments corresponding to the phytochrome-regulated genes examined, and suggests a good potential source of marker loci for studies involving this multilocus system. We hope to extend these studies to all of the pea cDNA clones of interest, using DNAs prepared from a series of experimental lines for which other morphological, quantitative, and biochemical markers already exist. After experimentally verifying the segregational integrity of these restriction endonuclease fragments with extant F2 lines, we hope to use a few of the most variable parental lines and corresponding tester strains to map these loci (i.e., the restriction fragments they generate) with respect to the numerous traditional marker loci previously mentioned.

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CHLOROPLAST GENES FOR COMPONENTS OF THE TRANSLATIONAL APPARATUS

John C. Watson, Jeffrey D. Palmer, and William F. Thompson

Chloroplasts contain the complete translational and transcriptional machineries necessary for chloroplast genome expression. Chloroplast DNA encodes only a portion of the translational apparatus; the remainder of the required genes are located in the nucleus. The ribosomal RNA and transfer RNA genes of chloroplasts have been widely investigated. However, study of the genes encoding protein components of the translational system is only beginning. The gene that encodes elongation factor Tu (*tufA*) has recently been mapped on the chloroplast genomes of *Chlamydomonas reinhardtii* (Watson and Surzycki, 1982) and *Euglena gracilis* (Passavant et al., 1983). The *tufA* gene was located in *C. reinhardtii* by heterologous DNA:DNA hybridizations, using cloned genes from *Escherichia coli* as hybridization probes. Homology between chloroplast DNA and *E. coli* genes for translational and transcriptional components is not limited to the *tufA* gene. Indeed, *C. reinhardtii* chloroplast DNA also shares homology with genes of both the S10 and alpha ribosomal protein operons, as well as with the genes encoding the beta and beta’ subunits of RNA polymerase (Watson and Surzycki, 1983). It seemed possible that cloned *C. reinhardtii* or *E. coli* gene probes might be similar enough to the corresponding higher-plant chloroplast genes to allow their identification in DNA:DNA hybridizations. Here we have concentrated on the chloroplast genomes of pea, mung bean, and spinach using as probes the *C. reinhardtii* *tufA* gene and part of the *E. coli* S10 r-protein operon.

The *tufA* gene probe used here, encoding approximately one-half of the 3’ end of *C. reinhardtii* *tufA* sequences, was isolated from plasmid pCp16 (pCp16 was the gift of J.-D. Rochaix; Rochaix, 1978). To begin an analysis of *tufA* expression, this cloned fragment, *Eco 25*’, was used to examine *tufA* transcripts in *Chlamydomonas*. After electrophoresis of *Chlamydomonas* RNA (gift of Arthur
Grossman) on a denaturing agarose gel, filter blots of the gel were reacted with the tufA probe. An abundant transcript, primarily present in the nonpolyadenylated RNA fraction and about 1600 bases in length, is the major transcript of the Chlamydomonas tufA gene. The E. coli tufA gene is about 1200 base pairs in length, so the Chlamydomonas gene may be approximately 400 base pairs longer, presuming the absence of large, untranslated regions in the Chlamydomonas transcript. The E. coli S10 r-protein operon probe, about 2.3 kb in length, was cloned from specialized transducing phage lambda fus3 (Watson and Surzycki, 1983) and carries genes for r-proteins L2, L3, L4, L23, and either L22 or S19 (Watson and Surzycki, 1983). It is the gene for either r-protein L22 or S19 which hybridizes to C. reinhardtii chloroplast DNA, about 10 kb from the tufA gene.

Regions of homology to these two probes were located on the physical maps of pea, mung bean (Palmer and Thompson, 1981a), and spinach (Herrmann et al., 1980) chloroplast DNAs by probing filter blots of agarose gels containing restriction digests of these chloroplast DNAs. This analysis identified a single Pst I fragment in each species that reacted with the tufA probe (Table 1). The S10 operon probe hybridized to a single Pst I fragment in pea, while in both mung bean and spinach two Pst I fragments reacted with this probe (Table 1). Restriction fragments produced by other enzymes, overlapping the Pst I fragments listed in Table 1, have also been identified. The coding regions have been further localized by analyzing cloned Pst I fragments (Palmer and Thompson, 1981b) digested with Pst I and a second enzyme. The restriction fragments to which the tufA probe hybridizes in the three different species have been found to share homology in previous chloroplast DNA rearrangement studies (Palmer and Thompson, 1982), and the same situation pertains to the fragments hybridizing to the S10 operon probe.

In mung bean and spinach, tufA is located within the large single-copy region of the chloroplast genome. About 30 kb separates tufA from the junction of the inverted repeat and large single-copy region. In pea, tufA is separated by 5–6 kb from the 3' end of the 23S rRNA gene. This placement suggests that, as in Chlamydomonas, tufA exists in a single copy per chloroplast chromosome in pea, mung bean, and spinach. However, we have not yet been able to demonstrate detectable homology between the E. coli tufA gene and higher-plant chloroplast DNAs, unlike the situation in C. reinhardtii (Watson and Surzycki, 1982). In mung bean and spinach, the smallest fragments that we have found to react with the E. coli S10 operon probe are 1–4 kb in length and are clearly present in two copies per chromosome. That is, the "S10 region" maps within or near the ends of the inverted repeats which border the large single-copy region, about 20 kb from the 5' end of the 16S rRNA gene. The "S10 region" is about 45–46 kb from the 23S rRNA gene in pea. Recently, the nucleotide sequence of a region near the end of the inverted repeat in tobacco (Sugita and Sugita, 1983) revealed the presence of a gene encoding a protein with substantial homology with E. coli r-protein S19. This evidence, together with the fact that the gene for either L22 or S19 hybridizes to Chlamydomonas cpDNA, and the analogous position of the fragments hybridizing to the S10 operon probe described here, suggests that it is the gene for r-protein S19 (rpsS) which we have detected here. Because

<table>
<thead>
<tr>
<th>Species</th>
<th>tufA Probe</th>
<th>S10 Operon Probe</th>
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<tbody>
<tr>
<td>Pea</td>
<td>5.7</td>
<td>9.2</td>
</tr>
<tr>
<td>Mung Bean</td>
<td>5.6</td>
<td>16.2, 17.2</td>
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<tr>
<td>Spinach</td>
<td>13.5</td>
<td>8.9, 8.1</td>
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*Individual Pst I fragments are identified by size in kb.
of its location, it is possible that transcripts through this region are altered due to the recombinational events leading to chloroplast DNA isomerization (Palmer, 1983).

A study of the transcripts arising from \textit{tufA} and the "S10 region" in pea and mung bean has been initiated. Cloned subfragments (of the Pst I fragments) overlapping these two regions hybridize to multiple transcripts in gel blots of total RNA from both plant species. When RNA from dark-grown and light-grown plants are compared in this way, it appears that light-grown plants have a higher steady-state level of these transcripts than plants grown in the dark. A preliminary analysis of the red light fluence response of \textit{tufA} transcript abundance indicates the presence of both very low fluence and low fluence responses (L. Kaufman, unpublished data). These data suggest that \textit{tufA} transcript abundance may be regulated by phytochrome, although we have not yet demonstrated far red reversibility.

In summary, the single gene encoding elongation factor Tu has been located on chloroplast genome maps of pea, mung bean, and spinach; regions homologous to the \textit{E. coli} S10 r-protein operon have likewise been located. In the coming year, we plan to investigate further the structures of the genes identified. Knowledge of gene structure will facilitate analyses of transcriptional organization and light regulation of transcript accumulation. The elongation factor and presumptive r-protein genes provide an important contrast to virtually all chloroplast protein-encoding genes now under study, since they are not directly involved in photosynthesis but rather are involved in the expression of chloroplast genes.

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curred in those legume species that have lost the inverted-repeat structure.

We have postulated two equally tenable hypotheses to explain the last two observations, above. (1) The inverted repeat may stabilize the chloroplast genome against rearrangements. In this case the loss of the inverted repeat in certain legumes would lead directly to an increased frequency of rearrangements. (2) The chloroplast genome of certain legumes somehow acquired the ability to rearrange, and the deletion of the inverted repeat simply represents one of many rearrangements following this acquisition.

During the last year we have concentrated our studies on several additional legumes which either retain or lack the inverted repeat (Palmer, 1983; Palmer et al., 1983a, 1983b, 1983c). The major findings of these studies follow. (1) Legume chloroplast DNAs that retain the inverted repeat (from mung bean, soybean, and common bean) are entirely co-linear in sequence arrangement. Thus, the legumes as a family are not characterized by an increased chloroplast genome instability relative to other groups of angiosperms. (2) Alfalfa chloroplast DNA has a "missing link" status among legume chloroplast DNAs in the sense that it has lost one entire segment of the inverted repeat but is otherwise unrearranged relative to legume chloroplast DNAs that retain the repeat. Together with our previous findings, this result allows the following additional conclusions. (3) The loss of the inverted repeat occurred prior to all the other rearrangements observed in the pea and broad bean lineages. (4) Broad bean chloroplast DNA probably has evolved from an alfalfa-like ancestral genome by two specific inversions. Thus, all characterized major chloroplast DNA rearrangements (five so far) are inversions. (5) There is a broad range of rearrangement frequencies in those genomes that lack the inverted repeat.

Our conclusion that the inverted-repeat loss occurred before all the other rearrangements observed in pea and broad bean allows us to choose between the two hypotheses previously advanced (see second paragraph) to explain why the pea and broad bean genomes are so rearranged. It now appears unlikely that the deletion of the inverted repeat occurred simply as one of many rearrangements resulting from a generally destabilized chloroplast genome. Rather, the evident antiquity of the inverted-repeat deletion relative to other pea–broad bean rearrangements considerably strengthens the possibility that there is a direct, causal link between these two sets of mutations. At the same time, the possibility still remains that the inverted-repeat deletion occurred as a very early event soon after some other genome destabilization event, such as a "transposon infection" or an alteration in chloroplast recombination systems.

We have developed a general model for chloroplast genome evolution which attempts to explain the origin of the various chloroplast genome types that we have described. The starting point for this model is the recent demonstration that inverted-repeattaining chloroplast DNAs from common bean (Palmer, 1983), soybean (Palmer et al., 1983a), three fern species (our unpublished data), and from the cyanelle of Cyanophora paradoxa (Bohnert and Loffelhardt, 1982) all exist as two equimolar populations of molecules differing only in the relative orientation of their single-copy sequences. From analogies to a number of well-characterized systems possessing similar or mechanistically related types of orientation heterogeneity to that found in the chloroplast genome, we can make two major predictions about the mechanism of chloroplast DNA isomerization (Palmer, 1983; Palmer et al., 1983). First, there is very likely a specific sequence, perhaps as little as 14 or 15 base pairs in size, located within the inverted repeat and which serves as the site for a conservative homologous recombination event that inverts the two single-copy regions. Second, inversion is probably mediated by a specialized recombination
system which acts independently of any generalized cellular recombination system. The site-specific recombination systems reviewed in Palmer (1983) and Palmer et al. (1983b) include specific recombination genes closely linked to the recombination site itself. Thus, one might predict that the chloroplast genome, and probably the inverted repeat itself, encodes its own site-specific recombination system. However, the possibility must also be considered that, as in the case of many other originally chloroplast-encoded chloroplast functions (Weeden, 1981), the genes for this system may have been transferred to the nucleus.

Essentially all the site-specific recombination activity in the chloroplast is postulated to occur between two strong recombination sites located in the inverted repeat (Fig. 9). These are not only the two strongest recombination sites in sequence but are also embedded within extremely large (10,000–25,000 base pairs) inverted repeats which will pair intramolecularly at high frequency. In addition, we postulate that there are several sites scattered throughout the genome which differ slightly in sequence from the two strong sites and which serve as substrates for the site-specific recombination reaction at considerably reduced frequencies (Fig. 9). In this case we make a direct analogy to the integration of lambda phage DNA into the E. coli chromosome. Deletion of the primary bacterial attachment site for integrative recombination leads to a low frequency of lambda integration at specific secondary sites possessing varying levels of homology to the 15-base pair core recombination site (Nash, 1981).

Chloroplast recombination will occur much more rarely between one strong and one weak site or between two weak sites. Most of these events are likely to create lethal alterations in the chloroplast genome. Recombination between directly repeated sites will lead to excision of the sequences between the sites; given the tight organization of the chloroplast genome into coding regions (Oishi et al., 1981), the frequency of these events will fall off dramatically as the distance between the two sites increases. Recombination between inversely oriented sites will produce inversions without the loss of DNA. However, many of these events will also be lethal, since on a probabilistic basis most of the sites will lie within transcribed or otherwise functional regions. Inversions between strong sites and those weak sites that do not interrupt functional domains may also be disfavored. These would generate direct repeats of a portion of the inverted repeat, allowing subsequent excision of intervening sequences, and would also destroy one of the two strong sites, abolishing the high-frequency inversion system that is probably necessary for chloroplast function when the inverted repeat is present (see below).

There are two types of recombination events that will be stable and nonlethal and that appear to have been detected by our comparative studies on chloroplast genome organization. A certain percentage of weak-site inversions will not interrupt functional regions. Putative examples of these in inverted-repeat genomes are the spinach–mung bean and spinach–corn inversions of 50 and 20 kilobase pairs, respectively (Fig. 9). Among genomes that lack the inverted repeat, broad bean appears to be derived from alfalfa by two inversions, while a smaller inversion (2–6 kb) has been mapped between two closely related pea ecotypes (unpublished data).

There is a broad range of rearrangement frequencies in those genomes that lack the inverted repeat, so that in pea, and to a lesser extent in broad bean, rearrangements have occurred significantly more often than in genomes that contain the inverted repeat (Fig. 9). We postulate that in inverted-repeat-containing genomes, essentially all the site-specific specialized recombination activity is channeled into the two strong sites embedded within the inverted repeat. Upon loss of the inverted repeat this activity is redistributed more evenly
Fig. 9. Model for chloroplast genome evolution. Molecules b and b' represent the two orientation isomers (Palmer, 1983) of an inverted-repeat-containing chloroplast genome, such as that of mung bean, thought to be derived from a spinach-like ancestral genome (molecule a) by a major inversion within the large single-copy region (Palmer and Thompson, 1982). Molecule c represents the loss of one-half of the inverted repeat from a mung bean–like ancestor, with subsequent evolution yielding the present-day alfalfa (molecule d), broad bean (molecule e), and pea (molecule f) genomes. Closed circles represent strong recombination sites located within the inverted repeat. Open circles represent weak recombination sites scattered throughout the genome. Arrows indicate the relative orientation of recombination sites. For simplicity, only one orientation is shown for both the linear and circular configurations of molecule a.

Throughout the genome, with the result that the overall level of permitted recombination events— inversions and small deletions—is markedly increased. The decreased rate of rearrangements in alfalfa, and to a lesser extent in broad bean, is then explained by some combination of the stochastic loss of recombination sites and, perhaps more importantly, by a decrease or even complete loss of recombination activity. Although the evolutionary conservation of recombination activity in inverted-repeat genomes indicates its maintenance (Palmer, 1983; Bohnert and Löffelhardt, 1982), loss of the inverted repeat may markedly reduce these pressures. It is thus logical to suppose that a marked decrease in re-
combination function may have taken place soon after the deletion event in a lineage leading to alfalfa and, later on, after two inversions, in the broad bean lineage.

We plan to test this model both in higher-plant systems, where sequence studies at the ends of very recent inversions should yield valuable clues concerning the nature of possible recombination sites, and in the green alga *Chlamydomonas reinhardtii*, where powerful genetic approaches are available that should allow the isolation of inverted-repeat recombination sites and recombination genes. In addition, we are currently using pea and mung bean as model systems to explore the relationship between chloroplast DNA sequence rearrangements and the organization of the genome into discrete transcription units.

**REFERENCES**


AUXIN UPTAKE BY SEALED PLANT PLASMA MEMBRANE VESICLES IS A SPECIFIC, SATURABLE TRANSPORT

Terri L. Lomax and Winslow R. Briggs

One of the major hurdles in understanding the mechanism of auxin action is a knowledge of how the hormone is actually transported through the plant. While auxin is produced only at the apical tip of the plant, it is involved in the control of cell division, growth, differentiation, and regeneration throughout the entire plant. Thus, the directed movement of auxin plays a major regulatory role in the development of plants.

Auxin is transported through the intact plant in a polar, specific manner. That its movement is facilitated is demonstrated by the fact that the natural auxin, indole-3-acetic acid (IAA), has been found to move through a plant segment much more quickly (1–2 cm h⁻¹) than do other weak acids, which are not active auxins, with similar pK's. Auxin also moves at a steady rate through tissue over extended periods of time: its movement does not decelerate, as would be expected if the transport were by simple diffusion. When auxin is applied to the basal end of an inverted segment, it is not transported, thereby demonstrating the exclusively polar transport of the molecule.

The current concept of auxin transport is that undissociated IAA is taken up into the cytoplasm when the pH of the cytoplasm is higher than that of the walls. The IAA dissociates in the more-basic environment and accumulates as a result of the reduced permeability of the anionic form. The IAA⁻ is secreted at the lower end of each cell by a localized, selective anion carrier which provides the polar nature of the transport. This theory is known as the chemiosmotic theory of po-
lar auxin transport (Rubery and Sheldrake, 1974).

Many elegant in vivo experiments have led to this model for the cellular mechanism for auxin transport, but until recently no in vitro system has existed with which to test its predictions. We recently presented evidence for such a system: Tightly sealed membrane vesicles, which are prepared from zucchini hypocotyls, can transport auxin in vitro using a pH gradient—inside basic, outside acidic—as the driving force (Year Book 81; Hertel et al., 1983). This ability to accumulate \([^{14}C]\text{IAA}\) was shown to be sensitive to the protonophore FCCP (carbonyl cyanide p-trifluoromethoxyphenyl hydrazone) as well as to osmotic shock, indicating that sealed vesicles are capable of holding a pH gradient. By separation on a linear Dextran T-70 gradient, IAA uptake was demonstrated to be restricted to vesicles of plasma membrane origin even though other sealed vesicles were present in the preparation (Year Book 81, 18-21).

It remained to be shown that this accumulation of \([^{14}C]\text{IAA}\) by sealed vesicles was facilitated by auxin influx and efflux via saturable, specific carriers, as predicted by Rubery and Sheldrake (1974) and Goldsmith (1982), rather than by the simple accumulation of a weak acid across a pH gradient. Here we present further evidence for the specific uptake of IAA by means of a saturable IAA\(^{-}/\text{H}^{+}\) symport for uptake and an anion efflux carrier.

**Materials and Methods**

Seeds of zucchini squash (*Cucurbita pepo* L., cv. Dark Green, Ferry Morse Seed Co., Mt. View, CA) and maize (*Zea mays*, cv. WF9 × Bear 88, Bear Hybrid Corn Co., Decatur, IL) were planted in moist vermiculite and grown in plastic boxes at 26°C and 95% relative humidity for five days in total darkness. Membrane particles were prepared from 2-m sections of hypocotyl (zucchini, cut 5 mm below the hook) or coleoptile (maize) and were tested following the procedures described in Hertel et al. (1983). Briefly, the vesicles were made by homogenization of the tissue in pH 7.9 medium (250 mM sucrose, 50 or 200 mM Tris base, 3 mM Na\(_4\)EDTA, and 0.1 mM MgSO\(_4\), titrated with 12 N HCl), followed by a series of centrifugations, after which the resulting microsomal pellet was resuspended in medium buffered at pH 5.5 (300 mM sucrose, 10 mM Na\(_3\)Citrate, and 0.1 mM MgSO\(_4\), titrated with 12 N HCl). This creates a pH gradient across any sealed membranes.

To start a transport assay, the vesicles were diluted 1:1 with pH 5.5 buffer containing radioactive auxin and any additions. The uptake was then stopped at any given time by addition of an aliquot (200 µl) of vesicle suspension to a large volume (10 ml) of ice-cold buffer which was immediately filtered over a Millipore filter under vacuum before any significant efflux could occur, the entire process taking <8 s. Alternatively, a centrifugation assay was used where many samples could be stopped simultaneously (see Hertel et al., 1983).

**Results and Discussion**

When the vesicles were given a 5-min pulse of radioactive auxin at various times after resuspension (after establishment of the pH gradient), there was an initial rapid rate of accumulation which fell off and became stable after approximately 4 min (Fig. 10). This initial instability may be the result of an initial rapid rate of disruption of vesicles, movement of \([^{14}C]\text{IAA}\) in and out of vesicles which are not as pH-tight, or a flux of buffer materials. Accordingly, all other experiments were started at least 5 min after resuspension, when a stable rate of uptake had been reached.

In Fig. 11, both FCCP, an electrogenic protonophore, and nigericin, an electroneutral \(\text{H}^{+}/\text{K}^{+}\) exchanger, are seen to induce very similar effects on the uptake of IAA, with both having a similar concentration dependence, becoming satu-
We had previously stated (Year Book 81, 18–21) that although we were able to demonstrate in vitro auxin transport by vesicles from several members of the Cucurbitaceae, we were unable to show pH-driven accumulation by membrane preparations from maize coleoptile tissue. We have since been able to resolve this discrepancy by demonstrating FCCP-sensitive $^{14}$C]IAA uptake not only by maize coleoptile vesicles but also, to a lesser extent, by vesicles prepared from pea epicotyl tissue (data not shown). These results were achieved by optimizing the buffer conditions and increasing the size of the pH gradient. The preparations from maize and pea may contain a smaller number of sealed vesicles, the vesicles may be sealed less tightly, or both. However, the fact that they exhibit a pH-dependent accumulation of IAA demonstrates that this is not a phenomenon restricted merely to the Cucurbitaceae.

Benzoic acid (BA), like IAA, is a lipophilic weak acid which has a pK and structure similar to that of IAA but which is inactive as an auxin. In studies with intact plant segments, benzoic acid has been shown to be taken up to an even greater extent than IAA, but it does not inhibit the polar movement of $^{3}$H]IAA through the tissue, as does IAA (Goldsmith, 1982). This result is consistent with the specificity of IAA transport in vivo and supports the hypothesis that transport consists of a saturable flux of auxin anions in parallel with a nonsaturable flux of undissociated IAA. In a similar experiment with membrane vesicles from zucchini hypocotyls we find that the uptake of $^{14}$C]IAA is indeed saturable by low concentrations of unlabeled IAA but is not inhibited by excess benzoic acid until quite high concentrations are reached (Fig. 12). The half-maximal level of IAA saturation of $^{14}$C]IAA accumulation by these vesicles appears to be approximately $2-4 \times 10^{-7}$ M. In comparison, the uptake of $^{14}$C]berzoic acid is inhibited by neither IAA nor BA until millimolar concentrations are present, consistent with competition for simple
diffusion of a weak acid across a pH gradient. This result provides further evidence that, while accumulation by diffusion is certainly a component, IAA accumulation in these vesicles is via a specific, saturable mechanism.

Figure 13 presents a time course for the FCCP-sensitive accumulation of both \(^{14}\text{C}\)IAA and \(^{14}\text{C}\)BA by zucchini hypocotyl membrane vesicles. Although the initial uptake kinetics appear to be similar, the \(^{14}\text{C}\)IAA is retained far longer at a high level than is the \(^{14}\text{C}\)BA. There are specific inhibitors of auxin transport such as TIBA (2,3,5-triiodobenzoic acid) and NPA (naphthylphthalamic acid) which block its movement through the tissue by blocking the efflux carrier (Goldsmith, 1982). While TIBA is present in both treatments here, it would appear to block only the efflux of IAA, not that of BA. This specificity is consistent with the observations of Sussman and Goldsmith (1981) that the transport inhibitor NPA stimulates the retention of IAA by intact cells but has no effect on the accumulation of benzoic acid. Since NPA and TIBA inhibit only the saturable efflux component of auxin transport (Goldsmith, 1982), the increased retention of auxin by the vesicles in the presence of TIBA is presumably due to the anion carrier being blocked. Thus, the anion carrier in these vesicles is not saturable at low concentrations by benzoic acid and is specific for the facilitated transport of IAA. The following article presents additional evidence for the facilitated nature of the accumulation.

REFERENCES

Quantitation of Sealed Vesicle Volume, pH Gradient, and Auxin Uptake of Zucchini Hypocotyl Membrane Preparations

Terri L. Lomax, Rolf J. Mehlhorn,* and Winslow R. Briggs

Having established that auxin uptake by zucchini hypocotyl membrane preparations is the result of a specific, saturable, and pH-dependent transport of auxin into sealed plasma membrane vesicles (see Lomax and Briggs, this Report), we wished to dissect the various components of this transport in order to elucidate its mechanism.

The transport system is postulated to operate via an uptake IAA-H⁺ symport and an anion efflux carrier, as well as by diffusion—all of which are dependent upon the size and direction of the pH gradient. Intact plant cells have previously been demonstrated to have a pH gradient across the plasma membrane, with the cytoplasm being basic or neutral (pH 7) and the wall space acidic (pH 5-6). The zucchini membrane vesicle system described here has a pH gradient of the same direction established by preparing the vesicles at pH 7.9 and testing them with pH 5.5 buffer on the outside (Hertel et al., 1983). Since these vesicles have been shown to perform all of the essential functions of auxin transport according to the chemiosmotic theory of auxin transport (Hertel et al., 1983), they provide a model system for approximating the contributions of the various transport components. However, although the zucchini vesicles have been demonstrated to maintain a pH gradient for an extended period of time, they are most likely not as tightly sealed as an intact plant cell is.

Figure 14 illustrates a typical time course for the accumulation of radioactively-labeled IAA by zucchini hypocotyl vesicles prepared as just described. Even in the presence of the protonophore FCCP, there is an initial small incorporation of auxin, indicating that FCCP acts slowly. However, after 40 min, a low, equilibrium-level association of auxin in the presence of FCCP is reached. The presence of the auxin transport inhibitor, TIBA, results in a higher level of accumulation than that seen when neither FCCP nor TIBA is present. This result indicates that TIBA is blocking the efflux site, as it is known to do in intact tissue (Goldsmith, 1977), thus increasing the amount of accumulation of IAA within the vesicles. The level of IAA association with the vesicles both in the presence and absence of TIBA declines after about 15 min (−TIBA) or 30 min (+TIBA), presumably because of the decay of the pH gradient. The decline approximates an exponential decay, as might be expected to result from a linear decay of the pH gradient. The addition of FCCP after 1 h (Fig. 14, open symbols) to vesicles not previously exposed to the protonophore resulted in a more-rapid release of [14C]IAA. Additional FCCP did not, however, change the level of IAA association for vesicles that had been maintained in FCCP. Thus, these data indicate that a pH gradient does indeed still exist after one hour and that the accumulation of auxin is maintained by that pH gradient.

The possibility existed that the decline after 15-30 min in the association of [14C]IAA with the vesicles was a result of gradual deterioration of their sealed nature over time. However, when the reaction was started by adding [14C]IAA to vesicles after 2 h, they were found to be still capable of accumulating IAA with kinetics similar to those started at 0 min.
Fig. 14. Time course of [14C]IAA uptake in the presence and absence of TIBA and FCCP. Each filtration assay sample contained 2 g fresh wt equivalents ml⁻¹ zucchini membrane vesicles prepared in pH 7.9 buffer (250 mM sucrose, 50 mM Tris base, 3 mM EDTA, and 0.1 mM MgSO₄, titrated with 12 N HCl), and resuspended in pH 5.5 buffer (300 mM sucrose, 10 mM Na₂Citrate, and 0.1 mM MgSO₄). At 0 min, 2 × 10⁻⁷ M [14C]IAA (6500 dpm sample⁻¹) was added along with either 10⁻⁵ M FCCP (circles), 3 × 10⁻⁶ M TIBA (diamonds), or no additions (triangles). Additional FCCP (10⁻⁵ M) was added to portions of each treatment at 1 h (open symbols).

Another possibility was that the decline in IAA accumulation was due to the metabolism or degradation of [14C]IAA. When additional [14C]IAA was added after 3 h in the experiment shown in Fig. 15, the same fraction of total dpm accumulated was reached as in those without the addition, indicating that the vesicles were accumulating the auxin at their maximum capability at that pH gradient (data not shown). Thus, degradation of IAA is not likely a significant contributor to these kinetics. The increased level of IAA accumulation and the longer retention time in Fig. 15 over those shown in Fig. 14 occurred because the vesicles used for the assay in Fig. 15 were made with 200 mM Tris buffer rather than with 50 mM, as for Fig. 14 (see below).

To calculate accurately the energetics and kinetics of auxin transport, and, most importantly, to demonstrate that the accumulation in these vesicles is a facilitated transport and not merely the result of diffusion across the pH gradient, reliable measurements of both vesicle volume and the size of the pH gradient were needed to correspond with the measurements of auxin uptake under various conditions. This has been accomplished using electron spin resonance (ESR). Nitrooxide spin labels are used to measure a variety of biological properties, including cell volumes, pH and electrical gradients, and membrane and surface potentials (Mehlhorn et al., 1982). The method is especially advantageous because of the ease of separating the ESR spectra of membrane-bound from aqueous probes, the availability of many different nitrooxide compounds which can be applied to different problems or conditions, and the very small amount of material required. (Determinations can be made with only 40 μl of a suspension containing less than 1 mg ml⁻¹ protein.)

The volume of sealed vesicles within a solution can be determined by quantitating the aqueous line heights of a membrane-permeable, yet highly watersoluble, nitrooxide probe, Tempone, in the presence and absence of an impermeable paramagnetic quenching agent, Na₂MnEDTA. The ratio of quenched to unquenched signal is directly related to the volume, which is sealed and unavailable to the quencher. Using zucchini membrane preparations identical to those used for IAA accumulation assays, we...
have demonstrated that increasing amounts of osmoticum in the external medium will reduce the volume of the vesicles. Conversely, decreasing the external osmoticum causes an increase in vesicle volume (data not shown). These experiments demonstrate that the vesicles are capable of expanding and contracting in response to external osmotic levels and provide further evidence as to their sealed nature.

Other nitroxide probes are either weak acids (Tempacid) or weak bases (Tempamine), which partition preferentially into basic or acidic environments, respectively. The concentration ratios of the amine and weak acid on both sides of the membrane can be measured and used to calculate the size of the pH gradient. Using only one probe, the “effective” volume can be calculated and related to the actual volume determined with Tempone, and this can again be related to the \( \Delta pH \). Using these methods we have determined both the vesicle volume and the size of the pH gradient in zucchini vesicles made with either 50 mM or 200 mM Tris buffer at pH 7.9. All vesicle preparations were resuspended in pH 5.5 buffer, exactly as for the auxin uptake assays described previously.

Figure 16 shows that the decay of the pH gradient over time is slow (approximately 0.03 pH units min\(^{-1}\)), and is similar under both of these conditions. The size of the pH gradient in both cases was about 0.8 pH units directly after resuspension, smaller than expected. This is caused in part by the elevated pH of the surrounding medium. Although the vesicles were resuspended in pH 5.5 medium originally, some breakage or leakage evidently took place during resuspension. Thus, the internal pH 7.9 buffer of higher strength (50 or 200 mM vs. 10 mM) was released from broken vesicles, raising the external pH to 5.73 for those vesicles prepared with 50 mM buffer and 6.09 for those prepared with 200 mM buffer. Nevertheless, the magnitude of the pH gradient observed here closely approximates that found in the intact plant.

The ESR measurements providing the data depicted in Fig. 16 were made at 4°C, the same temperature used for the auxin uptake assays. When the assay temperature was raised to 25°C the rate of decline of the pH gradient increased markedly (data not shown). This result suggests that the vesicles become less tightly sealed at higher temperatures and is in agreement with Hertel et al. (1983) who found that increased temperature decreased auxin uptake by zucchini membrane vesicles.

From our calculations, we found that the sealed vesicles made with 50 mM buffer were about 0.2% of the total volume of the membrane resuspension, or 8 \( \mu \)l mg\(^{-1} \) protein. Those prepared with 200 mM buffer were half again as large, 1% of the total volume, or 12 \( \mu \)l mg\(^{-1} \) protein. This difference is most likely because, while the 50 mM buffer is in osmotic balance with the external buffer, the osmotic concentration of the 200 mM buffer is higher than that of the external buffer (600 mOsm vs. 350 mOsm), which causes the vesicles to swell. This difference in vesicle size when loaded with different buffer strengths may explain the differences in total IAA accumulation between Figs. 14 and 15. The difference is illustrated more clearly in Fig. 17, where both conditions are compared in a single

![Fig. 16. Time course of \( \Delta pH \) in vesicles prepared as in Figs. 14 and 15. Calculated from aqueous line heights of nitroxide spin probes measured by ESR. Samples contained 1 mM Tempone or Tempacid in the presence or absence of 0.15 M Na\(_2\)MnEDTA and included 2 \( \times \) \( 10^{-7} \) M IAA and 3 \( \times \) \( 10^{-6} \) M TIBA as for an auxin uptake assay.](image)
Fig. 17. Kinetics of FCCP-sensitive IAA accumulation at 50 mM and 200 mM internal buffer strength. Conditions as described in Figs. 14 and 15.

The determinations of vesicle volume allow the calculation of the actual IAA concentration gradient generated by the sealed vesicles. With either internal buffer concentration, the maximal [IAA], is 5–10 μM. Thus, the differences observed in Fig. 17 are a consequence of variable vesicle volume and do not represent a difference in concentration of IAA accumulated. Since only 0.1–0.2 μM IAA was present in the external solution, this concentration represents at least a fiftyfold accumulation of the auxin. The actual figure is most likely much higher because we used the total sealed volume for these calculations and it has been shown that only one population of the sealed vesicles actually transports IAA (Goldsmith, 1977). The maximal accumulation of IAA at diffusional equilibrium with a pH gradient of the magnitude measured here would be only five times the external concentration of IAA (Goldsmith, 1977), rather than the fiftyfold accumulation obtained here. This difference is convincing evidence that there is indeed facilitated transport of IAA by these zucchini membrane vesicles and that the transport can enhance the concentration of IAA at least tenfold over the equilibrium distribution. These large accumulations are still within the physiological concentration range for IAA.

We have now demonstrated that we have an in vitro system with which to study auxin transport at pH gradients and IAA concentrations approximating conditions found in the intact plant, including all of the components of in vivo transport. Using ESR data for volumes and ΔpH in combination with [14C]IAA uptake and efflux kinetics, we will now be able to analyze in detail the cellular mechanism of auxin transport.

REFERENCES

AUXIN INCREASES COLEOPTILE SECTION SENSITIVITY TO RED LIGHT

James R. Shinkle and Winslow R. Briggs

Last year (Year Book 81, 39–43), we reported preliminary findings on the sensitivity of subapical oat coleoptile sections, excised from dark-grown seedlings, to red light–induced increases in growth. We found that adding IAA to the incubation buffer before irradiation increased the apparent sensitivity of the sections to red light by a factor of at least 1000. We have confirmed this finding, and have discovered that the nature and magnitude of the change in sensitivity to red light depends on the concentration of IAA applied. For example, certain
concentrations of IAA cause the sections to respond to red light in a manner very similar to the response of coleoptiles left intact on whole seedlings. This response is biphasic, consisting of a very low fluence response (VLF) and a low fluence response (LF), separated by a plateau (see Mandoli and Briggs, 1981). In sections, both responses appear as step functions rather than continuous gradual increases in growth.

Fluence-response curves for red light-induced increases in growth were performed on subapical coleoptile sections and coleoptiles on intact seedlings. Seedlings were grown in total darkness for 72 h after imbibition, as in Mandoli and Briggs (1981) and *Year Book* 78, 140–144. All manipulations other than actual irradiations on whole seedlings and sections were carried out in complete darkness.

For whole seedlings, plates of approximately 25 seedlings were placed, one at a time, in a foil-lined coffee can attached to a light source such that only diffuse light reached the seedlings. Seedlings were irradiated in an upright position. Irradiation with red light took place over 1–5000 seconds, depending on the fluence applied. All fluences of 1 nmol/cm² or less were given in a one-second irradiation. Each fluence was given to duplicate plates. Plants were returned to a dark cabinet after irradiation. Dark control plants were moved to and from the coffee can without being irradiated. Immediately after other plants were irradiated, two plates of seedlings were removed and measured for initial coleoptile length. At 24 h after irradiation, plants were removed from cabinets, placed on clear glass plates, and photocopied. Facsimile sections were digitized for length as for coleoptiles. Red light-induced increase in growth was determined and expressed as the percentage of maximal increment of increase induced by red light.

The red and far red light was obtained from an incandescent projector lamp, as described by Mandoli and Briggs (1981).

As seen in Fig. 18, the addition of 6 × 10⁻⁶ M IAA to the incubation medium decreases the fluence of red light required for both threshold and saturation of the R-induced increase in section growth by a factor of 10,000. In the whole-plant photobiology described by Mandoli and Briggs (1981), the two curves would be equivalent to an exclusively LF response (minus IAA) and an exclusively VLF response (plus IAA), respectively. Consistent with this interpretation, the R-induced increase in growth is far red reversible in the absence of added IAA, but is not FR reversible with IAA added. Also, the growth response is inducible by far red light alone in the presence of added IAA (data not shown).
The R-induced increase in coleoptile growth in whole seedlings irradiated 72 h after imbibition shows a biphasic fluence dependency, very similar to that found for seedlings irradiated at 56 h after imbibition, as in Mandoli and Briggs (1981). The biphasic fluence-response behavior is shown in Fig. 19.

Coleoptile sections cut from 72-h-old seedlings can be made to show a biphasic fluence-response curve for R-stimulated growth by the application of $6 \times 10^{-7}$ M IAA to the incubation medium. Figure 19 shows this treatment in comparison to the whole-tissue response. This lower applied IAA concentration is probably closer to the internal IAA level in whole tissue (see Bandurski and Schulze, 1974). The threshold for R-stimulated growth appears to be an order of magnitude higher for sections than for whole tissue. Otherwise, the ranges and magnitudes of the two responses to red light are quite similar.

Mandoli and Briggs explained both the LF and VLF red light-induced increases in coleoptile growth in terms of phytochrome action. The data presented here give no contrary indication. There are two current models to account for the separation of phytochrome responses into two phases. VanDerWoude (1983) has postulated that the two phases are the result of different binding affinities of a postulated phytochrome receptor/transducer for dimers of phytochrome which have either one or both molecules converted to Pfr. Blaauw-Jansen (1983) accounts for the two phases in terms of the attainment of a plateau level of Pfr by phytochrome destruction; further increases in Pfr are possible at fluences producing more Pfr than the destruction process reduces to the plateau level. It may now be possible to distinguish between these two models using the applied auxin to switch between the two responses.

VanDerWoude (1983) and Fredericq et al. (1983) have both shown that chemical treatment of seeds can lead to increased sensitivity to red light, similar to the shift found in Fig. 18. VanDerWoude also reported that prechilling of lettuce seeds could lead to a biphasic fluence-response curve for increased germination. Fred-
ERICQ ET AL. reported that the plant hormone gibberellin GA₃ was active in sensitizing the germination of seeds of Kalanchoe both to red light and far red light. These two reports indicate that alterations in phytochrome response by external factors may be common, but seed germination experiments do not allow for the precise changes in external conditions possible in the excised coleoptile system.

REFERENCES

FIBER-OPTIC CAPACITY OF PLANT TISSUES VARIES AS A FUNCTION OF TISSUE WATER STATUS
Dina F. Mandoli, John S. Boyer,* and Winslow R. Briggs

The light-carrying capacity of plant tissues is affected by anatomical structures, tissue damage (Mandoli and Briggs, 1982a), and the age of the cells (Mandoli and Briggs, 1982b). Since infiltration with distilled water causes a 60–80% increase in the light-piping capacity of oat mesocotyl tissue sections (Mandoli and Briggs, 1983), we attempted to discern if water loss in these tissues caused a concomitant decrease in the light-guiding capacity (Year Book 81, 24–25).

As plant tissue sections become dehydrated (evidenced by a loss in fresh weight), they lose the ability to guide light axially by total internal reflection (Table 2). For example, a 25% loss in the fresh weight of oat mesocotyl sections results in a 35% loss in their ability to guide light (Table 2). From these preliminary data, the light-guiding ability appears to decrease exponentially as fresh weight decreases.

The optimum angle for acceptance of a light beam applied to the side of a tissue section is species-specific rather than tissue-specific and is therefore largely independent of tissue geometry (Mandoli and Briggs, 1982b). The acceptance angle for oat mesocotyl and coleoptile tissue is 59°; for corn coleoptile and root it is 52–54°; and for mung bean hypocotyl it is 47° (Mandoli and Briggs, 1982b). Soybean (Glycine max) hypocotyls, like corn roots and coleoptiles, have a fairly broad acceptance angle with a peak centered at 55° with respect to normal (Fig. 20). When fully hydrated (100% water) there is a shoulder at 25° in the curve describing the angular dependence for soybean (Fig. 20). To date, the presence of a secondary peak in acceptance angle is unique to soybean. As the tissue becomes dehydrated,

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*Each value represents five sections each 25 mm in length. 100% fresh weight = 13.67 mg/25 mm section of mesocotyl. 100% light guiding 9,603 arbitrary photometer units or about 2.5 mW of monochromatic (632.8) red light from an He-Ne laser. Tissues were cut from 3.5-day-old etiolated Lodi oats, which were grown according to Mandoli and Briggs (1981). Light guiding of tissue sections was measured as in Mandoli and Briggs (1982a) with the laser input applied perpendicularly to the cut surface of the tissue sections.
Fig. 20. Acceptance angle in intact Glycine max seedlings plotted at three tissue water contents. Light emerging from the cut end of a tissue segment was measured with a photomultiplier (Mandoli and Briggs, 1982b). The maximum light output for a given tissue segment was designated as 100%, and that at an angle of incidence of zero as 0%. Ten seedlings were measured at each angle for all degrees of hydration and the mean and percent standard deviation were calculated for each angle for each hydration state. Fully hydrated tissues showed a peak of light-guiding capacity at 55 degrees. Overall light-guiding capacity of tissues dehydrated to 63% and 39% of their initial fresh weight decreased in their light-guiding capacity to 23% (maximum shifted to 63 degrees) and 3.9% (maximum shifted to 65 degrees) of the fully hydrated control. Curves have been adjusted vertically for clarity.

The sensitivity of the fiber-optic properties of plants to changes in water status (Table 2) may provide a means to measure water movements through plant tissues. Our initial attempts to discern the path of water through etiolated soybean hypocotyls and to correlate these movements with the light-guiding capacity of the cells was complicated by the apparent existence of two possible routes for water movement during tissue rehydration. Tissues rehydrated via the cuticle (ends occluded) regain their initial fresh weight roughly ten times faster than those allowed to rehydrate via the ends alone (sides occluded) (Table 3). The time required to rehydrate correlates roughly with the relative surface area of the exposed region.

The extent of tissue water recovery was clearly a function both of the degree of dehydration attained and the path of rehydration. Tissues dehydrated to 60% of their initial fresh weight recovered fully if allowed to rehydrate via the cuticle whereas even a decrease to 80% of initial fresh weight could not be fully regained

Table 3. Path of Rehydration in Soybeans*

<table>
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<th>Dehydrated to (approx. %)</th>
<th>Ends Occluded</th>
<th>Cuticle Occluded</th>
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<td></td>
<td>Time (min)</td>
<td>Recovered to (%)</td>
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<td>20–40</td>
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<td>40</td>
<td>150–300</td>
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</tbody>
</table>

*From three to four 10-cm etiolated hypocotyl sections were allowed to dehydrate to 80, 60, or 40% of their initial fresh weight. The ends or sides of these sections were then coated with vaseline and a layer of Saran Wrap and were immersed in distilled water for two days. The increase in fresh weight over this time was monitored.
over 48 h if rehydration occurred through the tissue ends (Table 3).

In summary, the endogenous light-guiding ability of plant tissues may prove useful as a quantitative measure of water movement and/or water status within the tissue. The promise of this concept depends on the uniformity of change in tissue light guiding as water is lost. The effects of possible local changes in hydration, such as more rapid dehydration of tissue ends than of the center or alteration in dimensions of radial tissues relative to the vascular system are unknown. However, if these changes have minor effects, light guiding may provide a means to study the kinetics of hydration and dehydration remotely and continuously.

REFERENCES


GROWTH DISTRIBUTION DURING FIRST POSITIVE PHOTOTROPIC CURVATURE OF MAIZE COLEOPTILES

Moritoshi Iino and Winslow R. Briggs

Phototropic curvature in higher plants is the consequence of growth differential induced across the plant axis. Despite this obvious aspect of the phototropic response, the nature of growth distribution during phototropic curvature has not been clearly characterized (see Firn and Digby, 1980). Recent reinvestigation of this problem by Franssen et al. (1981; 1982) with oat coleoptiles or other plant materials suggested that growth inhibition on the irradiated side of the plant axis is the primary growth response causing phototropic curvature. The authors interpreted their results in favor of Blaauw's hypothesis (1915) that the curvature is induced by differential growth inhibition. However, the results do not exclude the possibility that the observed growth responses during phototropic curvature are a mixture of growth redistribution, which is the basis of phototropic responses, and more-general growth responses to light, which cannot result in a growth differential. We obtained evidence that the first positive curvature of the maize coleoptile is caused by a redistribution of growth without change in net growth.

MATERIALS AND METHODS

Maize seeds were sown on moist Kimpack paper. Seedlings were selected for uniformity two days after sowing and transplanted into moist vermiculite. Seedlings were grown for another day for use in experiments. The growth of plants took place under red light (R, 0.15, \( \mu \text{mol m}^{-2} \text{s}^{-1} \)) from the time of sowing at 24°–25°C. Plants also received some R from above during phototropic induction and curvature development. The length of the coleoptile at the time of the experiments was about 2 cm. The entire coleoptile or the tip of the coleoptile was unilaterally irradiated with blue light (Corning blue glass filter, 5-60). When the coleoptile tip was to be irradiated, the coleoptile was covered during phototropic induction with a cylindrical tube made of black photographic tape, leaving the 1-mm tip portion uncovered. Duration of the irradiation was always 30 s, and the longest transverse axis of the coleoptile was parallel to the direction of the incident light.

To obtain increment in length for irradiated and shaded sides of the coleop-
DEPARTMENT OF PLANT BIOLOGY

tile, the coleoptile was marked 1.5 cm from the tip, just before phototropic induction, with India ink. In the fluence-response studies, the coleoptile was excised at the mark 100 min after phototropic induction, and the excised coleoptiles were photocopied. Images of coleoptiles excised immediately after the marking and those excised after 100 min without phototropic induction (control) were also obtained. In the time-course studies, the coleoptile was photographed using R-sensitive film (Kodak). In this case, the images in developed film were enlarged by means of a slide projector, and the enlarged images were traced on paper. Length measurements were obtained with a digitizer directly on line with a computer.

RESULTS AND DISCUSSION

The phytochrome-mediated growth responses in oat or maize seedlings have been shown to be so sensitive that even short exposure to dim green or blue light can cause substantial growth changes (e.g., Blaauw et al., 1968; Mandoli and Briggs, 1981; Iino and Carr, 1981; Iino, 1982). In the present study, growth of the seedlings, phototropic induction with blue light, and subsequent curvature development all took place under R. In this way, we hoped to minimize any effect of phytochrome phototransformation by the blue light itself. The coleoptiles of these seedlings showed a peak-shaped phototropic fluence-response curve, characteristic of the classical first positive curvature of the coleoptile. The threshold and peak fluences were also similar to those obtained by Chon and Briggs (1966) with dark-grown maize seedlings given only a brief exposure to R two hours prior to phototropic induction.

As illustrated in Fig. 21, phototropic curvature could be ascribed to growth stimulation on the shaded side and compensating growth inhibition on the irradiated side. At the high fluences, there was some evidence for overall stimula-

Fig. 21. Phototropic fluence–response curves for curvature (A) and growth increments on irradiated and shaded sides (B). Whole coleoptile irradiation. Each datum point is the mean obtained from 7–9 plants. Different symbols indicate separate experiments.

The above results for phototropic fluence–response curves obtained by irradiating the entire coleoptile as well as only the coleoptile tip agrees with the view that the extreme tip of the coleoptile is the site of photoreception for the first positive curvature of the coleoptile (Lange, 1927). The fact that the increase in the overall growth is shifted to higher fluences when only the tip of the coleoptile is irradiated indicates that this response to blue light is mediated by a
different mechanism than that mediating the phototropic response. (The photoreception site of the latter response is not restricted to the tip of the coleoptile.)

Time courses of the growth increment at shaded and irradiated sides were obtained for the blue light fluence causing the peak of the first positive curvature, together with the growth increment for the control plants that received no phototropic induction. As shown in Fig. 23, irradiation of the entire coleoptile and that of the coleoptile tip produced essentially identical time courses. Stimulation of growth on the shaded side and the inhibition of growth on the irradiated side began simultaneously about 30 min after the phototropic induction. The growth on the irradiated side almost ceased. However, since the growth rate on the shaded side is doubled relative to the control rate, it appears that the overall growth during phototropic curvature was not much affected.

The results obtained for the fluence-response relationship and the time courses show that the phototropic growth differential is essentially the result of growth redistribution and is not necessarily accompanied by changes in the net growth. The results are consonant with the view, known as the Cholodny-Went theory of tropism, that phototropic curvature is the consequence of a lateral redistribution of auxin (Went and Thimann, 1937).

**REFERENCES**


PHYTOCHROME-MEDIATED PHOTOTROPISM IN MAIZE SEEDLING SHOOTS

Moritoshi Iino, Winslow R. Briggs, and Eberhard Schafer

Action spectra for phototropic curvature in higher plants show characteristic shapes in blue light (B) and near-ultraviolet regions (e.g., Thimann and Curry, 1960). Ultraviolet-sensitive phototropism, the response site distinct from that for the B-type phototropism, has also been evidenced (Curry et al., 1956). A general conclusion reached earlier is that the wavelengths longer than about 520 nm are normally phototropically inactive in higher plants (Galston, 1959).

More recently, the question whether or not phytochrome-mediated inhibition of hypocotyl growth can result in phototropic curvature was tested by Shropshire and Mohr (1970) in etiolated seedlings of Sinapis and Fagopyrum. Under continuous irradiation (24 h) of the seedlings with unilateral R or far red (FR) light, they were able to produce gradients in anthocyanin production, known to be controlled by phytochrome, across the hypocotyl tissue, especially of Fagopyrum. However, no phototropic curvature could be observed, although the light fluence rates used were such not to result in saturation of growth inhibition.

While studying phototropic curvature of maize seedling shoots, we obtained evidence that the mesocotyls of these seedlings can bend positively toward R after a Pfr gradient is established across the plant axis. Results are summarized below.

Unilateral irradiation with R or B elicits positive curvature of the mesocotyl of maize seedlings (Zea mays L.) raised under R for two days from sowing and kept in the dark for one day prior to curvature induction. The fluence-response curve for R-induced mesocotyl curvature, obtained by measuring curvature 100 min after phototropic induction, shows peaks in two fluence ranges, designated first positive range (from the threshold to the trough, $10^{-4}$–$10^{-0.2}$ μmol m$^{-2}$) and second positive range (above the trough, $10^{-0.2}$–$10^{2.5}$ μmol m$^{-2}$). The fluence-response curve for B is similar to that for R but is shifted two orders of magnitude to higher fluences. Although the classical first positive curvature of the coleoptile is apparent with B, this response is not found with R. Positive mesocotyl curvature induced by either R or B is eliminated by immediate preirradiation with vertical R, unlike the B-induced coleoptile curvature, which is not eliminated. Mesocotyl curvature appears to be induced by R-sensitive photosystems.

Both R and B lead to negative curvature of the coleoptile; the B-induced negative curvature is found at fluences higher than those showing positive coleoptile curvature. Clinostat experiments show that the negative coleoptile curvature induced by either R or B is gravitropic compensation for positive mesocotyl curvature.

On the clinostat, the R-induced mesocotyl curvature develops after a lag and continues through two successive phases having different curvature rates, the late phase slower than the early phase. Durations of the lag and the early curvature phase become shorter as the fluence is increased in the first positive range,
whereas both are prolonged as the fluence is further increased to the second positive range.

Mesocotyl curvature in the second positive range is reduced by vertical FR applied after phototropic induction with R, but it is not affected by FR applied before. Unilateral irradiation with FR following vertical irradiation with a high R fluence leads to negative curvature of the mesocotyl. We conclude that mesocotyl curvature in the second positive range results from a gradient in the amount of Pfr established across the plant axis. Mesocotyl curvature in the first positive range is induced by unilateral irradiation with FR. This result is not inconsistent with the view that the curvature response is mediated by phytochrome, since the very small amount of phytochrome phototransformation induced by R in the first positive range could also be brought about by the FR. Red light–induced mesocotyl curvature in the first positive range is inhibited by vertical FR given either before or after the phototropic induction. This result indicates that the vertically applied FR reduces the response by adding Pfr at both sides of the plant axis.

REFERENCES


SPECTROSCOPY OF CHLOROPHYLL IN PHOTOSYNTHETIC MEMBRANES

Jeanette S. Brown

I. SPECTRA OF THREE NATIVE CHLOROPHYLL-PROTEINS

Our goal for many years has been to measure the absorption spectrum of chlorophyll a in its photosynthetically functional state. The problem has been to separate the chlorophyll-proteins from chloroplast membranes without altering the state of the pigment molecules. Earlier this year, Picaud et al. (1982) found a separation procedure which meets these requirements. They used digitonin for solubilizing chloroplast membranes and sodium deoxycholate (DOC) for a charged carrier during polyacrylamide gel electrophoresis (PAGE). Three bands were identified in the gel: a photosystem I chlorophyll-protein (CP1), which was the slowest migrating band; a photosystem II band (CPa); and an antenna Chl a-b protein (LHCP), which migrated the most rapidly. Other minor green bands were also found, but these were similar to one or the other of the three major bands.

We repeated the procedure with spinach chloroplasts, and measured absorption and fluorescence spectra at 77 K of the pigment-proteins extracted from the separate gel bands (Brown, 1983). Those spectra of the chlorophyll-proteins located close to the reaction centers of photosystems I and II and of the antenna are shown in Fig. 24. Spectra of washed spinach chloroplast membranes are shown at the top of the figure for comparison. After these membranes were solubilized with digitonin, their absorption spectra were unchanged but their fluorescence emission spectra (not shown) resembled the spectrum of LHCP, because energy
transfer from the antenna to the other chlorophyll-proteins was disrupted. When absorption spectra of the three isolated chlorophyll-proteins were added together in proportions corresponding to their density in the gel (35% CPI, 15% CPa, and 50% LHCP), the sum nearly matched the spectrum of the thylakoids. This result shows that the spectra of these three complexes account for essentially all of the chlorophyll absorption in the membranes, and each spectrum represents the absorption of the native state of each chlorophyll-protein.

This is the first time that such spectra of native chlorophyll-proteins isolated from the same chloroplasts in one procedure have been measured (Fig. 24). The maximum near 650 nm in LHCP is from Chl b. Obviously the shape of the Chl a absorption differs considerably between the three spectra. Because we had previously postulated that Chl a absorption in most plants may be represented by four major Gaussian-Lorentzian-shaped component bands, the RESOL curve-fitting computer program was applied to these spectra with input components similar to those previously found with both whole and partially fractionated thylakoid particles (French et al., 1972). Only the LHCP spectrum could be matched well with the four “universal” components, or “forms,” at approximately 660, 670, 677, and 684 nm. CPa has the 660-nm and 670-nm components; it also has a large, broad component at 675 nm and another at 682 nm. CPI has components at 662, 668, 679, and 687 nm; the 679 band is very large and all components are wider than those in the other spectra. Thus we may no longer consider that only four major absorbing forms of Chl a exist. The antenna chlorophylls dedicated to each photosystem have their own groups or pools of pigment molecules. Probably these differences reflect differences in the proteins and their binding to chlorophyll.

II. DETERGENT ACTION ON CHLOROPHYLL-PROTEIN ABSORPTION

Because sodium dodecylsulfate (SDS) and Triton X-100 are used so commonly for the solubilization and separation of intrinsic membrane proteins, the effects of these detergents on chlorophyll absorption should again be documented (Year Book 78, 189–194; 81, 38–40). Both the antenna Chl a-b protein (LHCP) isolated from higher plants and a similar complex from Mantoniella (see section III, below) have a sharp absorption maximum near 676 nm when they are isolated with digitonin, DOC, or Triton. Some years ago we isolated LHCP from spinach chloroplasts by solubilization with Triton followed by separation by centrifugation in sucrose density gradients (Year Book 78, 193–194). LHCP was precipitated from the sucrose gradient band by the addition of cations, lyophilized, and stored at −20°C. The absorption of this LHCP, resuspended in buffer by sonication, still retained the 676-nm maximum (Fig. 25). The Mantoniella LHCP was prepared with digitonin and DOC, as described in section III, below.
Fig. 25. Absorption spectra (77 K) of spinach and Mantoniella light-harvesting chlorophyll-proteins before and after treatment with 0.1% SDS or Nonidet.

Aliquots of each LHCP were diluted with 50 mM Tris buffer at pH 7.3 to about 30 μg/ml of chlorophyll and 0.1% of either SDS or Nonidet P40 (a detergent similar to Triton X-100). After mixing with the detergent, the samples were incubated for 10 min at room temperature in darkness before absorption spectra were recorded at 77 K. The spectra before and after detergent treatments are shown in Fig. 25. The most obvious change is a decrease in the 676-nm peak. That this change was caused by Nonidet is surprising because the spinach LHCP was isolated originally with this detergent. Probably the chlorophylls in the isolated complex were more exposed to detergent action than they were in the thylakoid membrane. The view that the 676-nm peak is an artifact caused by cation-induced aggregation of LHCP and therefore is lowered when the complexes are disaggregated by detergent action may be countered by the observation that the most rapidly migrating LHCP after electrophoresis with DOC is certainly not aggregated, but does have the 676-nm absorption maximum (Fig. 24). Spinach LHCP was also treated with 0.1% DOC in this experiment, but there was no change in its spectrum (not shown).

Another example of SDS action can be seen in Fig. 26 and Table 4, where spectral analyses of Mantoniella antenna chlorophyll-protein isolated by either sucrose gradient centrifugation with 0.05% DOC or by PAGE with 0.07% SDS are compared. Like the curves in Fig. 25, SDS caused a decrease in absorption at 676 relative to 670 nm. In addition, the curve resolutions show that several of the components changed. The long-wavelength bands at 680 and 687 nm...
TABLE 4. The Band Maxima, Half-widths, and Proportion (%) of Total Area from RESOL Spectral Analyses in Fig. 26*

<table>
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<tr>
<th>Band No.</th>
<th>DOC Max (nm)</th>
<th>DOC HBW (nm)</th>
<th>DOC %</th>
<th>SDS Max (nm)</th>
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*Spectra are of antenna chlorophyll-proteins prepared from Mantoniella membranes solubilized by either digitonin and DOC or by SDS only.

nearly disappeared. The 676-nm and 669-nm components retained the same peak positions and widths, but the band at 676 nm decreased by 1%, and the band at 669 increased by 3%. The 662-nm band shifted to 663 nm and became 1 nm wider—a small change but evidently enough to remove the shoulder near 660 nm on the spectrum. The 652-nm component became narrower by 6 nm but occupied 2% more of the total area under the curves after SDS treatment; also, it changed from 85% to 67% Gaussian in shape. The 643-nm Chl b band became narrower by 4 nm but accounted for nearly the same total area. The 632-nm band from the Chl c-like pigment and the composite 621-nm band remained essentially unchanged. Except for the 676-nm band, the proportions of Gaussian- and Lorentzian-shaped curves changed somewhat with all of the components, most notably with the 652-nm component.

In summary, SDS action caused subtle changes in most of the component bands comprising the chlorophyll absorption spectrum. If these component bands do indeed represent discrete groups of chlorophyll molecules bound to different sites on a protein, it is not surprising that a strong detergent can disturb several of these sites. These results emphasize that studies designed to investigate the state of chlorophyll in vivo using such techniques as resonance Raman, dichroism, or fluorescence polarization spectroscopy should be used with material that has not been exposed to SDS or Triton.

III. CHLOROPHYLL-PROTEIN COMPLEXES FROM PRIMITIVE GREEN ALGAE

Many times in the past we have compared absorption spectra of diverse algal species to gain a better understanding of the essential nature of chlorophyll in vivo. Because we now know that most of the chlorophyll is attached to protein, our goal has become the extraction of native pigment-protein complexes from the different algae. This year we have begun the study of a primitive group of green microalgae called Prasinophytes (Norris, 1980). This group contains a number of motile genera thought to be the antecedents of the better-known *Chlamydomonas* and *Dunaliella*. Two genera, *Micromonas* and *Mantoniella*, are at the bottom of the evolutionary scale, and both contain a chlorophyll c-like or protochlorophyll-like pigment—magnesium 2,4-divinylphaephorphyrin α₅ monomethyl ester—in addition to chlorophylls a and b (Ricketts, 1966a). *Micromonas* and *Mantoniella* also have an unusual carotenoid, which has been named micronone, or microxanthin in its reduced form (Ricketts, 1966b). Ricketts studied *Micromonas pusilla* only, but we have found that both *Micromonas pusilla* (Culture Collection, University of Texas, UTEX
No. 991) and *Mantoniella squamata* (UTEX No. 990) have identical absorption and fluorescence spectra. Cells of both species are highly motile spheres about one micron in diameter. We have found that *Mantoniella* grows much better than *Micromonas* in an enriched seawater medium, making it more useful for biochemical studies.

We routinely culture *Mantoniella* in 3-liter batches at about 20°C over a bank of daylight fluorescent lamps (continuous light) with air bubbling through the medium. It is our intention to try several, more-defined growth conditions in the future. The cells are harvested by centrifugation and broken by passage through a French press. The cell membranes are washed with 5 nM EDTA, pelleted, and stored frozen at -80°C.

An absorption spectrum of the cell membranes shows several features that may be unique to *Mantoniella* and *Micromonas* (Fig. 27). The peak of Chl b near 643 nm is shifted downward by nearly 5 nm compared to its maximum in spectra of higher green algae and plants. The band at 632 nm is probably from the Chl c-like pigment. The band near 520 nm may be one of the peaks of microxanthin. To estimate the chlorophyll concentration we used the equations of Jeffrey and Humphrey (1975), which were designed to measure Chls a, b, and c in mixed phytoplankton populations. The cells each had approximately $4 \times 10^{-8}$ μg chlorophyll, with ratios of Chl $ab = 3$ and $ac = 10$. These ratios have varied between 2.6 and 3.4 and between 8.9 and 11 because of yet undefined culture conditions.

We are trying various detergents for solubilization and sucrose density gradient centrifugation or gel electrophoresis for isolation of chlorophyll-proteins from the cell membranes. Thus far we have separated a green fraction in sucrose gradients from membranes solubilized in 0.5% Nonidet. This fraction is highly enriched in photosystem I (Chl/P700 = 89). Another brown fraction in the same gradient contained most of the antenna pigments (Chl $ab = 1.8$). Spectra of these fractions are shown in Fig. 27.

![Fig. 27. Absorption (solid lines), fluorescence excitation (400–600 nm), and emission (650–750 nm) spectra measured at 77 K of chloroplast membranes, antenna pigments, and PS I fractions isolated from *Mantoniella*. Excitation at 438 nm for emission spectra, slit-width = 3 nm; excitation slit-width = 4 nm for emission at 680 nm.](image-url)
We have not yet been able to subdivide further the brown antenna pigment fraction.

An important observation from fluorescence excitation spectra of both the membranes and fractions is the appearance of a band near 520 nm. Possibly, microxanthin can transfer absorbed light energy to Chl a and may be in a class of truly photosynthetic carotenoids similar to peridinin and fucoxanthin. Finding excitation bands at 470 nm from Chl a and near 520 nm from a carotenoid in spectra of the PS I fraction was unexpected. I have observed these bands even in PS I fractions separated by gel electrophoresis, which should be less contaminated by antenna fractions than those separated in sucrose gradients. The emission spectrum of Chl a in PS I fractions is also more like that in antenna-pigment fractions. The emission spectrum of either intact or broken cells does not have the long-wavelength bands seen in most green algae or higher plants at low temperature (Brown, 1969). Obviously Mantonella does have long-wavelength absorption bands (Fig. 27). Possibly the fluorescence yield of photosystem I in this alga is so low that a small amount of antenna pigment can mask its emission.

Micromonas-like phytoplankton have been isolated from the open ocean, sometimes at considerable depth (Jeffrey and Hallegraeff, 1980). The little light that penetrates to these depths is mostly in the blue-green spectral region. Our spectral studies show that these organisms are well adapted by their pigment content to harvest this light.

We have begun to study two other Prasinophytes, Platymonas sp. (UTEX No. 634) and Tetraselmis chui (CSIRO Culture Coll. No. CS26), which may actually be in the same genus (Norris, 1980). Their spectra are similar to each other and to those of most other green algae containing Chls a and b.

IV. CHLOROPHYLL-PROTEIN COMPLEXES FROM Euglena

Euglena has long been a favorite species for study by biologists, and at least two books are devoted exclusively to this organism (Wolken, 1967; Buetow, 1968). In our laboratory, French and Elliott (Year Book 57, 278–286) first observed the unique shape of the chlorophyll a absorption spectrum of Euglena with its three distinct maxima at about 672, 680, and 695 nm. Later we found (Year Book 59, 330–333) that the relative heights of these maxima as well as the proportions of Chl a and Chl b depended upon the light intensity during growth of the cells; the brighter the light, the more Chl b and the less longer-wavelength-absorbing Chl a forms.

Euglena was included in our first comparisons of isolated chlorophyll-proteins from diverse plants (Year Book 73, 694–706). Solubilization of Euglena chloroplasts with Triton X-100 or sodium dodecylsulfate, followed by separation of fractions with hydroxylapatite chromatography or polyacrylamide gel electrophoresis (PAGE), respectively, yielded chlorophyll-proteins that were only partly analogous to those from other algae and higher plants. One of these fractions, called CPAa, has been observed only from Euglena and has an unusual absorption peak at 683 nm in addition to the broad maximum at 670 nm. Later we were able to isolate three different chlorophyll-proteins from Euglena using Triton X-100 to solubilize the chloroplasts, and sucrose density gradient centrifugation to separate the fractions (Brown, 1980). By this procedure the lightest gradient band contained the Chl a-b antenna protein, a middle band was highly enriched in photosystem I (Chl/P700 = 50), and the heaviest fraction contained both photosystem I (Chl/P700 = 108) and CPAa.

In our early experiments with SDS and PAGE (Year Book 73, 694–706), we could distinguish the photosystem I band (CP1) near the top of the gel, but the other chlorophyll bands quickly broke down to free pigment. Ortiz and Stutz (1980) were able to separate the three major chlorophyll-proteins (CP1, CPA, and LHCP) with PAGE after solubilization of Eu-
*Euglena* chloroplasts with SDS. However, because SDS destroys the sharp absorption peak at 683 nm in CPa1, and Triton destroys the long-wavelength-absorbing Chl a (Brown, 1980), we tried the new procedure of Picaud et al. (1982), which uses digitonin for solubilization and sodium deoxycholate for electrophoresis. We still found considerable free pigment, but absorption and fluorescence spectra of three green bands isolated from the gel strongly suggested that they are CPI, CPa, and LHCP (Fig. 28).

The slowest migrating band in Fig. 28 (#1) has the long-wavelength absorption and emission characteristics of CPI in most higher plants. Band #2 has the absorption typical of CPa, with its prominent shoulder near 680 nm (Fig. 24) and emission band near 695 nm, but the emission near 720 nm may be unique to *Euglena*. Band #3 not only has most of the Chl b, like LHCP from other plants, but also has a sharp absorption band near 683 nm, like CPa1. This last result was unexpected because the CPa1 is isolated after Triton treatment and hydroxylapatite chromatography has no Chl b. Possibly, CPa1 is co-migrating with LHCP in this electrophoretic system. Further experiments will be necessary to clarify this last point, but the results suggest that definitive characterization of the chlorophyll-proteins of this very interesting alga will soon be possible.

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IS THE RED ABSORPTION BAND OF CHLOROPHYLL \textit{a} SKewed?

Jeanette S. Brown

In 1968, we first used a computer program called RESOL to deconvolute absorption spectra of chloroplast and algal fragments into Gaussian-shaped component curves (\textit{Year Book} 67, 536–546). When mixed Gaussian-Lorentzian components were used, the fit was much better, particularly in the long-wavelength tail region. Symmetrical component bands have always been assumed until now (French \textit{et al}., 1972). However, it was noted in 1968 that the red absorption bands of Chl \textit{a} dissolved in 80\% acetone-water and, later, bacteriochlorophyll \textit{a} dissolved in 80\% acetone-water (Philipson and Sauer, 1972) were not symmetrical but were skewed towards shorter wavelengths. This observation suggested that the complex spectra of chlorophyll in vivo might better be matched with asymmetric component bands.

This year we contracted with David Pasta of DMA Corporation to modify RESOL to permit the use of skewed components. In effect, the program can now vary the ratio between the half-widths of the left (short-wavelength) and right sides of each component band. We can set this ratio or allow the program to find the best one; the ratio is 1 for a symmetrical band.

To test the modified program, we recorded the absorption spectrum at 20\°C of an 80\% acetone-water extract of \textit{Anacystis nidulans}, a blue-green alga in which Chl \textit{a} is the only chlorophyll (\textit{Year Book} 80, 15–16). The optical density at the peak, 664 nm, was 0.45. We attempted to match the absorption band of this spectrum between 630 and 700 nm with either two or three components; the component added near 630 nm represented lower vibrational levels of the major band. Different peak wavelengths, bandwidths, Gaussian-Lorentzian ratios, and asymmetry parameters were tested.

Fig. 29. Absorption spectrum of Chl \textit{a} in 80\% acetone-water recorded at 20\°C resolved into (A) two or (B) three components. Band No. 1, that at the longest wavelength, represents lower vibrational levels of the major band. The measured data are plotted as points, while the line through them is the sum of the component curves. The error of fit is shown below each spectrum on a scale with the designated magnification.
TABLE 5. Four RESOL Analyses of an Absorption Spectrum (630–700 nm) of Chl a in 80% Acetone*

<table>
<thead>
<tr>
<th>Band No.</th>
<th>Max (nm)</th>
<th>HBW† (nm)</th>
<th>Gaussian (%)</th>
<th>Skew Factor</th>
<th>Total Area (%)</th>
<th>S.E.</th>
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<tr>
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<td>630</td>
<td>45</td>
<td>100</td>
<td>0.98</td>
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<td>.69†</td>
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<td>2</td>
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<td>20</td>
<td>84</td>
<td>1.03</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>630</td>
<td>46</td>
<td>93</td>
<td>1.00</td>
<td>25</td>
<td>.70</td>
</tr>
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<td>664</td>
<td>20</td>
<td>83</td>
<td>1.00</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>633</td>
<td>37</td>
<td>100</td>
<td>1.02</td>
<td>20</td>
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<td>2</td>
<td>648</td>
<td>8</td>
<td>100</td>
<td>1.00</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>3</td>
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<td>85</td>
<td>0.98</td>
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<td></td>
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<tr>
<td>1</td>
<td>633</td>
<td>39</td>
<td>98</td>
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<td>664</td>
<td>20</td>
<td>86</td>
<td>1.00</td>
<td>78</td>
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</table>

*The curve was analyzed with two or three components and with or without skewing. The S.E. of each analysis is expressed as percentage of peak height.
†Bandwidth at half height.
‡Figure 29A.
§Figure 29B.

and half-bandwidth ratios (skew factors) were tried for input to RESOL.

Figure 29A shows the spectrum resolved with two components, and Fig. 29B shows the spectrum with three. Table 5 shows the band parameters for the components when the RESOL program was allowed to skew each component (Fig. 29) and also for corresponding analyses where skewing was not allowed. The data show that the addition of only 1% of a band at 648 nm improved the match over that with one component, but skewing the components did not change the results significantly. The main component at 664 nm is about 85% Gaussian and 15% Lorentzian in shape. Apparently, the absorption spectrum of Chl a cannot be matched completely with a single Gaussian-Lorentzian component, skewed or not. Perhaps another type of skewed function may give a better approximation of chlorophyll absorbance.

The continuing interest and help of Glenn Ford for modifying RESOL is much appreciated.

REFERENCES
ADAPTATION BY THE RED ALGA *Porphyra perforata* TO CHANGES IN THE INTENSITY AND QUALITY OF LIGHT—A NEW MECHANISM

David C. Fork and Kazuhiko Satoh

Even though light is required for photosynthesis, the absorption of too much light can damage the photosynthetic machinery by photoinhibition (Powles and Thorne, 1981). By contrast, plants that live under low-light conditions often absorb light of a quality (color) that is not optimal for photosynthesis, thereby producing unbalanced excitation of the two photosystems.

Plants apparently have several mechanisms to aid them in overcoming limitations from absorption of too much light or light of an unfavorable wavelength. Last year, we reported (Year Book 81, 45–58) that in a blue-green and in a green alga we could clearly observe one of these mechanisms, the so-called state I-state II transition (Bonaventura and Myers, 1969; Murata, 1969; Wang and Myers, 1974). This year, we found two other mechanisms available to the red alga *Porphyra perforata* in overcoming unfavorable conditions of illumination. These mechanisms appear related to the ability of *Porphyra* to thrive under conditions of high light intensity, high salinity, and desiccation.

The plants obtained in these studies were collected at Half Moon Bay, California, and maintained under illumination in open dishes of sea water at 13°C. Samples were kept in the dark for 1 h before use.

Fluorescence spectra at 77 K and time courses at room temperature of fluorescence at 685 nm were measured with a fiber-optic system to excite and collect the fluorescence (Year Book 78, 196–199). Light-induced absorbance changes were measured with a single-beam spectrophotometer.

We could see marked changes in the intensity of chlorophyll fluorescence (Kautsky transients) when dark-adapted *Porphyra* was illuminated with light absorbed largely by photosystem II (light II, data not shown). Figure 30 shows fluorescence emission spectra measured at 77 K before and after illumination of dark-adapted *Porphyra* thalli with light II. The difference spectrum, given in the bottom curve of the figure, shows that light II produced a fluorescence decrease of both

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![Fluorescence emission spectra of *Porphyra perforata*](image-url)
REFERENCES

CHARACTERISTICS OF THE STATE II–STATE III TRANSITIONS IN THE RED ALGA Porphyra perforata

Kazuhiko Satoh and David C. Fork

As described in the previous report, excess light absorption by photosystem II in the red alga Porphyra perforata transforms it into a newly found state, termed state III. After conversion to this state, system I activity was unchanged, but there was a decrease in the light energy reaching photosystem II. Light absorbed by photosystem I was found to have a more antagonistic effect than system II light on the state II–state III transitions. The state II to III transition was related to the formation of ΔpH, and the state III to II transition was related to the formation of a membrane potential across the thylakoid membrane.

The Porphyra used in the experiments here reported was collected locally and maintained as described in the previous report. Green light (light II) was obtained by filtering the white light from a 150W, 21.5V projector lamp (DLS) through Corning glass filters 4-96 and 3-69 and a Califlex C heat-reflecting filter (Balzers). For light I, far red or blue light absorbed mainly by chlorophyll a of PS I was used. The output of the lamp was filtered through a Schott RG10 (A > 690 nm) cutoff filter (for far red light) or through Corning filters 4-96 and 5-60 (for

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TABLE 6. Effects of NH₄Cl, DCMU, DCCD, and Valinomycin on the State II to III Transition in Porphyra

<table>
<thead>
<tr>
<th>Added</th>
<th>F695, relative extent</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>25.9</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>5.1</td>
</tr>
<tr>
<td>DCMU</td>
<td>9.3</td>
</tr>
<tr>
<td>DCCD</td>
<td>28.8</td>
</tr>
<tr>
<td>Val. + KCl</td>
<td>28.1</td>
</tr>
</tbody>
</table>

*The samples were illuminated with light II for 3 min at 21°C, and the extents of the state II to III transition were measured by the decrease of F695 at 77 K. The concentrations of NH₄Cl, DCMU, DCCD, and Valinomycin and KCl were 30 mM, 20 μM, 10 μM, 10 μM, and 10 mM, respectively.

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Fig. 34. The continuation in the dark of the state II to state III transition after exposure of Porphyra perforata to green light absorbed by photosystem II. Green light (235 μW/cm²) was given for 5 s (at 21°C), after which the sample was cooled to 77 K and the emission spectrum measured (solid curve) using the same green light attenuated to 58 μW/cm². The dashed curve was obtained from a sample given 5 s of green light, 30 s dark, and then cooled to 77 K.
Fig. 35. The kinetics of the state III to II transition in the dark and in light I. State III was obtained by illuminating Porphyra with light II at 21°C for 3 min. The recovery to state II in the dark or in light I was then measured as the increase of F695 at 77 K. The intensity of the far red light was 1.40 mW/cm² (λ > 690 nm).

blue light). A Nicolet Signal Averager (Model 1010) was used as a transient time converter for measurements of fluorescence in the ms time range.

We found in the previous report that Porphyra exists in state II in the dark. Conversion of Porphyra from state II to state III upon absorption of light II gives rise to a decrease of system II fluorescence at 695 nm (F695). We measured fluorescence spectra at 77 K and studied the effects of several substances. The data presented in Table 6 show that the uncoupler of photophosphorylation NH₄Cl inhibited the state II to III transition. A similar effect was found using CCCP (carbonylcyanide m-chlorophenylhydrazone) (not shown). DCMU also inhibited the transition. The inhibitor of ATP formation, DCCD (N,N'-dicyclohexyl carbodiimide), and the ionophore valinomycin + KCl did not have an effect on the transition. These results suggest that proton translocation across the thylakoid membrane is related to the state II to III transition.

In order to convert dark-adapted Porphyra from state II to state III, system II light was given for 5 s and the sample was then immediately cooled to 77 K (Fig. 34, solid line). Another sample was also illuminated for 5 s, but it was then given a period of 30 s darkness before being frozen to 77 K (Fig. 34, dashed line). It is clear from this result that the state II to III transition proceeds in the dark after illumination with light II. Ried and Reinhardt (1977) also observed a dark step in light II effects in other red algae.

Figure 35 shows that the recovery from state III to II occurred slowly in the dark and that light I accelerated this transition. It is unlikely that the increase of F695 shown in Fig. 35 is produced by a state II to I transition, since we saw that this transition occurred very slowly and
required 30 min to show the maximum effect in Porphyra (see previous report).

In order to check further whether the state III to II transition was accelerated by light I, we measured fluorescence spectra at 77 K. Figure 36 shows the fluorescence spectrum obtained when Porphyra was illuminated for 2 min with green light absorbed by system II and then frozen to 77 K (dashed line, labeled G). The solid line (B) of Fig. 36 represents the spectrum obtained when Porphyra was illuminated with green light for 2 min as before, but it was then exposed for 1 min to blue light absorbed by system I. The difference spectrum (B-G) shows that system I light brought about the state III to II transition and not the state II to I transition, since there was a parallel increase of both system II fluorescence bands, at 685 and 695 nm, and an increase of the long-wavelength system I band—features characteristic of the state III to II transition (see Fig. 30). By contrast, the state II to I transition induces changes specific to one of

\[ \text{Fluorescence, rel} \]

\[ 12 \]

\[ 10 \]

\[ 8 \]

\[ 6 \]

\[ 4 \]

\[ 2 \]

\[ 0 \]

\[ 30 \text{ sec} \]

\[ \text{Light II off} \]

\[ \text{Light I on} \]

\[ \text{Light I off} \]

\[ \text{Light II on} \]

Fig. 37. The kinetics of the transitions from states II to III and from III to II in Porphyra at 21°C and the effects of NH₄Cl, valinomycin plus KCl, and antimycin A. The arrows indicate the times when light I and light II were switched. The concentrations of NH₄Cl, valinomycin, KCl, and antimycin A were 5 mM, 10 μM, 10 mM, and 50 μM, respectively. Blue light (682 μW/cm²) was used as light I.
the two system II fluorescence bands (F695) and a decrease of system I fluorescence.

Since light I accelerates the state III to II transition, an exposure to light I following an exposure to light II should increase the fluorescence level excited by system II light. The fluorescence kinetics given in Fig. 37 demonstrate these transitions and show the effects of various substances. After 3 min illumination with light II, the light was switched to light I for 1 min and then switched back to light II. The transitory high level of fluorescence following an exposure to light I is usually considered to result from a state II to state I transition in light I (Year Book 81, 45-49). But the transient high fluorescence level following an exposure to light I is an indication of the transition from state III to state II in light I, as shown in Figs. 35 and 36. The subsequent decline of fluorescence when light I was turned off and light II was turned on indicates the state II to III transition. Figure 37 also shows that the uncoupler NH₄Cl did not prevent the state III to II transition, but valinomycin + KCl or antimycin A strongly inhibited the change.

The state II to state III transition has both a light and a dark process (Fig. 34). Proton translocation across the thylakoid membranes induced by light seems to promote the transition (Table 6). The pH difference induced by the light reaction must decrease during the subsequent dark period. This dark process of the state II to III transition continued until reaching a certain level (Fig. 35). Prolonged storage in the dark brought about the slow conversion back to state II. The acceleration of the state III to II transition by light seems to be related to the formation of a membrane potential change across the thylakoid membranes produced by cyclic electron flow around PS I (Fig. 37), since antimycin A, an inhibitor of cyclic flow around PS I, strongly inhibited the transition, as did the potassium ionophore valinomycin.

Light I and light II have antagonistic effects on the state II to state III transition (Fig. 37). Thus the antagonistic effects of light I and II on the changes of chlorophyll fluorescence do not necessarily indicate that the change is related to a state I to II transition.

It was shown in the previous article that the state II to III transition reduced the light energy reaching photosystem II. This process would take place when there was an excess amount of light II. If, however, light I was present, the reverse process (state III to II transition) was accelerated. It would thus appear that the state II to state III transition may function to balance the light energy reaching photosystems I and II and thereby avoid photodestruction by absorption of excess amounts of light II.

REFERENCE

A REACTION THAT MAY PROTECT THE RED ALGA
Porphyra perforata AGAINST PHOTOINHIBITION

Kazuhiko Satoh and David C. Fork

Studies reported previously (Year Book 81, 45-58) described characteristics of the state I–state II transitions. These transitions appear to be mechanisms helping the red alga *Porphyra perforata* to maintain its two photosystems in an appropriate activity ratio by controlling the transfer of light energy from pigment system II to system I.

Since in its natural environment *Porphyra* is exposed periodically to desiccation and high light intensities as well
as to high salinity, it can be expected that this alga might have developed several other coping mechanisms. The effects of salinity on the photosynthesis of this alga are discussed by Smith et al. (this Report, p. 68). A new mechanism, found in Porphyra and termed the state II to state III transition, is also discussed in this Report, by Fork and Satoh (p. 55) and by Satoh and Fork (p. 58). This mechanism apparently decreases the amount of light energy reaching the reaction centers of PS II without causing any change in photosystem I (PS I) activity. Both mechanisms appear to protect the photosynthetic apparatus against damage by photoinhibition when Porphyra is exposed to high light intensities. If Porphyra becomes desiccated or exposed to high salt concentrations, conditions that occur commonly in the intertidal habitat, its water potential can become very low and the activity of PS II would be expected to be strongly inhibited. This report describes a third mechanism by which Porphyra can avoid photodamage to its reaction centers under conditions of high light and low water potential.

The Porphyra used in these studies was collected at Half Moon Bay, California, and maintained in the laboratory as described by Fork and Satoh on p. 55. Samples were kept in the dark for 1 h or more before use.

Figure 38 shows the kinetics of fluorescence when Porphyra was illuminated in the presence of DCMU with blue light absorbed by PS I and with green light absorbed by PS II. The blue and green actinic lights were adjusted initially to excite PS II equally. The initial reduction of Q was too rapid to be seen clearly on the time scale used in Fig. 38. Surprisingly, after the initial, rapid fluorescence increase attributable to Q reduction (Duysens and Sweers, 1963), fluorescence during illumination decreased more rapidly in blue light (PS I) than in green (PS II). This result suggests that PS I was responsible for this decrease. The decrease of fluorescence cannot be attributed to the reoxidation of Q by a PS I reaction because, at a concentration of 20 μM DCMU, this reaction was completely inhibited, as was O₂ evolution.

Porphyra was illuminated for 20 s and 7 min, respectively, with relatively weak blue light, and fluorescence kinetics were then measured in each case using a strong actinic light and on a faster time scale than in Fig. 38.

Figure 39 shows that 20 s of preillumination with PS I light in the presence of DCMU caused Q to become almost completely reduced, since the initial fluorescence (F₀) was high and there was very little variable fluorescence (Fᵥ). This result indicates that Q was almost completely reduced after the 20-s period of preillumination. But after 7 min of preillumination, Q was largely in its oxidized state.
state, since \( F_o \) was low and \( F_v \) large. It is important to note that both traces reached the same maximum fluorescence level. Thus the fluorescence decline during illumination in the presence of DCMU was produced by an oxidation of Q by a mechanism other than oxidation via plastoquinone because this reaction was completely inhibited by the DCMU. Since fluorescence spectra were the same before and after the fluorescence decline, it is clear that neither a state I-state II transition nor a state II-state III transition was responsible for this effect.

Three mechanisms may be responsible for the oxidation of Q: (1) oxidation by PS I via plastoquinone (not possible, however, because DCMU inhibits this reaction, as pointed out previously); (2) a back reaction of PS II where oxidation of Q is induced by oxidants produced on the water side of PS II (Bennoun, 1970; Homann, 1971; Ikekami and Katoh, 1973); or (3) donation of electrons from Q to unspecified redox substances.

Figure 40 shows the results of an experiment designed to distinguish the reaction(s) responsible for Q oxidation in the presence of DCMU. Trace b of Fig. 40 shows that \( \text{NH}_2\text{OH} \) prevented Q oxidation in the presence of DCMU. If Q were being oxidized by other unspecified redox substances (mechanism 3, previous paragraph), then this oxidation should be increased by \( \text{NH}_2\text{OH} \) since \( \text{NH}_2\text{OH} \) at 1 mM is an inhibitor of PS II (Bennoun and Joliot, 1969; Izawa et al., 1969). When functioning, \( \text{NH}_2\text{OH} \) keeps Q reduced. The uncoupler, CCCP (carbonyl cyanide m-chlorophenylhydrazone), strongly inhibited the fluorescence decline (Fig. 40c). At the concentration of CCCP used (5 \( \mu \text{M} \)), it acts as an uncoupler of photophosphorylation. Again, the oxidation of Q seems not to proceed via activity of PS I (mechanism 1) because the uncoupling action of CCCP would be expected to accelerate rather than retard the reaction. We also tested the effects of CCCP on the back reaction of PS II (mechanism 3) and found that low concentrations of CCCP and \( \text{NH}_2\text{OH} \) inhibited this reaction, as was noted previously by Homann (1971) and Ikekami and Katoh (1973).
Figure 40d shows that antimycin A, which inhibits cyclic flow around PS I (Tagawa et al., 1963), strongly accelerated the fluorescence decline in the presence of DCMU. Inhibition of cyclic electron flow may give rise to an accumulation of either oxidants or reductants produced by PS I.

Since the results of Figs. 38 and 40 suggested that PS I-produced oxidants or reductants accelerated the back reaction of PS II, we tested the effect of artificial electron donors to PS I on the fluorescence decline. Reductants such as reduced DCIP (2,6-dichlorophenol indophenol) or DAD (diaminodurol) decreased the rate of the fluorescence decline in the light. This result suggests, therefore, that an oxidant produced by PS I may give rise to an acceleration of the back reaction of PS II.

These results indicate that still another mechanism is available to *Porphyra* to consume the excess light energy absorbed by PS II: the acceleration of the back reaction of PS II. Such a mechanism could serve to prevent the formation of oxidants with high redox potentials in desiccated plants when oxidation of water is impossible. This mechanism, as well as other mechanisms reported previously (Year Book 80, 39-43; Fork and Satoh, and Satoh and Fork, this Report), apparently serve to prevent photooxidation under unfavorable physiological conditions. We can speculate that cytochrome *b*$_{559}$ may be the substance controlling the back reaction of PS II, since it is located close to the reaction center of PS II, and it can be photooxidized by both PS II and PS I. Further experiments will be necessary to verify this suggestion.

**REFERENCES**


Most fluorescence at room temperature emanates from photosystem II (PS II), while photosystem I (PS I) is very weakly fluorescent (Goedheer, 1972). Fork et al. (1982) reported an intense long-wavelength emission band at room temperature in the marine red alga Porphyra perforata, produced upon excitation of either PS I or PS II pigments.

In this study, we examined in some detail the changes in the yield and emission of this long-wavelength emission band at 715-730 nm in P. perforata and studied its possible associations with PS II and PS I.

P. perforata was collected from January to May at the Hopkins Marine Station, Pacific Grove, California, and at Pigeon Point, California, and was maintained in the laboratory as described earlier (Fork et al., 1982). Fluorescence emission spectra and transients were measured according to Fork et al. (1982). Delayed luminescence (DL) was measured in the ms time range. The cycle and the duration of the actinic light of the phosphoroscope were 5.9 and 0.8 ms, respectively. Light from an He-Ne laser (632.8 nm) was used as the actinic light.

Fluorescence emission of Porphyra at room temperature. Figure 41 shows the emission spectra obtained upon illumination of Porphyra with (A) low-intensity green light (550 nm) or (B) blue light (442 nm). Excitation with green light in the absence of DCMU (Fig. 41, curve a) induced a peak emission at 655 nm (allophycocyanin). A small hump was seen near 683 nm that probably represents the composite emission from allophycocyanin and Chl a. A long-wavelength emission of Chl a appeared as a broad shoulder around 720 nm. Addition of 50 μM DCMU (Fig. 41, curve b) enhanced the fluorescence yield at 683 nm approximately five-fold; this emission became the dominant peak in the spectrum. The long-wavelength band was also increased approximately threefold upon closure of PS II traps after the addition of DCMU. Curve d of Fig. 41 shows an emission spectrum...
obtained by using monochromatic blue light (442 nm) absorbed mostly by Chl a. Again, low-intensity excitation was used such that all PS II reaction centers remained open. This spectrum has the two characteristic peaks at 683 and 725 nm, and the ratio of long-wavelength to short-wavelength (F725/F683) bands was about 1.45. Addition of DCMU (Fig. 41, curve e) increased the fluorescence yield 3.75-fold at 683 nm and 2.5-fold at 725 nm. The differences in the emission spectra produced by DCMU show that the long-wavelength emission was relatively higher in blue light (Fig. 41, curve f) than in green light (Fig. 41, curve e). It is clear from these results that Porphyra exhibits an appreciable amount of a long-wavelength Chl a emission whose yield increases significantly upon closure of PS II traps after DCMU addition.

Since the long-wavelength emission band and the short-wavelength 683-nm emission band showed similar sensitivity to DCMU, especially when excited with blue light, we examined the effect of the inhibitor DBMIB (2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone), a quencher of Chl a fluorescence, on the room-temperature emission spectrum of DCMU-treated Porphyra. The difference spectrum obtained using blue excitation (Fig. 42) shows that DBMIB mainly quenched the fluorescence at 683 nm and had very little effect on the long-wavelength band. Thus DBMIB seems to affect preferentially the PS II-sensitized variable fluorescence at 683 nm.

**Fluorescence and delayed luminescence transients.** Most algae exhibit fluorescence transients (Kautsky effect) when dark-adapted cells are excited with bright PS II light. Porphyra, like other algae, shows complex transients depending on the physiological state and length of dark adaptation (Sato and Fork, 1983). Fluorescence-yield changes measured at 685 or 725 nm showed similar Kautsky transients (data not shown). Addition of DCMU increased the yield both at 685 and at 725 nm. We found that the fluorescence transients measured at 725 nm were similar whether they were obtained using blue actinic light absorbed mostly by Chl a of PS I, red (633-nm) light absorbed mostly by phycocyanin, or green light (PS II) absorbed mostly by phycoerythrin (data not shown).

Millisecond-delayed luminescence (DL) originates from re-excitation of PS II antenna Chl a as a consequence of charge recombination at the PS II reaction centers (Govindjee and Jursinic, 1979). As with Chl a fluorescence transients, dark-adapted Porphyra exhibits complex DL kinetics. Figure 43 shows the induction kinetics of DL measured at 685 nm and at 730–735 nm when Porphyra was excited with red light (632.8 nm) in the presence or absence of DCMU. The kinetics of DL observed at the long-wavelength band (720–730 nm) were similar to those observed at 685 nm, and the extent of DL measured in the 730-nm region was appreciable even in the presence of DCMU. We have observed similar DL transients using blue (442-nm)
The similarity in the fluorescence kinetics measured at the long-wavelength-emission band with those at 685 nm, and the extent of fluorescence-yield enhancement upon trap closure (Fig. 41) in blue light absorbed mostly by PS I, indicate that the long-wavelength emission possesses a component which is linked to the PS II antenna pigments. This idea is further supported by observation of a similarity in the kinetics at the long wavelengths and at 685 nm in the ms DL originating from charge recombination at the reaction centers of PS II (Fig. 43). The differential sensitivity of the 683 and 725 nm bands to DBMIB quenching, however, suggests that the long-wavelength emission also possesses a major component of Chl a of PS I, as suggested by Fork et al. (1982).

Although the spectral distinction between these two (or more) forms of the long-wavelength-fluorescing forms of Chl a in Porphyra remains to be elucidated, the results presented in this report argue in favor of the existence of a long-wavelength-emitting PS II band in the intertidal alga Porphyra. The presence of such a far red-emitting form of Chl a associated with PS II suggests that it may function as an antenna by way of reverse ("uphill") energy transfer and/or as a protective device against photooxidation of PS II centers in high light during periods of desiccation and other stresses, since excess light energy absorbed by the PS II reaction centers would be funneled to the long-wavelength-emitting forms.

**REFERENCES**


The intertidal marine red alga *Porphyra perforata* lives in an environment that, for most plants, is considered extremely unfavorable for efficient photosynthesis. *Porphyra* can tolerate severe drying whereby up to 91% of its water is lost (Smith, 1983). Under these conditions the photosynthesis of this alga can be completely inhibited. The alga also tolerates high light intensities and high salinity (from evaporative water loss). Even when exposed to high light intensities and when its photosynthesis is inhibited by desiccation, *Porphyra* is able to avoid permanent damage from photoinhibition.

Earlier studies on *Porphyra* dealt with the effects of desiccation on the photochemical reactions of photosynthesis (Year Book 72, 384–388) and on excitation energy distribution (Year Book 80, 39–43). Other studies reported in this Report (Fork and Satoh, Satoh and Fork, Mohanty and Fork) describe a newly found mechanism available to this alga for adapting to changes of light intensity and quality.

In the present work we investigated the effects of salinity on the primary processes of photosynthesis in *Porphyra*. By varying the salinity from normal sea water to saturating concentrations of NaCl we could impose an osmotic stress that mimicked the effects of air-drying, and thereby attain discrete levels of dehydration.

At least three sites of photosynthesis were affected by high salinity. The site most strongly affected was the photoactivation of electron flow on the reducing side of photosystem I (PS I).

The algae used in these experiments were collected at Monterey Bay, California, and maintained in open dishes of sea water at 13°C under illumination (8 µmol quanta/m² s). Samples were dark-incubated for 1 h or more in normal sea water and then incubated for 5 min in normal sea water or in NaCl solutions of various concentrations.

As shown in Fig. 44, *Porphyra* in normal sea water exhibits a typical fluorescence time course (Kautsky transient) with several maxima and minima (designated OIDPS by Govindjee and Paggio, 1971). The O to I rise is thought to represent the reduction of Q by photosystem II (PS II) (Duysens and Sweers, 1963; Kautsky et al., 1960). The dip (D) has been proposed to be produced by photooxidation by PS I of Q that was
initially reduced by PS II (Schreiber and Vidaver, 1974; Satoh and Katoh, 1981). The DPS transient is related to electron flow on the reducing side of PS I, since it can be eliminated by methyl viologen, which accepts electrons from PS I (Munday and Govindjee, 1969; Satoh et al., 1977). The DP rise was proposed to result from accumulation of photoreduced Q caused by the presence of a dark-inactivated site on the reducing side by PS I (Satoh et al., 1977). Photoactivation of this site (probably ferredoxin-NADP+ reductase, see Satoh, 1981) gives rise to the PS decline. The DP rise, therefore, is a reflection of the activity of PS II, and the PS decline in fluorescence corresponds to the photoactivation of PS I that was previously dark-inactivated.

The fluorescence kinetics shown in Fig. 44b demonstrate that the D to P rise is decreased by doubling the NaCl concentration of normal sea water from 32 to 70 parts per thousand (%). This result suggests that PS II activity was retarded by this treatment. (See also Wiltens et al., 1978.) Increasing the NaCl concentration did not seem to cause a further inhibition of the DP rise, but the P to S decline started to slow down at about 70 and stopped at 160% NaCl. The I to D dip disappeared between 70 and 115% NaCl. This latter change may result from a salt-induced retardation of electron flow between the two photosystems as suggested by Wiltens et al. (1978).

Figure 45 presents a summary of data obtained on the effect of NaCl on fluo-

Fig. 45. The effects of salinity on the rate of the P to S decline and on the extents of the I to P rise (triangles and circles) of the fluorescence transients in dark-adapted Porphyra. The samples were incubated with various concentrations of NaCl for 5 min, and fluorescence time courses were measured under low light (same conditions as for Fig. 44) and high light (235 μW/cm²). Circles and triangles were measured under the low light, and squares under high light.
rescence time courses. The PS decay (photoactivation of ferredoxin-NADP+ reductase) was decreased by one-half at a salt concentration of around 100% and was zero at 160%.

As mentioned earlier, the level of the peak (P) is determined by two factors: reduction of Q by PS II, and oxidation of Q by PS I. The effect of NaCl concentration on the difference between fluorescence at the P and I levels was measured at low or high light intensity. At a low light intensity, as used for Fig. 45 (triangles) and for Fig. 44, the $F_p - F_I$ extent was already noticeably reduced by an NaCl concentration of 44%. With higher light intensities (Fig. 45, squares), the decrease of $F_p - F_I$ was seen at higher NaCl concentrations. This decrease may be because photoactivation of electron flow on the reducing side of PS I is slower at the lower light intensity. In this case, photoactivation of PS I proceeds during the course of the reduction of Q, so that the level of P is highly sensitive to PS II activity. Since the I to P rise decreased and then increased with increasing salt concentrations, it is clear that salt inhibits electron flow on the water side of PS II and inhibits even more strongly electron flow on the reducing side of PS I by suppressing the photoactivation reaction.

Figure 46 shows the effects of NaCl concentration on the time courses of P700 oxidation (a PS I reaction). Illumination of dark-adapted Porphyra in sea water produced a rapid oxidation of P700 (decrease of absorbance). In this sample, electron flow on the reducing side of PS I would be initially dark-inactivated. Therefore, when the electron carriers between the inactivated site and the reaction centers of PS I are fully reduced, PS II becomes inactive. This results in a photoreduction of P700 by PS II (Fig. 46, trace a, increase of absorbance following initial decrease). After some period of illumination, photoactivation of the dark-inactivated site took place and photooxidation of P700 occurred once again. The rate of the dark reduction of P700 was determined by the activity of PS II. At a high salt concentration the initial transient P700 oxidation occurred as in normal sea water but the re-oxidation during illumination and dark reduction of P700 were both inhibited (Fig. 46b). At 160% NaCl, photoactivation was almost completely inhibited and P700 stayed reduced in the light (Fig. 46c). These results also suggest that electron flow on the water side of PS II and photoactivation of electron flow on the reducing side of PS I were inhibited by high salinity, the latter being the most strongly inhibited.

We measured a DPS transient in normal sea water and, after a dark interval of 5 min, measured the DPS transient again. During this period, dark inactivation occurred so that the extent of the second DPS transient was little more than
50% of the transient seen in the completely dark-adapted sample. If the sample was placed in 160% NaCl during the 5-min dark interval, there was almost no DPS transient (data not shown). This result suggests that dark inactivation of electron flow on the reducing side of PS I is inhibited by high salinity. In these experiments the steady-state levels of fluorescence were about the same for both samples, which indicates that the high salinity did not interfere with electron flow on the reducing side of PS I.

By measuring fluorescence induction in the presence of DCMU, we found that high salinity has another effect on the primary processes of photosynthesis. Figure 47 shows that a high concentration of salt did not affect the initial ($F_0$) fluorescence level. It did, however, decrease the variable fluorescence level ($F_v$) and retard the rate of reduction of Q. The ratio of fluorescence at its maximum level ($F_m$) to its $F_0$ level decreased linearly with increasing salinity (data not shown). This result could be explained by assuming that the back reaction of PS II is accelerated (re-oxidation of Q by electron donation to an electron acceptor on the water side of PS II). However, this possibility does not seem likely because we found that Q oxidation was slowed rather than accelerated by high salinity.

It seems likely that high salinity gives rise to a decrease of the light energy that is transferred to the reaction centers of PS II, since these algae were shown to have an increased energy transfer from PS II to I under adverse conditions (Year Book 79, 193–197; 80, 39–43). A decrease of the quantum yield of the photochemical activity of the reaction center of PS II itself by high salinity could not be ruled out by the present experiments.

The data presented here show that there are at least three sites in the primary processes of photosynthesis that are inhibited by high salinity: photoactivation of electron flow on the reducing side of PS I, electron flow on the water side of PS II, and transfer of light energy between pigment molecules. Perhaps an inhibition of electron flow at the different sites is necessary to prevent permanent damage to Porphyra by photoinhibition, since electron-flow inhibition is known to

![Fig. 47.](image-url)
occur when only one of these sites is inhibited. Inhibition of electron flow at three sites may help to prevent accumulation of large amounts of free reductants with very low redox potentials generated by PS I and of oxidants with very high redox potentials from PS II, both of which can quickly destroy the photosynthetic apparatus. A decrease in the light energy reaching the reaction center of PS II may also be a useful mechanism to avoid photoinhibition.

REFERENCES

REVERSIBLE INHIBITION OF PHOTOSYSTEM II PHOTOCHEMISTRY IN Anacystis nidulans BY REMOVAL OF Ca²⁺

Jerry J. Brand, Prasanna Mohanty, and David C. Fork

Intact cells of Anacystis nidulans lose the ability to evolve O₂ when Ca²⁺ is depleted from the growth medium (Becker and Brand, 1982). The Ca²⁺-depleted cells do not photoreduce benzoquinone coupled to O₂ evolution, but do carry out photooxidation of reduced diaminodurene (DAD) in the presence of methyl viologen. Thus Ca²⁺ depletion specifically inhibits photosystem II (PS II). Since the addition of Ca²⁺ to the depleted cells restores the capacity to evolve O₂ it appears that Ca²⁺ is required for the functioning of PS II. In this report we provide evidence that Ca²⁺ ions are involved in the primary photochemical charge separation of PS II reaction centers of A. nidulans.

A. nidulans was grown photoautotrophically in continuous culture at 39°C. Cells at late log phase were harvested and transferred to Cg-10 medium of Van Baalen (1967) lacking Ca²⁺ and supplemented with 1.5 times the normal amount of glycylglycine. As Ca²⁺ depletion requires light, the cells were incubated in red light (300 µmol, m⁻² s⁻¹) for several hours. Control cultures were treated identically except they contained 0.35 mM Ca(NO₃)₂. Aliquots of Ca²⁺-depleted and control cells were taken for O₂ evolution and fluorescence assays.

Chlorophyll a (Chl a) fluorescence-yield changes are indicative of photochemical functioning of PS II. The upper traces of Fig. 48 show that control cells exhibited typical Kautsky transients when dark-adapted cells were illuminated with bright monochromatic blue light (442 nm). We note that the fast Kautsky (OIDPS) transient is followed by a slow S-to-M rise in fluorescence yield in control cells (Mohanty and Govindjee, 1973). The Ca²⁺-depleted cells, however, did not show any
Fig. 48. Time course at 39°C of Chl fluorescence measured at 685 nm in control or Ca²⁺-depleted cells of A. nidulans illuminated with monochromatic blue light (442 nm) in the absence (upper traces) or presence (lower traces) of DCMU (20 μM). Cells were incubated for 8 h in red light in the presence (solid line) or absence (dashed line) of Ca²⁺. Curves were normalized to correct for small differences in Chl concentration between control and Ca²⁺-depleted cells. The arrow indicates the time when illumination began. Chl α = 4–6 μg/ml.

fluorescence transient; the fluorescence yield remained invariant throughout the period of illumination. The lower traces of Fig. 48 show the fluorescence transients of control and Ca²⁺-depleted cells in the presence of the inhibitor DCMU. The control cells showed a rise in fluorescence from a low \( F_0 \) level to a maximal \( F_m \) level upon illumination with light absorbed almost exclusively by Chl α, while Ca²⁺-depleted cells showed no timedependent change in fluorescence yield. The fluorescence yield remained at the \( F_0 \) level. Table 7 shows that the time-dependent loss of O₂ evolution parallels the loss of variable fluorescence \( (F_m - F_o) \), as a result of the withdrawal of Ca²⁺ from the growth medium. These results thus suggest that Ca²⁺ depletion stops the flow of electrons from \( \text{H}_2\text{O} \) to PS II.

Hydroxylamine (NH₂OH) at high concentration feeds electrons near the reaction center of PS II. Addition of 10 mM NH₂OH induced a rapid rise of Chl α fluorescence in control cells, but it did not change the fluorescence yield of Chl α in Ca²⁺-deprived cells (Fig. 49). These results suggest that Ca²⁺ depletion probably does not affect the O₂-evolving system of Anacystis but does affect the photochemical reaction of PS II itself. The loss of delayed luminescence measured in the millisecond region (Table 8) also confirms the suggestion that Ca²⁺ depletion interferes with the primary photochemistry of PS II.

**TABLE 7. Progressive Loss of O₂ Evolution Activity and Variable Fluorescence Yield in Ca²⁺-depleted Cells**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>O₂ Evolution Rate</th>
<th>% of Control</th>
<th>( F_v ) (%) of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>322</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>161</td>
<td>50</td>
<td>56</td>
</tr>
<tr>
<td>5</td>
<td>56</td>
<td>17</td>
<td>12</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Calcium depletion and fluorescence measurements were done as described in Fig. 48. Chl concentration was 3 μg ml⁻¹. Rates of O₂ evolution expressed in μmol O₂·mg Chl⁻¹·h⁻¹. O₂ evolution was measured at 39°C in saturating red light. \( F_v \) was calculated from the fluorescence transients as the difference in yield of \( F_m \) and \( F_o \).
Fluorescence-yield changes at 77 K reflect the photoreduction of the primary acceptor of PS II (Butler, 1978). In Fig. 50, it can be seen that illumination of control cells, frozen in the dark to 77 K, caused an increase in fluorescence yield measured at 695 nm. However, in Ca$^{2+}$-depleted cells the fluorescence yield remained invariable. The extent of variable fluorescence yield measured at 77 K diminished in parallel with the decrease in O$_2$ evolution (Table 9). These results strongly suggest that Ca$^{2+}$ plays a role in the primary photochemical charge separation of PS II in *Anacystis*.

Brand (1979) has previously shown that in order to obtain membrane fragments with high O$_2$-evolving capacity from *A. nidulans*, the cells required Ca$^{2+}$ during French-press breakage. Recently England and Evans (1983) showed that Ca$^{2+}$ addition enhanced the photooxidation of the artificial electron donor diphenylcarbazide (DPC) by *Anacystis* submembrane preparations pretreated with Pb$^{2+}$, the latter treatment being known to inhibit electron flow from H$_2$O to PS II.

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**TABLE 8. Effect of Ca$^{2+}$ Depletion on Delayed Fluorescence (DF) Emission in *A. nidulans*\(^*\)**

<table>
<thead>
<tr>
<th>O$_2$ Evolution Rate</th>
<th>% of Control</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>504</td>
<td>100</td>
</tr>
<tr>
<td>Partially depleted</td>
<td>184</td>
<td>37</td>
</tr>
<tr>
<td>Totally depleted</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^*\)The ms-delayed fluorescence was measured at 685 nm using an He-Ne laser (Spectra-Physics Model 124B) and a phosphoroscope, the rotating sector of which provided alternating light-dark cycle of 0.8 and 5.1 ms. The resulting signal was amplified by a lock-in amplifier tuned to the chopper. Height of the transient peak was measured and expressed as percentage of the height in control cells of the same chlorophyll concentration. Rates of O$_2$ evolution given in μmol O$_2$·mg Chl$^{-1}$·h$^{-1}$. 

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**Fig. 49.** Time course of Chl fluorescence in presence or absence of NH$_2$OH. Control and Ca$^{2+}$-depleted cells (20% of control O$_2$ evolution) were illuminated, and fluorescence was measured, as described in Fig. 48. NH$_2$OH (10 mM) was added directly to the sample and equilibrated in complete darkness prior to fluorescence measurements. Solid lines, no addition; dotted lines, NH$_2$OH addition.
CALCIUM DEPLETION AFFECTS ENERGY TRANSFER AND ALTERS THE FLUORESCENCE YIELD AND EMISSION OF PHOTOSYSTEM II IN Anacystis nidulans

Prasanna Mohanty, David C. Fork, and Jerry J. Brand

Incubation of cells of Anacystis nidulans in light in a growth medium lacking Ca\(^{2+}\) and supplemented with a Ca\(^{2+}\) chelator, glycylglycine, has been found to induce a loss of \(O_2\) evolution catalyzed by photosystem II (PS II), while photosystem I (PS I) was unaffected (Becker and Brand, 1982). In the previous report we presented data showing that Ca\(^{2+}\) depletion also induced loss of chlorophyll \(a\) (Chl \(a\)) fluorescence of variable yield both at the growth temperature (39°C) and at 77 K. We present here evidence that Ca\(^{2+}\) is required not only for efficient photochemistry but also for efficient energy transfer from phycobilins to Chl \(a\) and for efficient energy distribution between the two photosystems in Anacystis nidulans.

The phycobilins constitute the major light-harvesting pigments for PS II in cyanobacteria. We have therefore studied the effect of Ca\(^{2+}\) depletion on energy transfer from phycobilins to Chl \(a\). Figure 51 shows the time-dependent changes in fluorescence yield in control, Ca\(^{2+}\)-de-
Fig. 51. Time course of Chl a fluorescence measured at 685 nm at 39°C in control, Ca²⁺-depleted, and partially restored cells of *Anacystis nidulans* illuminated with monochromatic red light (633 nm) obtained from He-Ne laser. Cells were incubated in Ca²⁺-deprived medium in red light (>600 nm, 300 μmol m⁻² s⁻¹), as described earlier by Brand et al., 1983. Aliquots of cells were dark adapted for 4 min at 39°C and then illuminated with 633-nm red light obtained from the He-Ne laser (15 mW) attenuated with a 25% neutral density filter. Fluorescence at 685 nm was measured with a narrow interference filter. The partial restoration of Chl a fluorescence transient shown in the curve labeled “Partially restored” was obtained by adding Ca(NO₃)₂ (0.35 mM) to the Ca²⁺-depleted cells and incubating in red light for 1 h. The insert shows the change in the steady-state level of fluorescence yield at 685 nm at 39°C excited by the phycobilin-absorbing red light as a function of loss in the rate of O₂ evolution resulting from Ca²⁺ depletion.

Fluorescence yield, measured at 685 nm upon excitation with light absorbed by phycocyanin, increased (Fig. 51, inset). Addition of Ca²⁺ to the depleted cells lowered the fluorescence level and restored the fluorescence transient (Fig. 51) as well as O₂ evolution. We note that the extent of restoration depended very much upon the extent of depletion (as measured in terms of loss of O₂-evolution capacity and time of incubation in Ca²⁺-depleted medium) as well as the time of incubation in light after addition of Ca²⁺ to the depleted cells.

Addition of 20 μM DCMU increased the fluorescence yield in the control cells,
and the slow time-dependent change in fluorescence yield persisted in the presence of DCMU (Mohanty and Govindjee, 1973). However, in the case of the Ca\(^{2+}\)-depleted cells, the fluorescence yield remained insensitive to the addition of DCMU; the depleted cells showed no slow-fluorescence transients (data not shown). The large increase in fluorescence yield in Ca\(^{2+}\)-depleted cells when excited with light mainly absorbed by phycocyanin and not with light absorbed by Chl a thus suggests that Ca\(^{2+}\) depletion affects the energy transfer from phycobilins to Chl a.

We therefore investigated the effect of Ca\(^{2+}\) depletion on the emission characteristics of *Anacystis* excited by light absorbed either mostly by phycobilins or by Chl a. Ca\(^{2+}\)-depleted cells with no ability to evolve O\(_2\) did not show a change in the absorption spectrum measured either at 39°C or at 77 K (data not shown). Figure 52A shows emission spectra at 39°C of both control and Ca\(^{2+}\)-depleted cells using monochromatic blue excitation (442 nm) from an He-Cd laser. Control and Ca\(^{2+}\)-depleted cells showed almost identical emission spectra. However, excitation of cells with a broad band of blue-green light which excited both phycocyanin (PC) and Chl a produced an emission spectrum that showed an enhanced emission at 665 nm in Ca\(^{2+}\)-depleted cells (Fig. 52B). Furthermore, a peak around 700 nm with a shoulder around 720 nm was also seen in the spectrum of the Ca\(^{2+}\)-depleted cells. Control cells, however, showed a typical emission spectrum with a peak at 655 nm produced by PC and a peak at 685 nm produced by Chl a (Goedheer, 1968). The results given in Fig. 52 show that excitation of PC, and not Chl a, brought about the changes in the emission spectrum. Thus, Ca\(^{2+}\) depletion not only prevents Q reduction (seen as a loss of transients in fluorescence of variable yield in light absorbed by PC or by Chl a) but also causes the change in the emission spectrum seen only with illumination absorbed mostly by PC. It appears that Ca\(^{2+}\) depletion alters the energy transfer from phycobilins to Chl a. Excitation spectra measured at 39°C also showed that there was a relative increase in the effectiveness of 620-nm light in exciting fluorescence emission at 685 nm (F685) in Ca\(^{2+}\)-depleted cells, as compared to control cells (data not shown). This suggests that Ca\(^{2+}\) depletion affects the energy transfer.

The emission spectra of control and Ca\(^{2+}\)-depleted cells excited with blue-
green light and measured at 77 K are presented in Fig. 53. The control cells exhibited three characteristic emission bands, peaking at 655 nm (PC emission), 684 nm, 696 nm (Chl a2 emission), and 722 nm (Chl a1 associated with PS I) (Goedheer, 1968). In Ca2+-depleted cells, the PC and PS I emission bands remained almost unchanged, while a new band appearing at 681–683 nm (F683) became very distinct. The difference spectrum shown in Fig. 53 depicts positive peaks at 663 and 681 nm. Addition of calcium to the Ca2+-depleted cells suppressed this F683 seen at 77 K; the emission spectra resembled that of control cells.

The measurement of the development of this new low-temperature 683-nm emission peak in relation to the loss of O2 evolution due to Ca2+ depletion showed that the loss of O2 evolution and of variable fluorescence ensued much earlier than did the development of F683. However, when cells were allowed to remain in the medium deprived of Ca2+ for a long-enough period, the F683 became very intense (Fig. 54). This band is seen only when Ca2+-depleted cells were excited with light absorbed by PC and not with

![Figure 53](image1.png)

**Fig. 53.** Fluorescence emission spectra of (a) control, (b) Ca2+-depleted, and (c) partially restored cells of *A. nidulans* at 77 K. Control cells and cells depleted of Ca2+ for 6.5 h, which retained 15% of their original O2-evolving capacity, were preincubated in darkness at 39°C, then plunged into liquid nitrogen for fluorescence measurements. Ca(NO3)2 (0.35 mM) was added to the Ca2+-depleted cells and incubated in red light for 2 h to allow recovery (60%) of O2 evolution and restoration of the previously seen emission characteristics (c). Fluorescence spectra were determined from samples as described for Fig. 52 and in text. Chl a concentration was 6.0 μg ml−1 for both samples. The bottom part of the figure represents difference spectra of the (d) depleted minus control and (e) restored minus control.

![Figure 54](image2.png)

**Fig. 54.** Effect of long-term Ca2+ depletion on fluorescence emission of *A. nidulans* at 77 K. The spectra were measured using either monochromatic blue light (442 nm) or a broad band of blue-green light. The cells at a concentration of 4 μg Chl a ml−1 were depleted of Ca2+ for 15 h and used at this concentration for spectral measurements. These cells completely lacked both O2 evolution and the DCMU-induced increase of fluorescence yield.
monochromatic blue light, which is absorbed by Chl a. The latter illumination produced no intense emission at 682 nm, and the spectrum of the Ca²⁺-depleted cells looked identical to that of control cells (Fig. 54).

Blue-green algae exhibit characteristic state transitions in that the cells in the dark remain in low-fluorescent (high-spillover) state II, and change to highly fluorescent (low-spillover) state I upon adaptation in light absorbed by PS I (Murata, 1969; Fork and Satoh, 1983). Blue-green algae show these state transitions even in the presence of DCMU (Mohanty and Govindjee, 1973; Satoh and Fork, 1983). In the presence of DCMU, the cells remain in state II in the dark and adapt to state I in light absorbed by either PS I or PS II (Mohanty and Govindjee, 1973; Satoh and Fork, 1983). Besides changes in yield produced by dark or light adaptation, the cells of *Anacystis* also show characteristic changes in the emission at 77 K that are produced by each state (Ley and Butler, 1980; Fork and Satoh, 1983). Table 10 shows the results of dark or light adaptation on the yields of fluorescence in control and Ca²⁺-depleted cells in the presence of 20 μM DCMU. Adaptation of control cells both to monochromatic blue light (PS I) or to a broad-band blue-green light mostly absorbed by PS II produced the slow rise in fluorescence yield. The extent of increase in slow fluorescence yield at 39°C

![Image]

TABLE 10. Effect of Adaptation to Blue or Blue-green Light on State Transitions in Control and Ca²⁺-depleted *A. nidulans* Cells

<table>
<thead>
<tr>
<th>Slow-Fluorescence Rise (Relative Yield) at 685 nm</th>
<th>Adapted to blue-green</th>
<th>Adapted to blue (PS I)</th>
<th>Adapted to light (mostly PS II)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dark-adapted</td>
<td>+Ca²⁺ 24.0</td>
<td>41.0</td>
<td>33.7</td>
</tr>
<tr>
<td></td>
<td>-Ca²⁺ 2.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

*The state transitions were measured in the presence of 20 μM DCMU as the slow-fluorescence rise (the fluorescence intensity after 5 s of illumination minus the fluorescence level (F₇₁₁) attained after 200 ms of illumination) that is seen after Q reduction has taken place. The samples were incubated with 20 μM DCMU at 30°C in darkness for 4 min or they were preilluminated in monochromatic blue or blue-green light for 4 min. The preilluminating light was turned off and the fluorescence transient was recorded as described in text.

Fig. 55. The effects of state transitions on the low-temperature (77 K) fluorescence emission spectra of (b) control and (a) Ca²⁺-depleted *A. nidulans*. Cells were incubated in dark or in light (broad-band blue-green light) at 39°C for 4 min before freezing the cells in liquid nitrogen. Emission spectra were recorded with a broad band of blue-green light as described in text. The difference spectra given in the bottom of the figure show (d) the characteristic increase of the F₆₉₆ band in the control cells and (c) an absence of such increase in Ca²⁺-depleted cells. The difference curves were obtained by subtracting the spectrum for cells in state II from that in state I.
upon adaptation to light is slightly higher for blue than for blue-green light (Table 10). We note that light adaptation caused a substantial increase in fluorescence yield, suggesting that, in darkness, *Anacystis* cells remain in state II (Satoh and Fork, 1983) but are transferred to state I in the light even in the presence of DCMU. (See also Year Book 81, 50–54.) No such state changes were seen in the Ca$^{2+}$-depleted cells. Figure 55 shows the increase in the F696 band in the emission spectrum when control cells were adapted to state I before freezing to 77 K. No such change was observed when Ca$^{2+}$-depleted cells were used. Addition of Ca$^{2+}$ to depleted cells revived the capacity for the state adaptations, as was evidenced by the enhanced F696 band upon light adaptation (Fig. 55).

The results presented in this report thus confirm and amplify our observation that Ca$^{2+}$ depletion affects the primary photochemistry of PS II (Brand et al., 1983). These results also suggest that a long-term absence of Ca$^{2+}$ from intact cells of *Anacystis* disrupts energy transfer from PC to Chl $\alpha$ (Figs. 52, 53). Ca$^{2+}$ depletion induced a large alteration of the emission spectrum measured at 77 K when cells were excited with broad-band blue-green light; the 695-nm peak was suppressed and a new peak appeared at a shorter wavelength (683 nm), its intensity increasing with time of depletion. Since this intense F683 band is absent with blue excitation absorbed by Chl $\alpha$, it is evident that Ca$^{2+}$ depletion disrupts energy transfer from phycobilins to Chl $\alpha$. The fact that the F683 band is not seen either in control cells excited by light absorbed by PC or Chl $\alpha$, or in Ca$^{2+}$-depleted cells in blue light absorbed by Chl $\alpha$ suggests that this band originates from the emission of phycobilin. This long-wavelength emission indicates the fluorescence in vivo from the terminal form of allophycocyanin which transfers energy to Chl $\alpha$ (Gantt, 1980).

**REFERENCES**


**PREPARATION OF CHLOROPHYLL-PROTEIN COMPLEXES FROM THE CYANOBACTERIUM Anacystis nidulans BY SDS–SUCROSE DENSITY GRADIENT CENTRIFUGATION**

Satoshi Hoshina and David C. Fork

Following the initial work of Ogawa *et al.* (1966), who showed two distinct chlorophyll-protein complexes of spinach thylakoid membranes by using SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis), several kinds of chlorophyll-protein complexes have been prepared from thylakoid membranes of higher plants and algae. In a recent study of chlorophyll-protein complexes of cyanobacteria, six chlorophyll-protein complexes were separated from *Anacystis nidulans* by a method employing LDS (lithium dodecylsulfate)–PAGE (Gukema and Sherman, 1981, 1983). By contrast, Takahashi *et al.* (1982)
resolved eight chlorophyll-proteins from another cyanobacterium, *Synechococcus* sp., by SDS-PAGE.

Argyroudi-Akoyunoglou and Thomou (1981) reported the separation of chlorophyll-proteins from thylakoids of *Phaseolus vulgaris* by SDS–sucrose density gradient centrifugation. They showed that the SDS–sucrose density gradient centrifugation procedure could be used for all types of SDS-solubilized thylakoids and that this method provided a convenient alternative to SDS-PAGE, particularly when large amounts of chlorophyll-proteins for biochemical work were required.

In this report, therefore, we used the SDS–sucrose density gradient centrifugation method to separate three chlorophyll-proteins in high yield from *Anacystis nidulans* and characterized their spectral properties.

Continuous cultures of *A. nidulans* were grown at 39°C (Brand *et al.*, this Report). Cells in the logarithmic growth phase were harvested, suspended in 30 mM Na,K phosphate buffer (pH 7.0), and washed with 0.6 M sucrose and 30 mM Na,K phosphate buffer (pH 6.8). The washed cells were incubated with lysozyme (5 mg/30 ml) containing 2 mM EDTA, 0.6 M sucrose, and 30 mM Na,K phosphate buffer (pH 6.8) for 2 h at 30°C, as described by Murata *et al.* (1981). The spheroplasts were suspended in 10 mM NaCl and 10 mM Tricine-NaOH (pH 7.8), and centrifuged at 10,000 g for 30 min. The pellet was resuspended in the same medium and disrupted by passage through a French pressure cell at 14,000 psi. The disrupted cells were cooled in an ice-water mixture and centrifuged at 30,000 g for 30 min at 4°C to remove unbroken cells and debris. The supernatant (25 ml) was layered onto a medium (8 ml) containing 0.4 M sucrose, 10 mM NaCl, and 10 mM Tricine-NaOH (pH 7.8), and centrifuged at 117,000 g (ave) (T-865 rotor, 40,000 rpm) for 1 h at 4°C. The pellet (thylakoid membranes) was resuspended with 0.1 M Tris-HCl (pH 8.6).

The thylakoids were solubilized in 0.1 M Tris-HCl (pH 8.6)–4.5% SDS (750 μg Chl a/ml, SDS/Chl a = 60) for 30 min at room temperature. This solubilized sample (0.5 ml) was layered onto a linear sucrose density gradient (11 ml, 5–35% sucrose in 50 mM Tris-borate (pH 9.5)–0.1% SDS) and centrifuged at 258,000 g (max) (SW 41 rotor, 38,000 rpm) for 16 h at 15°C.

Figure 56 shows the separation pattern obtained using sucrose density gradient centrifugation. Five bands and an
orange-yellow pellet were observed after centrifugation. These bands have been designated as Ia, Ib, II, III, and IV (from top to bottom). In this condition, band II constituted the major fraction, comprising 40–50% of total chlorophyll. When the thylakoids were solubilized at a ratio of SDS/Chl $a = 40$ (3.0% SDS, 750 µg Chl $a/\text{ml}$), the major band was band III, and at SDS/Chl $a = 20$ (1.5% SDS, 750 µg Chl $a/\text{ml}$), band IV became the major fraction. The orange pellet seems to originate from cell envelopes contained in the thylakoid preparation.

Bands II, III, and IV exhibited P700 photooxidation and contained 80–90 Chl $a$ molecules per P700, as shown in Table 11. No photoresponse of P700 was detected in bands Ia and Ib.

Figure 57 shows the absorption spectrum of those bands and thylakoids measured at room temperature. The spectra of bands II, III, and IV showed a maximum at 675 nm, and the spectra of bands Ia and Ib had peaks at 671 nm. By contrast, thylakoids showed a maximum absorbance at 678 nm. Absorption spectra indicate that bands II, III, and IV have a very small amount of carotenoids.

### Table 11. P700 Content in Thylakoids and Chlorophyll-Proteins from A. nidulans*

<table>
<thead>
<tr>
<th>Band</th>
<th>Chl $a$/P700 (Molar Ratio)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thylakoids</td>
<td>242 ± 6</td>
</tr>
<tr>
<td>Band Ia</td>
<td>∞</td>
</tr>
<tr>
<td>Band Ib</td>
<td>∞</td>
</tr>
<tr>
<td>Band II</td>
<td>79 ± 3</td>
</tr>
<tr>
<td>Band III</td>
<td>81 ± 4</td>
</tr>
<tr>
<td>Band IV</td>
<td>89 ± 3</td>
</tr>
</tbody>
</table>

*Photooxidation of P700 was determined with a Perkin-Elmer 356 spectrophotometer. Sample and reference beams were 698 nm and 725 nm, respectively. The sample was illuminated with blue-green light (570 µmol quanta/m² s) obtained by using two Corning 4-96 filters. The photomultiplier was protected with a Schott RG-2 filter. The activity was measured at room temperature in a reaction mixture containing 10 mM Tricine-NaOH (pH 7.8), 10 mM NaCl, 2 µM DCPIP, 1 mM sodium ascorbate, 1 mM methylviologen, and thylakoids or complexes (about 5 µg Chl $a/\text{ml}$). An extinction coefficient of 64 mM$^{-1}$ cm$^{-1}$ (Hiyama and Ke, 1972) was used. Mean values of 4–9 measurements are given.

Figure 58 shows fluorescence emission spectra of thylakoids and bands Ia and II measured at 25°C. The spectra of thylakoids showed three peaks—at 682, 725, and 750 nm—and a shoulder near 695 nm. The band at 750 nm may be attributed to an emission from P750 of the cell envelopes. In band II, two peaks appeared at 682 and 725 nm with a shoulder around 695 nm. The spectra of bands III and IV were very similar to that of band II. Spectra of bands Ia and Ib showed two peaks at 680 and 730 nm. These bands are highly fluorescent when compared with bands II, III, or IV.

Figure 59 shows the fluorescence emission spectra measured at 77 K. Thylakoids showed three emission bands peaking at 683, 694, and 715 nm, and a shoulder near 750 nm. Band II had two peaks, at 683 (F683) and 720 nm (F1);...
Fig. 58. Fluorescence emission spectra of bands Ia, II, and thylakoids (T) measured at 25°C using the apparatus described in Year Book 78, 196-199. Samples excited by monochromatic blue light (442 nm, He-Cd laser, Lionix Model 4240). Chlorophyll concentrations were 5 μg/ml.

similarly, bands III and IV showed two peaks, at 683 (F683) and 715 nm (F1). The ratio of the peak height (F683/F1) varied among those bands, and showed the lowest ratio for band II. By contrast, bands Ia and Ib both showed peak emissions at 680 nm and 730 nm instead of at 683 and 715 nm.

The short-wavelength (F680) emissions of bands Ia and Ib and the lack of photochemical activity indicate that these bands contain mainly free pigments. Bands II, III, and IV are related to photosystem I. Thus, SDS-sucrose density gradient centrifugation is a useful prep-

Fig. 59. Fluorescence emission spectra of chlorophyll-proteins and thylakoids (T) measured at 77 K. Samples were excited at 442 nm (He-Cd laser). Chlorophyll concentrations were 5 μg/ml.

aration method for separating the three photochemically active chlorophyll-proteins associated with photosystem I from Anacystis nidulans. Further study is under way to clarify the interrelationships of these three chlorophyll-proteins, and to compare them with chlorophyll-proteins prepared by Guikema and Sherman (1981, 1983).
REFERENCES

ANALYSIS OF ABSORPTION SPECTRA OF CHLOROPHYLL a IN PHOSPHATIDYLCHOLINE LIPOSOMES AT DIFFERENT TEMPERATURES

Satoshi Hoshina

Several absorbing forms of chlorophyll a can be detected in the thylakoid membranes at liquid nitrogen temperature: Chl a – 662, Chl a – 670, Chl a – 677, and Chl a – 684, where the numbers represent the wavelengths in nm of the peak position (Brown, 1972). Several forms of chlorophyll a have also been detected in vitro, such as chlorophyll a in nonpolar solvents (Cotton et al., 1974), chlorophyll a in aqueous dispersions of lipids (Murata and Sato, 1978), or water-soluble chlorophyll-proteins (Sugiyama and Murata, 1978).

It was previously reported that the difference spectrum (B – A) between chlorophyll a in phosphatidylcholine liposomes at the phase transition temperature (A) and Chl a at a lower temperature (B) had a negative peak at 657–668 nm and a positive peak at 675–685 nm. A pronounced change in absorbance of chlorophyll a was seen when the lipid passed through the phase-transition temperature (Hoshina, 1981).

The purpose of the present study was to apply the curve-fitting method to the absorption spectrum of chlorophyll a in liposomes at temperatures above and below the phase transition of the lipid. The results suggest that the band with a peak at 662–663 nm is transformed into the band at 670–671 nm upon cooling below the phase-transition temperature of the lipid. Spinach leaf chlorophyll a and phospholipid liposomes containing chlorophyll a were prepared as described previously (Hoshina, 1981). The molar ratios of the lipid to chlorophyll a were 49. The absorption spectra at different temperatures were measured with a Shimadzu Recording Spectrophotometer UV-300 and digitally recorded with a Shimadzu Spectral Data Processor Sapcom-1. The curve analysis of the absorption spectrum was performed by using the RESOL Program (French et al., 1972).

The absorption spectra of chlorophyll a in phosphatidylcholine liposomes were analyzed at temperatures above and below the lipid phase transition. Figure 60 shows the results of the curve-fitting analysis on the absorption spectrum of chlorophyll a in liposomes having an equimolar mixture of dipalmitoyl and dimyristoyl phosphatidylcholine at 41°C. The absorption spectrum was composed of one major band with a peak at 669.9 nm and minor bands with peaks at 650.6, 662.6, and 684.8 nm. This result is essentially identical to the analysis of the absorption spectrum of chlorophyll a in an aqueous dispersion of a mixture of sulfatides and monogalactosyldiglyceride reported by Murata and Sato (1978). The results of curve analysis of the absorption spectrum of the same sample measured at different temperatures are shown in Table 12. At lower
temperatures, the width of the band at 662–663 nm tended to be narrower and that at 684–686 nm tended to be broader than width at higher temperatures, although the peak positions of bands were reasonably similar in the results at other temperatures. No change in the area of any band was detected upon cooling from 41°C to 31°C. Upon cooling from 31°C to 21°C, the area of the band at 662–663 nm decreased significantly and the band at 670–671 nm increased. Further cooling of the sample from 21°C to 6°C induced a little change in the bands at 662–663 nm and 670–671 nm. No change in the areas of the bands at 650–652 nm and

### TABLE 12. Curve Analysis of Absorption Spectra of Chlorophyll a in Liposomes having an Equimolar Mixture of Dipalmitoyl and Dimyristoyl Phosphatidylcholine*

<table>
<thead>
<tr>
<th></th>
<th></th>
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<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Temp.</td>
<td>HW (nm)</td>
<td>Area (%)</td>
<td>HW (nm)</td>
<td>Area (%)</td>
<td>HW (nm)</td>
</tr>
<tr>
<td>41°C</td>
<td>14.2</td>
<td>7.6</td>
<td>17.4</td>
<td>11.6</td>
<td>17.6</td>
</tr>
<tr>
<td>31°C</td>
<td>15.7</td>
<td>8.2</td>
<td>18.2</td>
<td>12.8</td>
<td>17.1</td>
</tr>
<tr>
<td>21°C</td>
<td>16.2</td>
<td>8.6</td>
<td>18.9</td>
<td>12.8</td>
<td>16.7</td>
</tr>
<tr>
<td>6°C</td>
<td>16.2</td>
<td>9.2</td>
<td>18.0</td>
<td>12.3</td>
<td>15.4</td>
</tr>
</tbody>
</table>

*Absorption spectra were measured at 41°, 31°, 21°, and 6°C. HW indicates half-bandwidth. The percentage of each band was calculated from the sum of the areas of the bands. Gaussian curves make up 77–100%, and Lorentzian curves the remainder.
684–686 nm was observed. According to the phase diagram for the mixtures of lipids measured with ESR or fluorescence method (Lee, 1977), the lipid in an equimolar mixture of dipalmitoyl and dimyristoyl phosphatidylcholine is in the liquid crystalline state at 41°C. When the temperature is reduced, lipid in the gel state appears at 30–38°C. Upon further cooling, the regions of the gel state of lipid increase and those of the liquid crystalline state of lipid decrease; finally, only the gel state of lipid is present. All of the lipid is in the gel state at 21° and 6°C.

Table 13 shows the results of the curve analysis of the absorption spectrum of chlorophyll a in dipalmitoyl phosphatidylcholine liposomes, measured at 48° and 4°C. At 4°C (the gel state of the lipid), the proportion of the band at 662–663 nm decreased and that at 670–671 nm increased, compared with results obtained at 48°C (the liquid crystalline state of the lipid). No change in the area of the other bands was detected.

Lee (1975) and Knoll et al. (1980) reported from results obtained by fluorescence measurements of chlorophyll a in phosphatidylcholine liposomes that both monomeric (fluorescent) and aggregated (nonfluorescent) forms of chlorophyll a were present in the gel phase as well as in the liquid crystalline phase of the lipid, and that the proportion of aggregated chlorophyll a increased at the expense of monomer when the lipid was in the gel state. The present study showed that the band at 670–671 nm increased at the expense of the band at 662–663 nm in the gel state of lipid. One of the possible causes of this interconversion of the band at 662–663 nm to the band at 670–671 nm may be a change in the aggregation state of chlorophyll a, as the lipids form a gel state at the temperature below the phase transition of the lipids. If this view is correct, the results suggest that Chl α–662 is a less-aggregated form (monomer) and that Chl α–670 is a more-aggregated form (dimer). However, further evidence will be required to test this hypothesis.

**REFERENCES**


INJURY AND REPAIR DURING REHYDRATION OF DROUGHTED CABBAGE LEAVES

Jacob Levitt

When onion bulbs are frozen, injury increases or decreases during subsequent thawing and post-thawing periods depending on the temperatures at which the bulbs were frozen (Palta et al., 1977). This kind of freezing is extracellular, resulting in cell dehydration. It is conceivable, therefore, that dehydration induced by other stresses, such as drought, may also be followed on rehydration by an increase or decrease in the injury.

In order to investigate this possibility, young (30–50 days from sowing) potted cabbage plants, raised from seed in a growth chamber, were allowed to wilt by withholding water. Leaves were excised after moderate to severe degrees of wilting, and floated on water. The rate and degree of dehydration were determined by weighing.

Moderate wilting, with a net loss of about 35% of the leaf’s water, had no harmful effects, and the leaf recovered its full turgor within about an hour (curve 3(4), Fig. 61). Severe wilting (loss of 78% of the leaf’s water) killed the leaves essentially completely, and there was little reabsorption of water even after floating on water for three days (curve 9(1), Fig. 61). Intermediate degrees of wilting (loss of 60–65% of the leaf’s water) yielded the most interesting results [curves 6(5) and 7(5), Fig. 61]. Damage must have occurred during the wilting, since the reabsorption of water was much slower (3–4 days) than by the moderately wilted leaf (1–2 hours).

These results suggest that moderate injury resulting from moderate wilting is repairable during the slow reabsorption of water. Sometimes, however, further injury occurs even after turgor is regained, for the turgor is later lost again (Fig. 61) even though the leaf is still floating on water. The rehydration period may therefore be as important as the dehydration period, as the reabsorption of water seems to involve either repair or additional injury. These results indicate that the same kinds of responses occur during the rehydration of dehydrated cells, whether the dehydration is drought-induced or freeze-induced.

REFERENCE


Fig. 61. Reabsorption of water by cabbage leaves floated on distilled water after wilting by withholding water for four days [leaf 3(4)], six days [leaves 6(5) and 7(5)], and ten days [leaf 9(1)].
PARTIAL INHIBITION OF PHOTOSYNTHESIS BY LOW NIGHT TEMPERATURES

C. Eduardo Vallejos and Olle Björkman

Tomato plants from early spring plantings are exposed to extreme diurnal temperature fluctuations only where night temperatures are generally below the critical chilling temperature (approximately 12°C). The effect of one-night, low-temperature treatment was evaluated in tomato plants (cv. VF36) grown in a phytotron cell at 25°/17°C. The distal leaflet of the first fully expanded leaf was selected for gas-exchange measurement. CO₂-dependence curves for assimilation were obtained before and after a single 12-h, low-temperature (5°C) treatment in the dark. In order to eliminate the possibility of water stress caused by cooling of the roots, the pot was wrapped with heating tape and insulated with dry sand in a styrofoam container. The temperature of the roots was kept at 17°C.

A single 12-h, low-temperature treatment at 5°C decreased the rate of CO₂ assimilation at CO₂-saturation conditions (Fig. 62) and lowered the mesophyll conductance. Fluorescence measurements at liquid-nitrogen temperature (Powles et al., 1983) indicated that no damage had occurred to PS II. These results disagree partially with those of Martin et al. (1981), who reported that, while the saturation rate decreased, the mesophyll conductance remained the same. In addition to strong reduction of stomatal conductance, an oscillation in the rate of assimilation was observed for more than nine hours after treatment. As time passed, the oscillations decreased in intensity. Such oscillations have not been reported before. One reason might be that although they are easily detectable in a continuous-flow system, used here, they are difficult to detect in a closed system. Although it is tempting to assign these oscillations to changes in stomatal aperture, due to induction of ABA synthesis by the treatment (Daie and Campbell, 1981), no direct evidence is available to support this assertion. Further experiments with a tomato mutant, “flacca,” which lacks ABA (Bradford et al., 1983), will probably help elucidate this question.

REFERENCES
Previous investigations with *Nerium oleander* have shown that water stress dramatically increases the susceptibility of the photosynthetic system of the leaves to photoinhibitory injury (*Year Book 80*, 57–59; *Year Book 81*, 76–77). It follows from these studies that any mechanism that minimizes the interception of radiant energy under conditions of water stress should alleviate injury to the photosynthetic system caused by accumulation of excess excitation energy.

Siratro (*Macropctilium atropurpureum*), a legume native to Mexico, is known to maximize the interception of radiant energy under conditions of ample water supply by continuously adjusting the leaf angle so that the leaves remain essentially normal to incident sunlight throughout the day (diaheliotropic leaf movement). However, under conditions of water stress the leaves of this species instead become paraheliotropic, i.e., the leaf angle adjusts so that the leaves remain parallel to the sun, thereby dramatically reducing the interception of radiant energy. Hence both the light absorbed by the photosynthetic system and the leaf temperature are reduced.

In our present studies, potted siratro plants were grown in the Stanford garden under full summer daylight (maximum photon fluence rate 2000 μmol m⁻² s⁻¹). After establishment under well-watered conditions, plants were gradually water-stressed to a leaf water potential (ψ) of −1.5 MPa by restricting the water supply. This resulted in complete stomatal closure and paraheliotropic leaf movements. Well-watered plants (ψ = 0.5 MPa) maintained a high stomatal conductance (400–500 mmol m⁻² s⁻¹), and the leaves continued to move diaheliotropically. When desired, leaf movements were prevented by restraining the leaves to a horizontal position with a grid of fine nylon wires. The light intensity received by the lower surfaces of horizontally restrained leaves was only about 8% of that impinging on the upper leaf surfaces.

To assess the damage to the photosynthetic system caused by the different treatments, chlorophyll fluorescence of the leaves at 77 K was followed at 692 nm and 734 nm, as described by Powles and Bjorkman (1982). All fluorescence measurements reported here were made in the early morning before sunrise. Hence, only those effects of the treatments that persisted after a full night of recovery are shown.

Figure 63 compares the fluorescence characteristics at 692 nm for the upper and the lower leaf surfaces of well-watered and water-stressed siratro leaves. Water stress had little or no effect on the

![Figure 63](image_url)

**Fig. 63.** Fluorescence emission characteristics of upper (left-hand bars) and lower (right-hand bars) surfaces of well-watered, unrestrained leaves, and of both restrained and unrestrained water-stressed leaves of siratro. Intrinsic fluorescence $F_o$, solid area; maximum fluorescence $F_m$, total height of bar; variable fluorescence $F_v$, open and hatched area.
fluorescence characteristics of either the upper or lower leaf surface as long as the leaves were permitted to move paraheliotropically. However, when the water-stressed leaves were restrained to a horizontal position, drastic quenching of the maximum \( F_m \) and the variable \( F_v \) fluorescence took place, indicating severe damage to the photosynthetic system. Although most pronounced for the upper leaf surfaces, the fluorescence characteristics of the lower leaf surfaces were also affected. This fact, and the fact that the intrinsic \( F_0 \) fluorescence of the lower leaf surface increased, suggest that the damage to the photosynthetic system caused by restraining the water-stressed siratro leaves resulted not only from excessive light but also from excessive heat. This may not be surprising, since restraining these leaves to a horizontal position resulted in a maximum leaf temperature of 44°C (in comparison with 33° and 30°C for the unrestrained water-stressed and well-watered plants, respectively). Although the restrained, water-stressed leaves and the unrestrained, well-watered leaves intercepted similar amounts of radiant energy, the loss of latent heat by high transpiration rates prevented the temperature of the well-watered leaves from rising to the point at which heat damage begins.

To permit a separation of the effects of excessive heat and of excessive light, leaves of water-stressed siratro leaves, restrained in a horizontal position, were subjected to a high light level over a range of leaf temperatures in a temperature-controlled leaf chamber. Each treatment lasted for 5.5 h, and the fluorescence characteristics of the upper and the lower leaf surfaces were measured the following morning. As shown in Fig. 64, there was no high-temperature damage between 31°C and 42°C because \( F_m,692 \) of the lower surface was unaffected by leaf temperature over this range. However, \( F_m,692 \) fell sharply as temperature increased above 42°C and high temperature became progressively more severe. In contrast, \( F_m,692 \) of the upper, exposed leaf surface fell linearly with temperature between 31° and 42°C. Over this range of temperature, therefore, there was a marked interaction between light and leaf temperature, such that increased temperature exacerbated photoinhibition. Moreover, at temperatures above 42°C, which cause high-temperature damage in the dark, the exacerbation by temperature was even greater, probably reflecting functional and physical dissociation of the pigment-protein complexes of the photosynthetic membranes.

The interaction between temperature and light on fluorescence characteristics was also determined by enclosing leaves in a temperature-controlled chamber and exposing them to a range of leaf temperatures and photon flux densities for 5.5 h. Fluorescence characteristics determined the following morning are shown in Fig. 65. Even at a quite moderate temperature of 31°C, variable fluorescence \( (F_v,692) \) falls, and hence photoinhibition increases as photon flux density increases. Moreover, the sensitivity of photoinhibition to photon flux density increases with temperature until 42°C, when damage to the pigment-protein complexes of the photosynthetic membranes...
Fig. 65. Interactive effects of photon fluence rate and leaf temperature (°C) on the variable fluorescence ($F_v, 692$) of the upper surfaces of water-stressed siratro leaves.

by high temperature exacerbates the effect of photon flux density on photoinhibition. Thus over the range of temperatures normally experienced by siratro leaves in the field, the probability of photoinhibitory damage is high unless paraheliotropic leaf movements reduce the light incidence-fluence ratio to a low level.

Paraheliotropic leaf movements therefore protect water-stressed leaves of siratro from (1) high-temperature damage, (2) photoinhibition, and (3) the interactive effects of high temperature and excess light.

**REFERENCE**


**CONTROL OF PHOTOSYNTHESIS BY RuP$_2$ CONCENTRATION: STUDIES WITH HIGH- AND LOW-CO$_2$-ADAPTED CELLS OF Chlamydomonas reinhardtii**

Susanne von Caemmerer, John R. Coleman, and Joseph A. Berry

The photosynthetic fixation of CO$_2$ by C$_3$ plants is dependent upon internal production of the substrate ribulose-1,5-bisphosphate (RuP$_2$). The concentration of this substrate is a function of the balance between light-dependent reactions that produce it and the reactions of RuP$_2$ carboxylase/oxygenase that consume it. These latter reactions are a function of the concentrations of the second substrates CO$_2$ or O$_2$, the amount of enzyme present, and the state of activation of the enzyme as influenced by cofactors and effectors such as H$^+$, Mg$^{2+}$, and phosphorylated compounds (Lorimer, 1981). Berry and Farquhar (1978) and Farquhar et al. (1980) developed models for the kinetics of C$_3$ photosynthesis which make the assumption that the rate might alternatively be limited either by the rate of production of RuP$_2$ or by the enzymatic reactions that consume it, the choice depending upon environmental factors such as temperature, CO$_2$ concentration, and light intensity, and upon plant characteristics that determine the capacity of these reactions.

As pointed out by Farquhar (1979), the steady-state concentration of RuP$_2$ present in vivo should provide an index of the nature of the limiting step. When the rate of supply of RuP$_2$ is limiting, it follows that the concentration of RuP$_2$ [RuP$_2$] present in vivo should be such that the enzyme active sites are not fully occupied. The concentration of enzyme active sites [E$_t$] in the chloroplast is quite high (>1 mM) and the Michaelis constant of RuP$_2$ for these sites is very low. Therefore, when [RuP$_2$] > [E$_t$] the active sites should be nearly saturated with RuP$_2$ molecules. This leads to the interpretation that at any particular CO$_2$ and O$_2$ concentration when [RuP$_2$] < [E$_t$], the reaction is limited by the supply of RuP$_2$, and when [RuP$_2$] > [E$_t$], all of the active sites of the enzyme present in the chloroplast are probably occupied by RuP$_2$. 
molecules, and the rate of catalysis is limited by the amount of enzyme, or its state of activation.

Collatz (Year Book 78, 248–251; 1982) reported simultaneous measurements of photosynthetic O_2 exchange and [RuP_2] of isolated spinach leaf cells and Chlamydomonas reinhardtii in response to changes in CO_2, O_2, light intensity, and temperature. In the dark, [RuP_2] was undetectable and increased to concentrations exceeding the estimates of binding-site concentration at high light intensity and low concentrations of CO_2 and O_2, providing evidence that the photosynthesis of these organisms was limited by the RuP_2 supply under some conditions and by the amount of activated RuP_2 carboxylase/oxygenase under other conditions. Other investigators (Perchorowicz et al., 1982; Perchorowicz and Jensen, 1983) show that [RuP_2] is often higher than the presumed active site concentration of wheat leaves; they argue that light- and CO_2-dependent regulation of the activation state of RuP_2 carboxylase plays an important role in regulating the rate of photosynthesis even when light is strongly limiting for photosynthesis. The present studies were conducted to reexamine and extend the studies of Collatz, and to investigate the influence of the inducible CO_2-concentrating system of the alga C. reinhardtii (see Coleman et al., this Report) on the local environment of the RuP_2 carboxylase reaction in these cells as indicated by changes in the environmental control of [RuP_2].

**MATERIALS AND METHODS**

*Chlamydomonas reinhardtii* 2137 mt+ was grown as described (Coleman et al., this Report) and bubbled either with air or with 5% CO_2 in air. Cells were harvested during log-phase growth and resuspended in 30 mM MOPS buffer, pH 7.2. Assays of net O_2 production were conducted in a Clark-type oxygen electrode (Rank Brothers) with 2.0 ml of cell suspension containing 20–30 μg of chlorophyll ml⁻¹. After a constant rate of photosynthesis was achieved at a given condition, cells were drawn into a syringe containing HClO_4⁻ (final concentration 10%) to sample the steady-state RuP_2 concentration. The RuP_2 was assayed as described by Collatz (Year Book 77, 248–251). RuP_2 carboxylase active-site density was assayed as described by Collatz et al. (Year Book 78, 171–175), and catalytic activity was assayed as described by Seemann and Berry (Year Book 81, 78–83) with cell lysates obtained with a French pressure cell. Carbonic anhydrase was assayed as described by Coleman et al. (this Report).

**RESULTS AND DISCUSSION**

Cells used in these experiments were routinely analyzed for chlorophyll, RuP_2 carboxylase activity, RuP_2 carboxylase protein, and carbonic anhydrase activity (Table 14). The activity of carbonic anhydrase was about 40 times higher in cells cultured at low rather than high CO_2 concentrations, but there were only small differences in the amount of chlorophyll per cell or in the amount of RuP_2 carboxylase on a chlorophyll basis. Kinetic studies of the carboxylase obtained from freshly lysed cells indicated a higher k_cat (6.6 s⁻¹ per active site vs. about 3 s⁻¹) and a higher K_m (55 μM vs. about 10 μM) for the algal as opposed to the higher-plant enzyme. As reported by Badger et al. (1980), there was no detectable difference in the kinetic properties of RuP_2 carboxylase from high- or low-CO_2-grown cells.

Studies of photosynthetic exchange of oxygen (Fig. 66, bottom) verified that at constant and rate-saturating illumination there was about a tenfold difference between high- and low-CO_2-grown cells in the concentration of total inorganic carbon required to achieve half-saturation of photosynthesis. As discussed elsewhere in this Report (Coleman et al.), this difference in the apparent requirement for carbon is thought to be related to the existence in low-CO_2-adapted cells of a transport system that provides CO_2 to the site of the RuP_2 carboxylase reaction at a concentration several times...
TABLE 14. Comparison of High- and Low-CO₂-Grown Cells of Chlamydomonas reinhardtii

<table>
<thead>
<tr>
<th>Property</th>
<th>High CO₂</th>
<th>Low CO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorophyll content, μg 10⁷ cells</td>
<td>20.1 ± 7</td>
<td>15.7 ± 4</td>
</tr>
<tr>
<td>RuP₂ Carboxylase activity, μmol mg⁻¹ Chl hr⁻¹</td>
<td>418 ± 48</td>
<td>336 ± 60</td>
</tr>
<tr>
<td>active site density, nmol mg⁻¹ Chl</td>
<td>17.3 ± 2</td>
<td>14.1 ± 3.5</td>
</tr>
<tr>
<td>protein content, g/g Chl</td>
<td>1.2 ± 0.1</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>Carbonic Anhydrase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>activity, Wilbur-Anderson units</td>
<td>50 ± 58</td>
<td>2194 ± 991</td>
</tr>
<tr>
<td>ratio to RuP₂ carboxylase</td>
<td>7.3</td>
<td>390</td>
</tr>
</tbody>
</table>

higher than that in the surrounding medium. The high-CO₂-grown cells, lacking this mechanism, have internal CO₂ concentrations similar to that in the medium.

The intracellular pool of RuP₂ present in algae rapidly killed while photosynthesizing at a constant rate (Fig. 66, top) also responded strongly to the carbon concentration. The highest [RuP₂] was observed at low carbon concentrations, and [RuP₂] fell as carbon was increased. This pattern is consistent with a changing balance between the rates of production and consumption of RuP₂. At high carbon concentrations the RuP₂ carboxylase reaction can occur rapidly and draws the steady-state [RuP₂] down to a low level. At low carbon concentration the rate of the carboxylation reaction is much slower, and the potential rate of RuP₂ synthesis should remain about the same as it was at high carbon. Consequently the steady-state pool of RuP₂ builds up to a high level.

These studies at saturating light intensity should have permitted the maximum rate of RuP₂ production to occur, while the potential rate of carboxylation was altered by changing the carbon concentration. At low carbon concentrations [RuP₂] (Fig. 66) was greater than [Eɪ] (17.3 nmol mg⁻¹ Chl, Table 14), indicating that the concentration of activated RuP₂ carboxylase was limiting the rate of carboxylation under this condition. As the carbon concentration was increased, the [RuP₂] fell, reaching equivalence with the active-site concentration at a total carbon concentration of about 1.5 mmol and 0.5 mmol for the high- and low-CO₂-grown cells, respectively. Above these carbon concentrations, [RuP₂] was lower than [Eɪ] indicating that carboxylation of RuP₂ was limited by the capacity for RuP₂ production.

If, as proposed, the low-CO₂-grown algae have a higher internal concentration of CO₂ than do the corresponding high-
CO₂-grown algae, then the [RuP₂] should be lower at any given carbon concentration in the low-CO₂ cells, since the carboxylation reaction would be limited to a lesser extent by CO₂. This is indeed what was observed (Fig. 66, top), a result providing strong additional support for the inference that the low-CO₂ cells have a CO₂-concentrating mechanism. One additional observation was a tendency for [RuP₂] to fall at very low external carbon concentration (Fig. 66) with the low-CO₂ cells, but [RuP₂] did not fall to a concentration that would be rate-limiting.

Another way to study the balance between RuP₂ production and consumption is by controlling the light intensity while keeping the carbon concentration constant. With high-CO₂ cells at 0.2 mM carbon, [RuP₂] varied from about 2 to about 70 nmol mg⁻¹ Chl when the light was increased from darkness to 1600 μmol quanta (400–700 nm) m⁻² s⁻¹. Data collected over this range of light intensity are plotted (Fig. 67) as rate of O₂ production vs. [RuP₂]. It is clear that the data are biphasic, with a region of strong dependence of photosynthesis on [RuP₂] and another region where rate appears to be independent of [RuP₂]. The transition between these regions seems to be at about 20 nmol mg⁻¹ Chl, which is close to the estimate of [E₅].

This pattern of response is similar to that predicted (see fig. 1 of Farquhar, 1979) from a model for the kinetics of the reaction of RuP₂ with the carboxylase when the concentration of active sites is high, as occurs in vivo. We should, also, be able to predict the rate of photosynthesis in vivo from the model and from measurements of the quantity of enzyme present in these algae and its kinetics in vitro. For this comparison we selected measurements of photosynthesis for which [RuP₂] > E₅, as these rates should be rate-saturated with respect to RuP₂. For the calculation we used E₅ = 17.3 nmol mg⁻¹ Chl and kₘ = 6.6 s⁻¹ (from this study); Kₘ(CO₂) = 55 μM (Badger et al., 1980); Kₗ(O₂) = 480 μM (Jordan and Ogren, 1981), and a Michaelis-Menten expression for the RuP₂-saturated rate of the carboxylation reaction as a function of O₂ and CO₂ (equation 5, Farquhar, 1979). The CO₂ and O₂ concentrations were 20 and 250 μM, respectively. We also took into account the finding of Kaplan and Berry (1981) that net O₂ production under these conditions

![Fig. 67. The dependence of net O₂ production on the RuP₂ pool size. Inorganic carbon concentration was constant at 0.2 mM, and light intensity was varied to cause variation in [RuP₂]. Cells were grown at high CO₂, oxygen was 21%, and the temperature was 25°C.](image-url)
was only about 80% of the rate of gross CO₂ uptake (presumably because these high-CO₂-grown cells form glycolate as an end product of photosynthesis). These calculations yield a theoretical estimate of the rate of O₂ production (ignoring respiration) of 63.4 μmol mg⁻¹ Chl h⁻¹ which is slightly higher than the actual rate measured in vivo (mean = 42.4 μmol mg⁻¹ Chl h⁻¹). The rate of respiration observed with these cells in the dark (30 μmol mg⁻¹ Chl h⁻¹) is similar to the difference between the actual and the calculated rates. If there was substantial respiratory CO₂ production under the measurement condition, then the estimated and actual rates may agree nearly perfectly. Taken together, the good quantitative and qualitative agreement obtained in this study with predictions based on the model of Farquhar (1979), provide strong experimental support for the validity of that model as a basis for analyzing the kinetics of C₃ photosynthesis in vivo.

Studies of the control of [RuP₂] by carbon concentration conducted with low-CO₂-grown cells (data not shown) yielded about a fivefold steeper initial dependence of O₂ production on [RuP₂] at the same external carbon concentration as that shown for high-CO₂-grown cells (Fig. 67). This might be explained by assuming that the kcat for the carboxylase was much higher than for the corresponding high-CO₂ cells (not supported by measurements conducted in vitro), or that the CO₂ concentration at the site of the carboxylase reaction was much higher. High-CO₂-grown cells measured at tenfold higher external carbon concentration yielded a similarly steeper slope, supporting the latter interpretation.

CONCLUSIONS

The data obtained in these studies with *C. reinhardtii* provide additional support for the postulate that [RuP₂] is a factor regulating the rate of the RuP₂ carboxylase reaction, and the observed dependence of photosynthesis upon [RuP₂] was qualitatively similar to that predicted from the analysis by Farquhar (1979). Quantitative analysis of the responses showed reasonably good agreement between the responses observed in vivo and those predicted from measurements of the RuP₂ carboxylase of these cells in vitro. The comparison of high- and low-CO₂-adapted cells gave results completely consistent with the proposals that, given the same external CO₂ concentrations, the CO₂ concentration at the site of the RuP₂ carboxylase reaction was substantially higher than in air-adapted cells in high-CO₂-grown cells of *Chlamydomonas*.

REFERENCES


GENETIC CONTROL OF THE KINETIC PARAMETERS OF RuP$_2$ CARBOXYLASE: STUDIES OF A C$_3$ AND A C$_4$ SPECIES AND THEIR F$_1$ HYBRID

Joseph A. Berry, Malcolm Nobs, Bernardita Osorio, Jeffrey D. Palmer, James Tepperman, and William F. Thompson

The enzyme ribulose-1,5-bisphosphate (RuP$_2$) carboxylase (EC 4.1.1.39), which catalyzes the initial CO$_2$-fixation reaction, is a major limiting factor in the photosynthetic metabolism of C$_3$ plants. Previous reports (Year Book 80, 67–72; Year Book 81, 78–83) have shown that significant differences may exist between species in the catalytic efficiency of this enzyme on a protein basis, suggesting that it might be possible to improve the photosynthetic performance (at least on a protein basis) of some plants through genetic manipulation of the genes coding for this enzyme. For example, the rate of photosynthesis of leaves of spinach under CO$_2$-limiting conditions was about 1.7 times higher per unit RuP$_2$ carboxylase protein than it was for similar leaves of soybean plants under identical conditions, and, since this enzyme is a large fraction of the total leaf protein of these species (about 20%), the rate of photosynthesis per unit total leaf nitrogen also differed by a factor of about 1.5. These differences in the efficiency of photosynthesis could be related quantitatively to a corresponding difference in the rate of CO$_2$ uptake, predicted to occur from studies of the kinetics of the corresponding enzymes assayed in vitro. In this instance, the $K_m$ (CO$_2$) was about the same but the $k_{cat}$ was higher for the spinach enzymes than for the soybean enzymes.

If these differences in the apparent catalytic efficiency of these enzymes are indeed intrinsic and not the result of an artifact, then it should be possible to manipulate the enzymatic properties genetically. Genetic barriers between species of different families prevent the testing of this hypothesis with soybeans and spinach. Thus, in order to extend this line of investigation we chose to examine the RuP$_2$ carboxylases of C$_3$ and C$_4$ species of Atriplex and their F$_1$ hybrids. A previous study (Yeoh et al., 1981) demonstrated that the $K_m$ (CO$_2$) of RuP$_2$ carboxylase from C$_4$ plants was consistently about double that of C$_3$ plants, and the C$_3$ species Atriplex hastata L. (synonyms, A. patula ssp. hastata Hall and Clements, A. triangularis Wild.) could be hybridized with the C$_4$ species A. rosea, yielding a plant with many characteristics intermediate between the two parents but lacking a functional C$_4$ photosynthetic pathway (Year Book 68, 620–633; Year Book 69, 624–649; Year Book 70, 507–511). We reasoned that these species of Atriplex might provide the opportunity to test the heritability of kinetic differences. Preliminary experiments verified that the kinetic properties of the RuP$_2$ carboxylase protein from A. rosea and A. hastata were indeed different, and Malcolm Nobs was persuaded to repeat the tedious hybridization process.

MATERIALS AND METHODS

Atriplex rosea and A. hastata seeds were collected from the vicinity of the Palto Alto yacht harbor, and seedlings were maintained in a greenhouse. Hybridizations were conducted as previously described (Year Book 69, 624–629). Hybrid seeds were germinated in moist sand and in chambers, as described above. These plants were morphologically and cytologically similar to the F$_1$ hybrids obtained in the earlier experiments (Year Book 69, 624–649). RuP$_2$ carboxylase was extracted and assayed essentially as de-
scribed previously (Year Book 81, 78–83) except that RuP₂ (0.4 mM) was enzymatically generated in the assay medium immediately prior to the assays from ATP and ribose-5 phosphate using phosphoribose isomerase (Sigma) and phosphoribulokinase (RuP₂ carboxylase–free) generously supplied by George Lorimer. Nuclear and chloroplast DNAs were extracted and purified from leaves of mature plants as described in Palmer (1982). Chloroplast and nuclear DNA heterogeneities were analyzed as described previously (Year Book 81, 96–97; Year Book 81, 98–101).

RESULTS AND DISCUSSION

The $K_m$ (CO$_2$) of RuP₂ carboxylase from the C₄ species was about double that of the C₃ species (Table 15), as reported for other C₃ and C₄ species (Yeoh et al., 1981). In addition, we observed that the $k_{cat}$ of the C₄ species was also significantly higher. This is in agreement with the findings of a recent survey of other plants (M. R. Badger and J. R. Seemann, personal communication). The higher $k_{cat}$ of the enzyme from C₄ species would, in the presence of the high concentration of CO$_2$ thought to occur within the bundle sheath cells of these plants, yield a higher rate of carboxylation per unit protein than would the corresponding enzyme from the C₃ plant. It is of interest that the carboxylase of the green alga Chlamydomonas reinhardtii, which is thought to possess a CO$_2$-concentrating system (Coleman et al., this Report), also has higher $k_{cat}$ and $K_m$ than the carboxylases of C₃ higher plants. It seems likely that the changes in $K_m$ and $k_{cat}$ are linked (see Ulmer, 1983), and that the major advantage to these organisms possessing CO$_2$-concentrating systems accrues from the higher $k_{cat}$. The change in $K_m$ is not likely to have any significant effect at the high CO$_2$ concentration provided to these enzymes.

The hybrid plants had kinetic properties (Table 15) closely resembling those of the C₄ parent, A. rosea. Highly replicated studies with different hybrid plants indicated no significant difference between individual plants or between the hybrid and the C₄. The hybridization was accomplished with the C₄ species as the female parent. The fact that the hybrid enzyme properties resembled those of the female parent suggests that these traits are inherited maternally. It is well established that the gene specifying the large subunit of RuP₂ carboxylase is located on the DNA of the chloroplast and that this subunit contains the active site of the enzyme (Kung, 1977). In contrast, the small subunit is coded by the nuclear genome. No function is yet known for the small subunit, and no additional insight into this puzzle is provided from these studies.

In order to check that the purported hybrid was indeed a hybrid and that the chloroplast of the hybrid was derived from the maternal parent as expected, DNA was prepared from the two parents and three hybrid individuals, and analyzed for restriction-site heterogeneity. The fragments obtained after digesting the chloroplast DNAs with NruI and SacI (Fig. 68) showed a great deal of homology between the two genomes, but A. hastata had fragments at about 2 and 7 kb (NruI) and at 15 kb (SacI) that were lacking in the genome of A. rosea, while A. rosea had fragments at about 9 and 14 kb (NruI) and at 8.5 kb (SacI) lacking in A. hastata. The genomes of the hy-

<table>
<thead>
<tr>
<th>Species of Atriplex and Their F₁ Hybrid.</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}$ (s⁻¹ per active site)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. hastata</td>
<td>13.7 ± 0.5</td>
<td>4.4 ± 0.2</td>
</tr>
<tr>
<td>A. rosea</td>
<td>22.3 ± 1.0</td>
<td>6.0 ± 0.2</td>
</tr>
<tr>
<td>F₁ hybrid</td>
<td>19.6 ± 0.7</td>
<td>6.0 ± 0.2</td>
</tr>
</tbody>
</table>
Fig. 68. Electrophoresis in 0.8% agarose gels of Nru I and Sac I digests of chloroplast DNA from *Atriplex rosea* (R), *A. hastata* (H), and three F₁ hybrid plants (R × H). Fragment size differences attributable to restriction site changes between the parental genomes are evident at approximately 2, 7, 12, and 14 kilobases (kb, left axis) with the Nru I digest, and at 8.5 and 15 kb with the Sac I digest. The hybrids possessed all of the fragments characteristic of *A. rosea* and lacked those characteristic of *A. hastata*.

Hybrids were each identical to that of *A. rosea*. These differences in the restriction sites of chloroplast DNAs permitted a clear distinction of the two parental species and confirmed that the chloroplast DNA of the hybrid was derived from the maternal parent.

The nuclear DNA was much more complex (Fig. 69), and no heterogeneity could be resolved by fragment size alone. Increased resolution was obtained by Southern blot analysis of heterogeneity in those fragments that showed homology to the rDNA repeat unit of *Pisum sativum*. A clear difference in the parental genomes was resolved with a fragment of about 2.3 kb (*A. rosea*) and 1.9 kb (*A. hastata*). The hybrids contained both fragments, indicating biparental inheritance. These studies confirm that hybrids were obtained and that the chloroplasts were maternally inherited. Since the small subunit of RuP₂ carboxylase is coded by the nuclear DNA, it seems likely that the enzyme present in the hybrids was heterogeneous with a mixture of small subunits derived from either parent (Rhodes et al., 1980).
large subunit, however, could only be from A. rosea. Attempts to achieve the hybridization with the C3 species as the female parent, the reciprocal cross, have not been successful.

CONCLUSION

A hybrid between A. rosea and A. hastata possessed an RuP2 carboxylase that was clearly similar to the maternal parent. The large and easily resolved differences in the kinetic properties of the parental types and their hybrid provide strong evidence that these differences are under genetic control. Examination of the nuclear and chloroplast DNAs confirmed that these were inherited in the hybrid according to a biparental and a maternal pattern, respectively. Since the large subunit of the hybrid enzyme was derived only from the maternal parent, these results indicate that the major determinants of the catalytic properties are resident on the large subunit and are coded for by the chloroplast DNA. Not only have these studies confirmed the feasibility of genetic modification of RuP2 carboxylase kinetic properties, but the chloroplast genome has been identified as the relevant target for such modifications.

REFERENCES


LOCATION AND IDENTIFICATION OF CARBONIC ANHYDRASE IN Chlamydomonas reinhardtii

John R. Coleman, Joseph A. Berry, Robert K. Togasaki,* and Arthur R. Grossman

The photosynthetic characteristics of the green alga *Chlamydomonas reinhardtii* are dependent upon the CO2 concentration experienced by the alga during growth (*Year Book* 75, 423–432). Cells grown at air levels of CO2 (0.03%) have a much higher affinity for CO2 during photosynthesis than do algae grown at high CO2 concentrations (3–5%). Since the CO2 concentration at which the algae are grown has no effect on either the mechanism of photosynthetic CO2 fixation or the $K_m$ (CO2) of the principal CO2-fixing enzyme, ribulose-1,5-bisphosphate (RuBP) carboxylase (*Year Book* 75, 423–432), another process for improving the efficiency for CO2 utilization must be present in air-grown cells and absent in high-CO2-grown cells. We now know that the appearance both of carbonic anhydrase activity and of the mechanisms for the active transport and accumulation of bicarbonate enables air-grown algae to photosynthesize efficiently at low levels of inorganic carbon (Nelson *et al.*, 1969; Badger *et al.*, 1980). Growth at high levels of CO2 suppresses the activity of carbonic anhydrase and the bicarbonate transport system, and if these cells are transferred to low CO2 concentrations they are unable to fix carbon photosynthetically. The presence of carbonic anhydrase and bicarbonate transport enables air-grown cells to form a large, intracellular inorganic carbon pool, which provides sufficient CO2 for saturation of RuBP carboxylase and elimination of the oxygen inhibition of photosynthesis (*Birmingham et al.*, 1981; Coleman and Coleman, 1980). *C. reinhardtii* is also able to adapt to changes in the CO2 concentra-
tion during growth. When high-CO₂-grown cells are transferred to air levels of CO₂ and illuminated, their photosynthetic capacity at these low levels of carbon gradually increases such that 5 h after the transfer the algae display photosynthetic characteristics similar to those observed in air-grown cultures. During this adaptation period the induction both of carbonic anhydrase activity and of bicarbonate transport occurs. Exactly how carbonic anhydrase and the transport system coordinate inorganic carbon accumulation and, indeed, where this process is located in the cell are not fully known.

We have studied the induction of carbonic anhydrase activity in *Chlamydomonas reinhardtii* in an attempt to localize and identify the protein responsible for this activity and to gain some understanding of the mechanism of its regulation. We have also examined the effect of the change in CO₂ concentration on the regulation of synthesis of other proteins.

**MATERIALS AND METHODS**

*Chlamydomonas reinhardtii* 2137 mt+ (obtained from M. Spalding, Michigan State University) and the cell wall-less mutant, CW-15 (obtained from R. K. Togasaki, Indiana University), were cultured axenically in the minimal medium described by Spalding et al. (1982) at 28°C and a light intensity of 300 μmol m⁻² s⁻¹ (400–700 nm). Cultures were vigorously shaken and bubbled with either 5% CO₂ in air or with air alone. All experiments were performed with cells in early-to-midphase exponential growth.

Carbonic anhydrase activity, expressed in Wilbur-Anderson units (WA), in cell pressates was determined electrometrically, as previously described (Wilbur and Anderson, 1948).

For the determination of $K_{i/2}(CO_2)$, as well as for the maximal rate of photosynthesis, $P_{\text{max}}$, algae grown under the appropriate conditions were harvested by centrifugation (4,000g), resuspended in CO₂-free 20 mM MOPS buffer, pH 7.2, and the rates of O₂ evolution at varying HCO₃⁻ concentrations were measured at saturating light intensity and 25°C with a Clark-type O₂ electrode.

Autolysin was isolated according to Tamaki et al. (1981). Titrations were performed with the crude autolysin preparation to determine both the time of incubation and the concentration most effective in removing the *Chlamydomonas* cell wall. Since the effectiveness of these preparations varied, titrations were required in each case.

Purification of carbonic anhydrase released in the medium by air-grown CW-15 cells or autolysin-treated wild-type cells was achieved by a combination of DEAE cellulose and agarose gel column chromatography. Proteins synthesized during growth at 5%, 0.03%, and after transfer from 5% to 0.03% CO₂ were analyzed following in vivo labeling with $^{35}$SO₄. After a labeling period of 3–5 h in the light, the cells were quickly broken in a pre-chilled French press (25,000 psi) and centrifuged briefly at 2,000g to remove unbroken cells and debris. The supernatant was then centrifuged at 43,000g for 30 min to pellet the thylakoid fraction. The resulting supernatant was subjected to high-speed centrifugation (150,000g) for 3 h to produce a pellet and supernatant fraction. The supernatant was made 10% trichloroacetic acid to precipitate the protein. The three fractions—thylakoids, high-speed supernatant, and pellet—were treated with sodium dodecylsulfate (to 1.7%), and the proteins were analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (12–18% linear acrylamide gradient containing 8 M urea). The gels were stained with Coomassie brilliant blue G-250, dried, and the newly synthesized polypeptides were visualized by autoradiography.

**RESULTS AND DISCUSSION**

As shown in Table 16, the transfer of *C. reinhardtii* from growth at high CO₂
TABLE 16. Adaptation of 5% CO₂-Grown Chlamydomonas to Air Levels (0.03%) of CO₂

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>(K_{i/2}(\text{CO}_2)) (μM)</th>
<th>(P_{\text{max}}) (μmol·mg Chl⁻¹·h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>56.7</td>
<td>226.4</td>
</tr>
<tr>
<td>0.75</td>
<td>39.5</td>
<td>221.3</td>
</tr>
<tr>
<td>1.50</td>
<td>17.0</td>
<td>206.9</td>
</tr>
<tr>
<td>2.25</td>
<td>8.1</td>
<td>180.8</td>
</tr>
<tr>
<td>3.00</td>
<td>3.6</td>
<td>192.5</td>
</tr>
<tr>
<td>4.00</td>
<td>2.8</td>
<td>179.0</td>
</tr>
<tr>
<td>5.00</td>
<td>2.6</td>
<td>185.1</td>
</tr>
<tr>
<td>24.00</td>
<td>2.2</td>
<td>183.7</td>
</tr>
</tbody>
</table>

Concentrations to air levels results in a substantial increase in the whole-cell affinity for CO₂. The inorganic carbon concentration required for half-saturation of photosynthesis by high-CO₂-grown cells is similar to that required for RuBP carboxylase isolated from the alga (Year Book 75, 423–432). These results suggest that high-CO₂-grown Chlamydomonas lacks a CO₂-concentrating mechanism. In contrast, the \(K_{i/2}(\text{CO}_2)\) of photosynthesis for high-CO₂-grown cells transferred to air decreases rapidly with time; after a 5-h exposure to low CO₂, the \(K_{i/2}(\text{CO}_2)\) is twenty times lower than either that of high-CO₂-grown cells or the \(K_{m}(\text{CO}_2)\) of the isolated carboxylase. Carbonic anhydrase activity was measured in Chlamydomonas at various times after transferring cultures from high CO₂ to air (Fig. 70). After a 5-h induction period, the level of carbonic anhydrase activity approaches 50% of the total activity, measured after 24 h of induction. A comparison of the increase in carbonic anhydrase activity with the increase in affinity for CO₂ during photosynthesis (Table 16) suggests that only a portion of the total activity induced is needed for the initial decline in the \(K_{i/2}(\text{CO}_2)\) observed after a 5-h exposure to air. The continued increase in carbonic anhydrase activity past this point may be required for maximal reduction in the \(K_{i/2}(\text{CO}_2)\) attained after extended periods of air exposure.

The induction of carbonic anhydrase activity after transfer from high to air levels of CO₂ was also examined in the cell wall–less Chlamydomonas mutant, CW-15. In this mutant the kinetics of carbonic anhydrase induction were similar to that observed in the wild-type cells (data not shown). However, the enzyme does not remain in the cell but is excreted into the medium. The export of carbonic anhydrase into the medium by this mutant suggests that most of this enzyme is normally located within the periplasmic space (an area between the cell wall and the plasmalemma) or within the cell wall itself. In the absence of the cell wall, the enzyme is lost to the surrounding medium. Further evidence for the location of the enzyme was obtained by treating air-adapted wild-type cells with autolysin, a cell wall–degrading enzyme.

Fig. 70. Induction of carbonic anhydrase activity in Chlamydomonas reinhardtii after transfer of cultures from 5% CO₂ to air levels of CO₂.
synthesized by *Chlamydomonas* during mating. This treatment resulted in the release of more than 80% of the total assayable carbonic anhydrase activity into the medium. Carbonic anhydrase was not being released as a result of cell lysis, since less than 0.1% of the total RuBP carboxylase, a soluble intracellular protein, could be detected in the medium following autolysin treatment. These data indicate that although some carbonic anhydrase may be located within the cell (presumably inside the chloroplast), most of the enzyme passes through the plasma membrane, its appearance regulated by the CO$_2$ concentration in the growth medium.

Since the cell wall-less mutant of *Chlamydomonas* excretes carbonic anhydrase into the medium during growth at air levels of CO$_2$, this cell type was used as a source of the protein for identification and purification. Following a 24-h period at air levels of CO$_2$, the CW-15 cells were removed from the medium by low-speed centrifugation. The carbonic anhydrase-containing medium was then fractionated by column chromatography (DEAE cellulose and agarose gel filtration), and the proteins in fractions containing carbonic anhydrase activity were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (Fig. 71). Carbonic anhydrase was also identified by reacting an aliquot of ammonium sulfate-concentrated growth medium with dansylamide (5-dimethyaminonapthalene-1-sulfonamide), which forms a highly fluorescent complex with carbonic anhydrase (Drescher, 1978). This sample was applied to a native polyacrylamide gel and electrophoresed at 4°C. A single fluorescent band was located by exposure of the gel to ultraviolet illumination. This band was excised from the gel, electroeluted, and re-electrophoresed. Sodium dodecylsulfate polyacrylamide gel electrophoresis is described in text.) Lanes 2-10 show stained polypeptides from fractions exhibiting carbonic anhydrase activity after a combination of DEAE cellulose and agarose gel column chromatography. The peak activity was found in the fractions of lanes 3-6. The molecular weight standards indicated in this figure were phosphorylase b (92,500), bovine serum albumin (66,200), ovalbumin (45,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400).

![Fig. 71. Isolation and identification of carbonic anhydrase (CA) by sodium dodecylsulfate, polyacrylamide gel electrophoresis (as described in text). The sample in lane 1 was prepared from a band exhibiting carbonic anhydrase activity on a non-denaturing Laemmli gel. The activity on the gel was monitored by ultraviolet fluorescence of dansylamide, which associates tightly with carbonic anhydrase. The non-denatured sample was excised from the gel, electroeluted, and re-electrophoresed. (Sodium dodecylsulfate polyacrylamide gel electrophoresis is described in text.) Lanes 2-10 show stained polypeptides from fractions exhibiting carbonic anhydrase activity after a combination of DEAE cellulose and agarose gel column chromatography. The peak activity was found in the fractions of lanes 3-6. The molecular weight standards indicated in this figure were phosphorylase b (92,500), bovine serum albumin (66,200), ovalbumin (45,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400).](image)
described carbonic anhydrase of *Chlamydomonas* as a hexamer in the native state (Bundy and Cote, 1980). The monomer was reported to be 27,000. While it is difficult to reconcile our results relating to monomer size with those reported by Bundy and Cote, the monomeric species might be susceptible to proteolytic degradation.

In vivo labeling of proteins of wild-type *Chlamydomonas* cells with $^{35}S$ has demonstrated the synthesis of this same 37,000 polypeptide when the cells are grown continuously in air or allowed to adapt for 5 h to air levels of CO$_2$. This polypeptide is not synthesized when the cells are maintained at high CO$_2$ concentrations (Fig. 72, compare lanes 1, 2, and 3). The 37,000 protein is most easily visualized in the high-speed pellet fraction where approximately 60% of the total carbonic anhydrase activity is located. This band is also visible in the high-speed supernatant fraction which contains approximately 40% of the carbonic anhydrase activity. However, in this latter fraction, the labeled protein profile is much more complex, and carbonic anhydrase represents only a minor component. The distribution of carbonic anhydrase between the pellet and the supernatant may be due to the high molecular weight of the multimeric enzyme, its association with other cellular components, or non-specific adhesion to other proteins (perhaps to RuBP carboxylase, the major protein of the high-speed pellet fraction).

In both the high-speed supernatant and pellet, carbonic anhydrase is such a minor species it is only apparent in the autoradiogram and not in the profile of stained polypeptides. These results are not surprising, since carbonic anhydrase is a very efficient enzyme (Tobin, 1970) and therefore little protein is required for high levels of catalysis.

Also shown in Fig. 72, the transfer of *Chlamydomonas* from high to low CO$_2$ concentrations affects the rate of synthesis of a number of polypeptides and, in particular, RuBP carboxylase (compare lanes 1 and 2). A more-detailed ex-

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Fig. 72. Newly synthesized polypeptides during the transfer of cultures of *Chlamydomonas reinhardtii* from 5% CO$_2$ to air. Samples prepared from the high-speed pellets (lanes 1–3) and high-speed supernatants (lanes 4–6) following in vivo labeling were electrophoresed on polyacrylamide gels (as described in text). The gels were dried and exposed to Kodak XAR-5 film to visualize the newly synthesized polypeptides. Cells were labeled for 3 h during growth at 28°C on 5% CO$_2$ (lanes 1 and 4), air (lanes 3 and 6), or following a transfer from 5% CO$_2$ to air (lanes 2 and 5). All lanes in the gel received equal protein loads, as determined by staining with Coomassie brilliant blue G-250. Molecular weight markers are described in Fig. 71. The large subunit (LS) and small subunit (SS) of RuBP carboxylase and the carbonic anhydrase monomer (CA) are indicated in the figure.
amination of this response is presented elsewhere in this Report (pp. 109–111).

Additional evidence that the 37,000 polypeptide is carbonic anhydrase has been obtained by analyzing $^{35}$S-labeled proteins secreted into the medium by CW-15 cultures grown either in air or high levels of CO$_2$, and comparing the secreted proteins with those released from wild-type cells (also grown in air or high levels of CO$_2$) by autolysin treatment. The results of such an experiment are presented in Fig. 73. With either cell type, the 37,000 polypeptide is synthesized only by air-grown cells or by those exposed to low levels of CO$_2$ for 5 h. Maintenance of the cells at high levels of CO$_2$ inhibits the synthesis of this polypeptide. In the samples prepared from the autolysin-treated wild-type cells, a small amount of cell lysis, as indicated by the release of RuBP carboxylase into the medium, occurs. However, the prominence of the 37,000 protein after autolysin treatment is in complete agreement with the distribution and regulation of carbonic anhydrase activity demonstrated in the experiments with cultures of CW-15.

It is interesting to speculate on the role of carbonic anhydrase in Chlamydomonas reinhardtii. The enzyme acts to maintain the concentrations of the various inorganic carbon species in rapid equilibrium with one another; the relative abundance of each species is a function of the relative pH of the system. The rapid influx of bicarbonate into the cell by a transport system on the plasma membrane may require the action of carbonic anhydrase to keep the bicarbonate concentration at equilibrium levels. The location of carbonic anhydrase in the periplasmic space would be of particular advantage to a soil organism, such as Chlamydomonas, where the aqueous environment is limited to a thin layer of water. The maintenance of the bicarbonate concentration at equilibrium levels would allow for a more-rapid solubilization of gaseous CO$_2$ and would provide sufficient inorganic carbon for the transport system.

Fig. 73. Newly synthesized polypeptides released to the medium by the cell wall-less mutant (CW-15) of Chlamydomonas reinhardtii, or following autolysin treatment of wild-type cells. Conditions for growth, labeling, and autoradiography were as described in the legend of Fig. 72. Lane 1 shows newly synthesized polypeptides in the high-speed pellet of air-grown wild-type cells. Lanes 2–4 show newly synthesized polypeptides released into the growth medium by CW-15 grown on high CO$_2$ (lane 2), air (lane 4), and following transfer from high CO$_2$ to air (lane 3). Lanes 5–7 show newly synthesized polypeptides released into the growth medium after autolysin treatment of wild-type cells grown on high CO$_2$ (lane 5), air (lane 7), or following transfer from high CO$_2$ to air (lane 6). Molecular weight markers are described in the legend of Fig. 71. The large subunit (LS) and small subunit (SS) of RuBP carboxylase and the carbonic anhydrase monomer (CA) are indicated in the figure.
EFFECT OF INHIBITORS OF PROTEIN SYNTHESIS AND GLYCOSYLATION ON THE INDUCTION OF CARBONIC ANHYDRASE

John Coleman and Arthur Grossman

The expression of carbonic anhydrase activity in *Chlamydomonas* and other chlorophytes is regulated by the CO$_2$ concentration of the growth medium (Berry et al., *Year Book* 75, 423–432; Findenegg, 1976; Hogetsu and Miyachi, 1977). Cells grown at or adapted to air levels of CO$_2$ exhibit both carbonic anhydrase activity and the capacity to transport actively and accumulate inorganic carbon. In contrast, algae grown at high levels of CO$_2$ (3–5%) have little carbonic anhydrase and only a limited capacity to transport inorganic carbon. The concomitant expression of carbonic anhydrase and bicarbonate transport activities enable air-grown algae to photosynthesize much more efficiently than high-CO$_2$-grown algae at low levels of inorganic carbon. In the previous report we demonstrated that synthesis of a specific protein, which appears following the transfer of *C. reinhardtii* from high to low CO$_2$ concentrations, is responsible for carbonic anhydrase activity. This protein is a diffuse band of approximately 37,000 on sodium dodecylsulfate polyacrylamide gels, and is localized in the periplasmic space (the area where the majority of the carbonic anhydrase activity is found). In this study we examined the effect of inhibitors of protein synthesis and protein glycosylation on the expression of carbonic anhydrase.

MATERIALS AND METHODS

*Chlamydomonas reinhardtii* 2137 mt+ and the cell wall-less mutant, CW-15, were grown as described previously (Spalding and Ogren, 1982). For in vivo labeling studies, the cells were harvested, resuspended in SO$_4$-free growth medium containing the appropriate inhibitor, and preincubated in the dark for 15 min prior to the addition of $^{35}$SO$_4$.$^3$. The cultures were then placed at the appropriate CO$_2$ concentration and incubated under growth conditions for 5 h. The concentrations of inhibitors used were: 100 $\mu$g/ml chloramphenicol, 2 $\mu$g/ml cycloheximide, 2 $\mu$g/ml tunicamycin. After labeling, the cells were fractionated (Coleman et al., this Report), and the newly synthesized polypeptides were analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis followed by autoradiography. Proteins released into the medium by CW-15 cells were concentrated on DEAE cellulose prior to their preparation for electrophoresis. Carbonic anhydrase activity was assayed electrometrically as described previously (Wilbur and Anderson, 1948).

RESULTS AND DISCUSSION

As shown in Table 17, an inhibitor of translation on 80S cytoplasmic ribosomes, cycloheximide, blocks the induc-
TABLE 17. Effect of Inhibitors of Protein Synthesis and Glycosylation on the Induction of Carbonic Anhydrase Activity

<table>
<thead>
<tr>
<th>Growth Conditions</th>
<th>Carbonic Anhydrase Activity WA·mg Chl−1</th>
</tr>
</thead>
<tbody>
<tr>
<td>High CO₂</td>
<td>26.1</td>
</tr>
<tr>
<td>Air</td>
<td>1295.7</td>
</tr>
<tr>
<td>5 h on Air</td>
<td>610.3</td>
</tr>
<tr>
<td>5 h on Air + Chloramphenicol</td>
<td>583.3</td>
</tr>
<tr>
<td>5 h on Air + Cycloheximide</td>
<td>31.3</td>
</tr>
<tr>
<td>5 h on Air + Tunicamycin</td>
<td>117.4</td>
</tr>
</tbody>
</table>

Fig. 74. Effect of chloramphenicol and cycloheximide on the synthesis of carbonic anhydrase in C. reinhardtii. Cells were labeled with 35SO₄ for 5 h during growth at 5% CO₂ (lanes 1, 2, 3 and 8, 9, 10) after transfer from 5% CO₂ to air (lanes 4, 5, 6 and 11, 12, 13) and during growth at air levels of CO₂ (lanes 7 and 14). Chloramphenicol (lanes 2, 5 and 9, 12) was used at 100 μg/ml while cycloheximide (lanes 3, 6 and 10, 13) was used at 2 μg/ml. After labeling, the cells were lysed and fractionated as described in text. Samples prepared from the high-speed pellet were subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis. The profiles of the Coomassie blue-stained polypeptides obtained after each treatment are shown in lanes 1–7. The gels were then dried and exposed to Kodak XAR-5 film for visualization of the newly synthesized polypeptides (lanes 8–14). Carbonic anhydrase (CA) and the large (LS) and small (SS) subunits of RuBP carboxylase are indicated in the figure. Molecular weight markers were phosphorylase b (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400).
tion of carbonic anhydrase activity in *Chlamydomonas*. Chloramphenicol, an inhibitor of translation on the 70S plastid ribosomes, has no effect on the expression of activity. Since we have demonstrated that a 37,000 polypeptide is responsible for carbonic anhydrase activity, we examined the effect of inhibitors on its synthesis (Fig. 74). As shown previously, synthesis of both the 37,000 polypeptide and carbonic anhydrase activity is repressed by growth in high CO$_2$ (Fig. 74, lane 8) but induced if the cultures are grown continuously in air (Fig. 74, lane 14) or if they are transferred from high CO$_2$ to air (Fig. 74, lane 11). Cycloheximide inhibits the synthesis of this polypeptide (Fig. 74, lane 13), while chloramphenicol has no significant effect (Fig. 74, lane 12). The impact of these inhibitors on the synthesis of the large subunit (LS) and small subunit (SS) of RuBP carboxylase indicates that they are working properly. Chloramphenicol blocks the translation of LS (Fig. 74, lanes 9 and 12) but does not inhibit SS synthesis, while cycloheximide blocks the translation of SS (Fig. 74, lanes 10 and 13) but does not eliminate LS synthesis. These data demonstrate that carbonic anhydrase is synthesized on cytoplasmic ribosomes and is therefore encoded in the nuclear genome.

Since most of the carbonic anhydrase is localized to the periplasmic space, it must traverse the plasmalemma to reach its site of function. This fact, in conjunction with the observation that the polypeptide responsible for carbonic anhydrase activity is a diffuse band on a polyacrylamide gel, suggests that glycosylation may be an important step in the enzyme’s biosynthesis. Tunicamycin, a potent inhibitor of protein glycosylation (Ericson *et al.*, 1977), limits the induction of carbonic anhydrase activity after a 5-h exposure to air to approximately 20% of the control level (Table 17). Examination of the polypeptides synthesized during the induction period, in the presence or absence of the tunicamycin, revealed that this inhibitor blocks the synthesis of the 37,000-molecular-weight polypeptide (Fig. 75, lanes 5 and 7). No synthesis of this polypeptide blocks the synthesis of the 37,000-molecular-weight polypeptide (Fig. 75, lanes 5 and 7). No synthesis of this polypeptide

![Fig. 75. Effect of tunicamycin on the synthesis of carbonic anhydrase by C. reinhardtii. Cells grown at the appropriate CO$_2$ concentration were incubated with $^{35}$SO$_4$ and tunicamycin (2 µg/ml) for 5 h. The newly synthesized polypeptides of the high-speed pellet fraction were determined as described in Fig. 74. A profile of the Coomassie blue-stained polypeptides is in lane 1. Inhibitor treatment did not lead to noticeable alterations in this profile. Newly synthesized polypeptides from CO$_2$-grown cells (lanes 2 and 3), air-grown cells (lanes 6 and 7), and cells transferred from high CO$_2$ to air levels of CO$_2$ (lanes 4 and 5) are presented. Cultures treated with tunicamycin are in lanes 3, 5, and 7. Untreated cultures are in lanes 2, 4, and 6. Carbonic anhydrase (CA) and large (LS) and small (SS) subunits of RuBP carboxylase are indicated in the figure. Molecular weight markers are described in the legend of Fig. 74.](image-url)
occurs at high levels of CO₂ in either the presence or absence of tunicamycin (Fig. 75, lanes 2 and 3). The synthesis of other polypeptides in the high-speed pellet fraction is not affected by tunicamycin.

Since tunicamycin inhibits the induction of carbonic anhydrase, a major periplasmic protein, we examined the effect of this inhibitor on the synthesis and/or export of the general population of proteins of the periplasmic space. For this purpose we employed the cell wall-less Chlamydomonas mutant, CW-15. Electrophoretic analysis of polypeptides released into the medium by CW-15 is presented in Fig. 76. The 37,000 polypeptide is not found in the medium of cultures maintained on high CO₂ in the presence or absence of tunicamycin (Fig. 76, lanes 1, 2, 4, and 5). Air-grown cells excrete large amounts of carbonic anhydrase (Fig. 76, lanes 3 and 7) unless tunicamycin is included in the culture medium (Fig. 76, lanes 4 and 8). In addition to blocking the appearance of carbonic anhydrase, tunicamycin causes a reduction in the total number of exported polypeptides, regardless of the CO₂ concentration. In cultures grown under high CO₂ almost no polypeptides seem to be exported, while air-grown cultures do export some polypeptides in the presence of tunicamycin. However, many of these polypeptides do not co-migrate with exported polypeptides from cultures grown in the absence of tunicamycin. These results suggest that even if some export is occurring, the exported polypeptides may not be properly or completely processed. In yeast, tunicamycin also blocks the synthesis of several exported glycoproteins such as invertase and acid phosphatase, but it has no effect on the synthesis of intracellular polypeptides (Kuo and Lampen, 1974).

The reason for the absence of 37,000 protein in the presence of tunicamycin is unclear. The addition of the carbohydrate moiety may be required for export or resistance of the newly synthesized polypeptide to proteolytic degradation (either within the cell or in the periplasmic space). The low levels of carbonic anhydrase activity found following induction in the presence of tunicamycin may be the result of another form of the enzyme which does not require glycosylation (perhaps localized to the chloroplast), or may represent the activity of the nascent polypeptide (localized within the cell prior to degradation).
REGULATION OF PROTEIN SYNTHESIS DURING ADAPTATION OF *Chlamydomonas reinhardtii* TO LOW CO₂

John Coleman and Arthur Grossman

The transfer of *Chlamydomonas reinhardtii* from high to low concentrations of CO₂ during growth results in marked changes in the photosynthetic characteristics of the organism (Berry et al., Year Book 75, 423-432). The induction both of carbonic anhydrase and of a bicarbonate transport capacity following the transfer, enable the alga to photosynthesize efficiently at CO₂ concentrations well below that required for saturation of either isolated RuBP carboxylase or most terrestrial plants.

In this study we examined the effect of adaptation to low CO₂ concentrations on the synthesis of polypeptides other than carbonic anhydrase. We note transient changes in the synthesis of a few polypeptides, including both the small and large subunits of RuBP carboxylase.

**MATERIALS AND METHODS**

*Chlamydomonas reinhardtii* 2137 mt+ was grown at the desired CO₂ concentration, as previously described (Coleman et al., this Report). ³⁵S⁰₄ was used for in vivo labeling of proteins. Cells grown at high CO₂ concentrations were transferred to air levels of CO₂ at the appropriate time and labeled for 2 h. The cells were then harvested, broken in a pre-chilled French pressure cell, and fractionated, as described previously (Coleman et al., this Report). Proteins in each fraction were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis, and the newly synthesized polypeptides were visualized by autoradiography.

**RESULTS AND DISCUSSION**

*Chlamydomonas reinhardtii* grown at high CO₂ concentrations was allowed to adapt in air for 2, 4, 6, 12, and 24 hours. Prior to the final 2 h of adaptation, the cells were harvested and resuspended in sulfate-free medium to which ³⁵S⁰₄ was added. Controls were maintained at high CO₂ concentrations over the labeling period (Fig. 77, lanes 1 and 7). In this manner we were able to examine newly synthesized polypeptides from cells at different stages of adaptation. The stained gel and the autoradiogram of the polypeptides in the high-speed pellet fraction are shown in Fig. 77. This high-speed pellet contains much of the RuBP carboxylase holoenzyme plus other, less-prominent polypeptides. Although each lane received equal amounts of protein (as shown by the stained gel profile, Fig. 77, lanes 1-6), the autoradiogram shows substantial differences in the rate of synthesis of both the large (LS) and the small (SS) subunits of RuBP carboxylase.

Following the 4-h time
Fig. 77. Newly synthesized polypeptides in the high-speed pellet fraction after transfer of *Chlamydomonas* from high CO$_2$ to air for various times. Stained protein profiles are presented in lanes 1–6, while labeled polypeptides are shown in lanes 7–12. Cells were incubated in $^{35}$S0$_2$ during growth at 5% CO$_2$ (lanes 1 and 7) or during exposure to air levels of CO$_2$ for 2 h (lanes 2 and 8), 4 h (lanes 3 and 9), 6 h (lanes 4 and 10), 12 h (lanes 5 and 11), and 24 h (lanes 6 and 12). Labeling was for 2 h. Breakage and fractionation are described in text. Samples were prepared from the high-speed pellet fraction and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by autoradiography. Large subunit (LS) and small subunit (SS) of RuBP carboxylase are indicated on the figure. Molecular weight markers are phosphorylase b (92,500), bovine serum albumin (66,000), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400).

At point, synthesis of both subunits begins to increase. Other polypeptides in this fraction, in a similar manner, show a transient decrease in synthesis (2–4 h) during the induction period. Carbonic anhydrase, a diffuse band of approximately 37,000 in the high-speed pellet, is not easily seen in this autoradiogram because of its low rate of synthesis with respect to other newly synthesized polypeptides.

Changes in protein synthesis during adaptation are also observed in the high-speed supernatant fraction from *Chlamydomonas* (Fig. 78). As indicated on the stained gel (Fig. 78, lanes 1–6), each lane received equal amounts of protein. However, the autoradiogram clearly shows transient changes in the rates of synthesis of both large and small subunits of RuBP carboxylase (this enzyme is found in both the high-speed pellet and supernatant), as well as other soluble proteins (indicated by arrows). After a 2-h exposure to air, two prominent polypeptides are synthesized (lane 8, indicated by arrows on the left-hand side of the autoradiogram). The rate of synthesis of these polypeptides quickly decreases and essentially no new synthesis occurs after a 6-h exposure to air. A polypeptide of approximately 20,000 (indicated by arrow) is also synthesized during the induction period. This species is similar in molecular weight to the precursor of the small subunit of RuBP carboxylase and may represent the immature polypeptide. A pre-sequence (or transit peptide) of approximately 4,000 is present at the N-terminal of the primary translation product of the small subunit. The pre-sequence is thought to enable the small subunit to traverse the double membrane of the chloroplast envelope. (For a review of this subject see Grossman et al., 1982.) The observed decline in the rate of synthesis of RuBP carboxylase holoenzyme could be the result of a specific decrease in the translation of the large subunit messenger RNA. (Some evidence, not presented here, does indicate that the control of large subunit synthesis is at the level of translation and not transcription.) A decreased rate of large subunit synthesis may result in elevated levels of the small subunit precursor if the unassembled large subunit is required for the processing and/or transport of the immature small subunit. Immunological characterization of this presumptive precursor is in progress.
Fig. 78. Newly synthesized polypeptides in the high-speed supernatant fraction after transfer of *Chlamydomonas* from high CO₂ to air for various times. Stained protein profiles are presented in lanes 1–6, while labeled polypeptides are shown in lanes 7–12. The cells were labeled in vivo (see legend of Fig. 77), and the high-speed supernatant fraction was prepared as described in text. Cells grown at 5% CO₂ (lanes 1 and 7) or exposed to air levels of CO₂ for 2 h (lanes 2 and 8), 4 h (lanes 3 and 9), 6 h (lanes 4 and 10), 12 h (lanes 5 and 11), and 24 h (lanes 6 and 12), were used for labeling. Large subunit (LS) and small subunit (SS) of RuBP carboxylase as well as other polypeptides with altered rates of synthesis during the induction are indicated in the figure. Molecular weight markers are described in the legend to Fig. 77.

In conclusion, the rates of synthesis of a number of polypeptides are affected by the transfer of high-CO₂-grown algae to air levels of inorganic carbon. There is a transitory decline in the rate of synthesis of pre-existing proteins such as the holoenzyme of RuBP carboxylase, with perhaps a decline specifically in the rate of synthesis in the large subunit. There is the transient, rapid synthesis of polypeptides with undetermined function. And finally, the decreased synthesis of the large subunit might lead to the accumulation of the small subunit precursor in the cytoplasm of the cell. This last point is important to our understanding of the biosynthesis of plastid components and suggests a lack of coordination between the synthesis of the large and small subunits of RuBP carboxylase.

**REFERENCE**

BIOSYNTHESIS OF PHYCOBILISOME POLYPEPTIDES OF
Porphyridium aerugineum AND Cyanophora paradoxa

Arthur Grossman, Lawrence Talbott, and Thomas Egelhoff

Phycobilisomes are major light-harvesting complexes in both prokaryotic and eukaryotic algae (Bogorad, 1975; Gantt, 1981). The pigmented polypeptides, or phycobiliproteins, of this complex (phycoerythrins or phycoerythrocyanin, phycocyanin, and allophycocyanin) serve to harvest light energy and transfer it to the photosynthetic reaction centers. Each of these pigmented polypeptides is composed of an α and a β subunit (Gantt, 1981). The α subunits of the different phycobiliproteins are related, as are the β subunits (Glazer, 1977). Furthermore, the α and β subunits are related to each other and probably arose via a gene duplication (Glazer, 1977). In addition to phycobiliproteins, nonpigmented polypeptides are integral constituents of the phycobilisome (Tandeau de Marsac and Cohen-Bazire, 1977). These nonpigmented polypeptides (linker proteins) function in the maintenance of phycobilisome structure (Glazer, 1982). The linker proteins have been most thoroughly studied in cyanobacteria (Lundell et al., 1981a, 1981b). In Synechococcus 6301 there are four linker polypeptides (see Glazer, 1982, for a review). These polypeptides are located in specific positions along the phycobilisome and serve to hold the hexameric arrays of pigmented polypeptides together.

We are studying the biosynthesis of the phycobilisomes in eukaryotic algae and hope to determine the compartment in which each phycobilisome constituent is synthesized, the arrangement of the genes encoding these constituents on both the plastid and nuclear genomes, and the factors important in the regulation of these genes. In this report, we identify the sites of synthesis of the specific phycobilisome polypeptides in two eukaryotic algae, Porphyridium aerugineum and Cyanophora paradoxa. P. aerugineum is a red alga thought to have evolved following the invasion of a unicellular protist by a cyanobacterium (Harington and Thornley, 1982). An intermediate in this evolutionary process may be represented by Cyanophora paradoxa (Jaynes and Vernon, 1982). The plastid of Cyanophora paradoxa closely resembles a cyanobacterium. This organelle, termed "cyanelle," is surrounded by a thin peptidoglycan cell wall and is located in the vacuole of the host organism (Aitkin and Stanier, 1979). While this arrangement suggests a symbiotic association, the cyanelle cannot be cultured independently, and the DNA within the cyanelle is the size of chloroplast DNA and not the size of blue-green algal DNA (Herdman and Stanier, 1977; Mucke et al., 1980). The reduced size of the DNA within the cyanelle suggests that the functional aspects of this organelle depend upon genetic information located in the nucleus of the host organism. We have compared the origin of phycobilisome constituents in P. aerugineum and Cyanophora paradoxa, and we find that these two organisms exhibit many similarities with respect to the sites of synthesis of phycobilisome polypeptides.

METHODS

Porphyridium aerugineum (UTEX 755) was grown in a modified Bristol's medium (Egelhoff and Grossman, 1983), and Cyanophora paradoxa, kindly provided by L. Provasoli, Yale University, was grown in Schenk's medium (Schenk, 1977). Both organisms were grown at 22°C and bubbled continuously with a mixture of 5% CO₂ and air. Illumination was from fluorescent tubes (100 μmol m⁻² s⁻¹).
For in vivo labeling of \textit{C. paradoxa}, 100 ml of mid-log-phase cells were centrifuged at 4,000 rpm (Sorvall SS34 rotor) and the pellets washed with 20 ml of 20 mM MOPS, pH 7.7, 10 mM NaCl, 1 mM MgCl$_2$, and resuspended in 40 ml of the wash medium. \textit{P. aerugineum} cultures were labeled in modified Bristol's medium minus sulfate and soil extract. We used 300 $\mu$g/ml chloramphenicol for inhibition of translation on 70S ribosomes and 1 $\mu$g/ml cycloheximide for inhibition of translation on 80S ribosomes. The cultures, both with and without inhibitors, were bubbled with air for 10 min in the light at 22°C prior to the addition of labeled SO$_4$\. We used 0.5 mCi of $^{35}$SO$_4$ per tube of \textit{P. aerugineum} and 1.0 mCi of $^{35}$SO$_4$ per tube of \textit{C. paradoxa}.

Following in vivo labeling, the cells were washed in 0.65 M phosphate buffer, pH 7.0, and the phycobilisomes isolated according to the method of Williams \textit{et al.} (1980), as modified (see preceding report). After collecting the intact phycobilisome band from sucrose gradients, the phycobilisomes were diluted 4–5 times with 0.75 M phosphate buffer, pH 8.0, and pelleted for 2 h at 45,000 rpm in a 50 Ti rotor. The pellets were solubilized as before, and treated with $\frac{1}{2}$ vol of 5% sodium dodecylsulfate, 30% sucrose, 0.1% bromphenol blue, and then electrophoresed on 12–18% polyacrylamide gradient gels containing 8 M urea. Molecular weight standards were also run. After staining with Coomassie brilliant blue G-250, destaining, and fluorography (Bonner and Laskey, 1974), the gels were dried and exposed to Kodak XAR-5 film.

\section*{RESULTS}

Several polypeptide constituents of phycobilisomes of both \textit{P. aerugineum} and \textit{C. paradoxa} were resolved by sodium dodecylsulfate, polyacrylamide gel electrophoresis. The profile of phycobilisome polypeptides of \textit{P. aerugineum} is presented in Fig. 79, lane 1. These polypeptides have molecular weights between 15,000 and 95,000. The band with the highest molecular weight (band 1) is thought to be the anchor protein (Red-

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig79}
\caption{Synthesis of phycobilisome polypeptides of \textit{Porphyridium aerugineum}. Growth of \textit{P. aerugineum}, in vivo labeling with and without inhibitors of translation, phycobilisome isolation, and polyacrylamide gel electrophoresis and fluorography are described in the text. (1) Stained profile of \textit{P. aerugineum} phycobilisome polypeptides. (2) Phycobilisome polypeptides labeled in vivo. (3) As in (2) but in the presence of cycloheximide. (4) As in (2) but in the presence of chloramphenicol. (5) As in (2) but in the presence of cycloheximide and chloramphenicol.}
\end{figure}
linger and Gantt, 1982), while the major bands toward the bottom of the gel (bands 9–12) are the pigmented components of the complex (α and β subunits of the phycoobilinproteins). The anchor protein is involved in the attachment of the phycobilisome to the external surface of the thylakoid membranes, while the low-molecular-weight pigmented polypeptides are the major light-absorbing components. Since the pigmented polypeptides have similar molecular weights, some of these broad bands may represent more than one species. All of the stained polypeptides of the phycobilisomes of P. aeruginosum do incorporate label in vivo. Some bands that do not correspond to phycobilisome polypeptides are also labeled. Generally, these polypeptides are less-intensely labeled than phycobilisome constituents and may represent contamination of the isolated light-harvesting complex with heavily labeled cytoplasmic polypeptides. Minor stained bands such as bands 3, 4, and 5 of Fig. 79 may also be cytoplasmic contamination of phycobilisome preparations. Some bands may also result from a small amount of proteolysis (in spite of the inclusion of protease inhibitors in all solutions used in the isolation). For example, band 2 of P. aeruginosum phycobilisomes may result from proteolytic degradation of the anchor protein. This band, like band 1, has very faint pigmentation (both are blue), and while we generally find that band 1 predominates over band 2, in other published profiles the lower band (band 2) appears to predominate (Mörschel, 1982).

Phycobilisome polypeptides labeled in the presence of cycloheximide and chloramphenicol are presented in lanes 3 and 4 of Fig. 79, respectively, while lane 5 shows labeling in the presence of both inhibitors. Cycloheximide inhibits translation on 80S cytoplasmic ribosomes, while chloramphenicol inhibits translation of 70S chloroplast ribosomes. We observe a reciprocal labeling pattern of the phycobilisome polypeptides in the presence of these inhibitors. If both inhibitors are included during in vivo labeling, no phycobilisome constituents are synthesized (Fig. 79, lane 5). Comparing lanes 3 and 4 suggests that the anchor protein (band 1) and the major pigmented bands (bands 9–12) are translated on chloroplast ribosomes, while band 6, a linker polypeptide (Mörschel, 1982), is synthesized in the cytoplasm of the cell. While the synthesis of polypeptides 7 and 8 is lowered in the presence of either inhibitor, longer exposures of the fluorographed gel reveal that both of these polypeptides are made on the 70S ribosomes of the chloroplast. The decreased labeling of those polypeptides that we observe in the presence of cycloheximide may be the result of poor integration of these components into intact phycobilisomes without the synthesis of cytoplasmic phycobilisome polypeptides.

In Fig. 80, the polypeptide profiles of the phycobilisomes of C. paradoxa are presented. The profile of the stained phycobilisome polypeptides is presented in lane 1, while labeled polypeptides synthesized in the absence and presence of protein synthesis inhibitors are in lanes 2–5. The anchor protein of C. paradoxa appears to be degraded into bands 1 and 2 during the isolation procedure, and the amount of each species varies depending upon the speed of isolation. The major pigmented polypeptides are bands 7–10. At least some of the polypeptides localized between the anchor protein and the pigmented polypeptides, and ranging in molecular weight from 34,000 to approximately 55,000, may represent linker polypeptides. Although there appears to be contamination of the C. paradoxa phycobilisome preparation with some stainable bands (note the high-molecular-weight band above the anchor protein), the origin of phycobilisome polypeptides of C. paradoxa seems to be similar to those of P. aeruginosum as well as to those of P. cruentum and C. caldarium (Egelhoff and Grossman, 1983). The anchor protein is synthesized in the chloroplast (represented by band 1 and probably a proteolytic product of this polypeptide, band 2), since it is made in
Fig. 80. Synthesis of phycobilisome polypeptides of *Cyanophora paradoxa*. Procedures used are in the legend of Fig. 79. (1) Stained profile of *C. paradoxa* phycobilisome polypeptides. (2) Phycobilisome polypeptides labeled in vivo. (3) As in (2) but in the presence of cycloheximide. (4) As in (2) but in the presence of chloramphenicol. (5) As in (2) but in the presence of cycloheximide and chloramphenicol.

The characteristics of the photosynthetic apparatus of the cyanelle are strikingly similar to those observed for cyanobacteria (Klein *et al.*, 1981). On the other hand, the cyanelle cannot be cultured as an independent organism, and the size of the cyanelle DNA is similar to the size of DNA found in plastids of higher plants (Jaynes *et al.*, 1981).
1981). Therefore, the cyanelle has lost the genetic potential to exist independently of its biflagellate host. Since it has been suggested that C. paradoxa may represent an evolutionary bridge between cyanobacteria and chloroplasts (see Jaynes and Vernon, 1982, for review), we thought that the sites of synthesis of the phycobilisome polypeptides might be different from the sites of synthesis of phycobilisome constituents of red algae and Cyanidium caldarium. In this report we show that the biosynthesis of C. paradoxa phycobilisomes does not differ in general aspects from the biosynthesis of phycobilisomes of other eukaryotic algae (Egelhoff and Grossman, 1983). In spite of serious proteolysis of the anchor protein, our data indicate that both the anchor protein and the major pigmented polypeptides are synthesized within the cyanelle and are probably encoded on the plastid DNA. Other polypeptides possibly analogous to linker polypeptides (extensively studied in blue-green algae) are synthesized in the cytoplasm of the organism. The location and arrangement of the genes for these polypeptides and the specific changes that these genes have undergone during their transfer from the invading endosymbiont to the nucleus of the host organism will strengthen our understanding of gene transfer and the development of eukaryotic chloroplasts.

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A RAPID PROCEDURE FOR THE ISOLATION OF INTACT PHYCOBILISOMES

Arthur Grossman and Jerry Brand

Only a few different methods have been employed in the isolation of intact phycobilisomes from blue-green and red algae. The procedure used most extensively involves the breakage of cells in high concentrations of phosphate buffer (0.65–0.75 M, NaH₂PO₄/K₂HPO₄, pH 7.0–8.0) followed by incubation in 1–2% Triton X-100 and sucrose gradient centrifugation (Gantt and Lipschultz, 1972; Gantt et al., 1979; Williams et al., 1980). The method is time-consuming, involves a long ultracentrifugation step, and is limited in the amount of sample that can be processed. There are other, less frequently used methods to isolate phycobilisomes. So
Dium sulfate has been used in place of phosphate buffer when CaCl₂ was required in the isolation medium (Yamanaka et al., 1982). Another method involves the pelleting of phycobilisomes with 15% polyethylene glycol 6000 (Rigbi et al., 1980; Rosinski et al., 1981). This procedure, like the one described below, is relatively rapid and does not require prolonged ultracentrifugation.

A similarity among these procedures is the reduced water activity to which the phycobilisome is exposed, as has been pointed out by Glazer (1982). With increased water activity, energy transfer between the different classes of pigmented polypeptides is uncoupled as the phycobilisome begins to dissociate. Low temperature also promotes dissociation of phycobilisomes (Gantt et al., 1979). Exposure of phycobilisomes to low-phosphate buffer and/or low temperature for short periods of time has been used to cause partial dissociation of the phycobilisome and has yielded information about the structural organization of this light-harvesting complex (Morschel, 1982; Myeong-Hee Yu et al., 1981). Furthermore, by careful adjustment in the concentration of phosphate buffer, reconstitution of phycobilisomes (both homologous and heterologous) from individual constituents has been achieved (Canaani and Gantt, 1982).

By varying the concentrations of phosphate buffer to which the phycobilisomes are exposed, we have developed a simple and rapid method for the isolation of this light-harvesting complex. The yield obtained using this technique is either as high or higher than yields obtained using standard procedures. The isolation can be performed in 3–4 hours and does not require ultracentrifugation. We have employed this technique in the isolation of phycobilisomes from the prokaryotic organism Anacystis nidulans (Synechococcus 6301) and the eukaryotic organisms Porphyridium aerugineum and Cyanidium caldarium, but have not found it useful in the isolation of phycobilisomes from Porphyridium cruentum.
membranous material and the phycobilisomes to pellet. The supernatant, containing the soluble cytoplasmic polypeptides as well as the stromal polypeptides, was discarded, and the pelleted material was resuspended in 0.6 M phosphate buffer, pH 7.5. For homogeneous resuspension the pellet was dispersed with a ground-glass homogenizer. This suspension was incubated for 30 min after the addition of Triton X-100 to 1% and then centrifuged at 20,000 rpm for 30 min. During this centrifugation the membranes became pelleted while a large proportion of intact phycobilisomes remained in solution. The pellet was discarded and the supernatant diluted tenfold with 1.0 M NaKPO₄, pH 7.5, and spun at 20,000 rpm for 1 h. Intact phycobilisomes became pelleted under these conditions.

Phycobilisome pellets obtained by either method described above were solubilized in 0.1 M Na₂CO₃, 0.1 M dithiothreitol, and then treated with ½ vol of 5% sodium dodecylsulfate, 30% sucrose, and 0.1% bromphenol blue. The samples were boiled for 1 min prior to electrophoresis on 12–18% polyacrylamide gradient gels containing 8 M urea. Molecular-weight standards were phosphorylase b (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400). After electrophoresis the gels were stained with Coomassie brilliant blue G-250.

Fluorescence emission of phycobilisome preparations was analyzed following excitation of phycocyanin with broadband blue-green light, produced by passing light from a type DTS 125W tungsten lamp (21.5V) through two CS 4-96 (Corning) filters and one Calflex C (Balzers) heat-reflecting filter. Fluorescence emission spectra (600–850 nm) were measured with a microprocessor-based spectrofluorimeter equipped with a trifurcated fiber-optic bundle (Fork, Ford, and Catanzaro, Year Book 73, 196–199) which permitted illumination and fluorescence detection at the same surface of the sample. The photomultiplier was protected from the exciting light by the cutoff filter CS 2-64.

RESULTS

Polypeptide profiles of the phycobilisomes of A. nidulans, P. aerugineum, and C. caldarium isolated by sucrose gradient centrifugation or via the rapid, pelleting procedure are presented in Fig. 81. The polypeptide profiles obtained for

![Fig. 81. Polypeptide profiles of phycobilisomes isolated from Porphyridium aerugineum, Cyanidium caldarium, and Anacystis nidulans. The isolation procedures and electrophoretic analyses are described in the text. Phycobilisomes of P. aerugineum, C. caldarium, and A. nidulans were isolated by sucrose gradient centrifugation (lanes 2, 4, and 5, respectively) or by the rapid procedure described in the text (lanes 1, 3, and 5, respectively).]
each organism by the two different methods of isolation are similar. For *C. caldarium* we find that the rapid-pelleting method eliminates some of the minor bands often observed after isolation by ultracentrifugation (compare Fig. 81, lanes 3 and 4). Furthermore, the phycobilisome yield is at least as high as for the standard method of isolation (data not shown).

Although the polypeptide profiles of phycobilisomes obtained by the two methods are similar, we used fluorescence emission spectroscopy to demonstrate the integrity of the complex after the rapid-pelleting procedure. This method of determining phycobilisome integrity makes use of the observation that the intact light-harvesting complex efficiently transfers energy from the short-wavelength-absorbing biliproteins (phycocyanin or phycocyanin) to allophycocyanin and ultimately to the pigmented anchor protein or allophycocyanin B (see Glazer, 1982, for review). These latter two proteins fluoresce between 675 nm and 680 nm. Therefore, a phycobilisome preparation which absorbs wavelengths of light below 600 nm and fluoresces between 675 and 680 nm has coupled energy transfer. This indicates that the in vivo arrangement of the phycobilisome polypeptides has not been altered during the isolation procedure.

Fluorescence emission analysis of *Porphyridium aerugineum* phycobilisomes obtained by the pelleting method is presented in Fig. 82. Similar preparations from *A. nidulans* and *C. caldarium* gave comparable results. A broad-band filter with a cutoff at 620 nm (CS 4-96) and very low transmission above 600 nm was used to excite the isolated phycobilisomes, and fluorescence emission was measured between the wavelengths of 600 and 850 nm. As a control, the pelleted phycobilisomes were dissociated in low-phosphate buffer (30 mM, pH 7.0) prior to the measurement of fluorescence emission. The fluorescence emission from the phycobilisome preparation maintained in high-phosphate buffer had a peak at 677 nm.

Following dissociation in the low-phosphate buffer, the emission broadened and peaked at 658 nm. This fluorescence emission data, in conjunction with the electrophoretic analysis of pelleted phycobilisomes, clearly indicates that this rapid isolation procedure is effective for the preparation of intact phycobilisomes from certain eukaryotic and prokaryotic algae. The phycobilisomes obtained are comparable to phycobilisomes isolated via sucrose gradient centrifugation and in some cases show less cytoplasmic contamination.

**DISCUSSION**

We have described a method for the rapid isolation of intact phycobilisomes from both prokaryotic and eukaryotic algae. This procedure does have some limitation, however, and we have not been able to use it for isolating the larger, more-complex phycobilisomes of *P. cruentum*. The isolation entails lysis in 1.0 M phosphate buffer followed by a series of low-speed centrifugations, resulting in a phy-
Phycobilisomes are macromolecular aggregates which function in harvesting light energy and transferring it to the photosynthetic reaction centers. They are composed of colored proteins and linker proteins. The linker proteins are required for assembly of the pigmented polypeptides and are essential structural components of the complex (Glazer, 1982). Phycobilisomes are generally isolated as intact complexes on sucrose gradients in high-phosphate buffer. Upon electrophoresis of the intact phycobilisome on denaturing polyacrylamide gels, the constituent polypeptides dissociate and the molecular weights of the individual components generally range in size from 12,000 to 95,000 (Gantt, 1981). The highest-molecular-weight polypeptide (anchor protein) serves in the attachment of the complex to the thylakoid membranes (Redlinger and Gantt, 1981), while the low-molecular-weight polypeptides (12,000–20,000) are the major pigmented polypeptides. In the phycobilisomes of Porphyridium cruentum, three or four other pigmented polypeptides with molecular weights between 25,000 and 35,000 have been visualized (Redlinger and Gantt, 1981). While the association of various components has been examined by several methods (partial dissociation followed by sucrose gradient centrifugation [Mörschel, 1982], analysis of phycobilisome mutants [Gingrich et al., 1982]), no report of high-molecular-weight aggregates of phycobilisome polypeptides on sodium dodecylsulfate polyacrylamide gels has appeared. Here we note that if the large hemi-ellipsoidal phycobilisomes...
of the red alga *P. cruentum* are electrophoresed on 7.5–15% polyacrylamide gradient gels in the cold, large complexes containing phycobilisome polypeptides can be seen. While only preliminary work has been done on the nature of these aggregates, they appear to contain both the major pigmented polypeptides and the less-prominent pigmented components, which range in molecular weight from 25,000 to 35,000.

**METHODS**

Phycobilisomes of *P. cruentum* were isolated according to the method of Williams *et al.* (1980) except that protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine-HCl, 5 mM e-aminon-caproic acid) were added to all solutions. The concentrations of sucrose used in the step gradients were 0.5 M, 0.75 M, 1.0 M, and 2.0 M. Centrifugation was overnight at 18°C in an SW27 rotor. The phycobilisomes, which collected at the 1.0/2.0 M sucrose interface, were diluted 3–4-fold with 0.75 M phosphate buffer, pH 7.5, and then pelleted for 2 h at 45,000 rpm in a Beckman Ti50 rotor. The pellet was resuspended in 0.1 M Na$_2$CO$_3$, 0.1 M dithiothreitol, and treated with 1/3 vol of 5% sodium dodecylsulfate, 30% sucrose, 0.1% bromphenol blue. Some samples were boiled for 1 min prior to loading them onto the gel. The electrophoresis system used Laemmli buffers (Laemmli, 1970) and 7.5–15% polyacrylamide slab gels. The samples were electrophoresed at 4°C and, at the completion of electrophoresis, stained with Coomassie brilliant blue G-250.

**RESULTS AND DISCUSSION**

In Fig. 83 we show a polypeptide profile of *P. cruentum* phycobilisomes. There is some contamination of the phycobilisome preparation with cytoplasmic components. The phycobilisomes were either heated (Fig. 83, H) or nonheated (Fig. 83, NH). Two phycobiliprotein aggregates are present in the nonheated sample toward the top of the gel. These aggregates are labeled A and B (Fig. 83, NH) and are bright-red bands prior to Coomassie-blue staining of the gel. Frequently, component B can be resolved into two distinct bands. Upon heating the phycobilisome sample (1 min of boiling), these aggregates dissociate. In the figure
presented, dissociation of B is incomplete and the two distinct pigmented components (perhaps even three) of the band are resolved. Furthermore, following dissociation a number of components (C, D, E, F, and G in the figure) intensify. These polypeptides are all pigmented, with the components between 25,000 and 35,000 (C and D) being only faintly colored. Component E contains several polypeptides and represents the major pigmented polypeptides of the complex. Therefore, it appears that at least the major pigmented aggregate (component B) contains primarily colored polypeptides. Since A represents such a minor species, it is uncertain what components (other than at least some colored polypeptides) are contained within it.

While we have not been able to observe phycobiliprotein aggregates following electrophoresis of phycobilisomes isolated from a number of other organisms, it is clear that high-molecular-weight phycobiliprotein complexes from P. cruentum are stable to electrophoresis under these conditions. The apparent molecular weights of these aggregates, over 200,000, may not be accurate, since the protein-protein interactions of each complex may cause anomalous detergent binding. The nature of these complexes has not been clearly defined but the major constituents (at least for the B complexes) are pigmented polypeptides of both low and intermediate molecular weights. These aggregates may represent trimers and/or hexamers of the phycobiliproteins, although we cannot eliminate the possibility that they are artificial associations which occur during sample preparation. The isolation of stable aggregates of phycobilisome polypeptides on sodium dodecylsulfate polyacrylamide gels and the subsequent analysis of these aggregates may prove helpful in elucidating the molecular architecture of the phycobilisome.

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