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Contents

Introduction ................................................. 561

Biochemical Investigations ................................. 566
Studies on fractions of chlorophyll complexes from a variety of plants ..... 566
Absorption and fluorescence of chlorophyllide a in vivo .................. 570
Photosystem 1 and 2 particles from leaves of diverse ages ............... 572
An action spectrum for methyl viologen reduction by fractionated spinach chloroplasts .......................... 574
The forms of chlorophyll a in fractions of chloroplasts from different sources . 578
A comparative study of the light-induced carotenoid change and fluorescence in the chlorophyll-b-less alga Botrydiopsis alpina (Xanthophyceae) . 587
The effect of ultraviolet irradiation on the carotenoid change, electron transport, and photosynthesis of Botrydiopsis alpina .................. 595
Electron transport and degradation of chloroplasts by hydrolytic enzymes and ultraviolet irradiation .......................... 598
Effects of N-methylphenazonium methosulfate and pyocyanine on delayed light emission in Chlorella cells and spinach chloroplasts . 603
A test of fiber optics for fluorescence spectroscopy .................... 607
Use of the ACME computer for analysis of real-time data ................ 608

Experimental Taxonomy Investigations ........................ 609
The Mimulus investigations .................................. 609
Growth, photosynthetic, and biochemical responses of contrasting Mimulus clones to light intensity and temperature .................. 614
Comparative studies of Atriplex species with and without β-carboxylation photosynthesis and their first-generation hybrid .......... 620
Leaf factors affecting the rate of light-saturated photosynthesis in ecotypes of Solanum dulcamara ................................ 633
Application of a new O₂ sensing device to measurements of higher plant photosynthesis .................. 636
Intercontinental crosses in Solidago ......................... 640
Vegetation of the Harvey Monroe Hall Natural Area .................... 643

Staff Activities .............................................. 644
Bibliography ............................................... 645
Speeches ...................................................... 646
Personnel ..................................................... 648

Carnegie Institution Year Book 88, 1988–1989
INTRODUCTION

In recent years photosynthesis investigations have centered on the kinetic relations between the substances that make possible the flow of electrons from water to those carbon compounds whose reduction is the significant function of the whole process. These oxido-reduction reactions are coupled to phosphorylation systems that also store chemical energy as adenosine triphosphate. Thus by a linked series of complex reactions carbon dioxide is turned into the required organic components of living matter, and power for their further interconversion is provided in usable form.

The main tide of scientific effort in the study of photosynthesis flows increasingly toward the more precise refinement of a theoretical picture describing the interrelations between the pigments, enzymes, and intermediate compounds that make up the photosynthetic system. The drawing power of this tide had for some years left an ebb in the field of knowledge from which the main tide originated. This was the descriptive and comparative type of plant physiology through which the general significance and biological function of photosynthesis became apparent over a century ago. Such types of investigation have now been revived by many vigorous groups.

Experimental taxonomy. One aspect of the work of the Experimental Taxonomy Group of the Department might now be described as an effort to bring the advances in the detailed understanding of the mechanisms of photosynthesis to bear on explanations for the diverse physiology of contrasting kinds of plants. To apply effectively the relevant parts of the vast body of intricate concepts about photosynthesis to broader biological problems—such as a determination of the physiological basis of adaptation and of natural selection and evolution in plants—almost requires that investigators themselves be active contributors to the main body of theoretical progress as well as users of that new information for the clarification of fundamental ecological questions. A strong collaborative effort on these lines has been developed by Drs. Björkman, Hiesey, and Nobs with several members of the Stanford faculty and their graduate students.

In the Experimental Taxonomy Section, this year's activities include continuing studies at the altitudinal transplant stations at Stanford, Mather, and Timberline as well as intensive laboratory investigations directed toward further penetration into unknown mechanisms underlying natural selection and plant evolution.

In 1947 the Experimental Taxonomy Group met with university colleagues having similar interests at the Department Laboratory and at the mountain stations. The purpose of this conference was to select the type of plant material best suited for long-term studies of the adaptation mechanisms of plants to contrasting environments. The resulting choice, the Erythranthe group of *Mimulus* (monkey flower), has amply proved its anticipated value as an experimental group of species for such investigations. Cross-fertilization experiments have been combined with studies of growth characteristics both at the Department's three field stations and in controlled environments. Field work has been correlated to laboratory measurements of the photosynthetic characteristics of first-, second-, and third-generation progeny. Each generation was studied for several years.

A strong correlation was found between the inheritance of certain morphological characters and the ability to survive in specific environments. The action of combinations of genes caused nearly all of the significant characters to
be inherited in groups, that is, a purely random assortment of characters did not take place. Hybrid vigor was found to depend as much upon the environment in which it was tested as upon the genetic inheritance of the plants. The genetic recombinations of some of the various steps of the photosynthetic process suggest the mechanisms underlying the ability of hybrids to flourish in contrasting environments.

This long series of studies on the basic question of biological quality, as determined by the interplay of genetic and of environmental influences, is being prepared for publication as an Institution monograph. This fifth volume in the series Experimental Studies on the Nature of Species appropriately marks Dr. William M. Hiesey's retirement after forty-four years devoted to basic research on this problem of the comparative influences of environment and of heredity on the performance of an individual.

Many of the questions arising from the work of the Experimental Taxonomy Group under Dr. Clausen's and Dr. Hiesey's leadership can now be investigated in a more definitive manner than was possible at the time their significance was first perceived. As Dr. Björkman now has assumed responsibility for experimental taxonomy work at the Department, the biochemical basis for physiological characteristics of plant adaptation to contrasting environments is receiving greater emphasis. Thus there is an increasing amount of collaboration, and of similarity in the experimental techniques, of the two groups in the Department, although their objectives remain distinct.

Recently Dr. Björkman and Mr. Eckard Gauhl, an Institution Research Fellow from Professor Egle's laboratory at Frankfurt, have been able to measure simultaneously with high precision not only the rates of carbon dioxide uptake and water vapor release, but also the rate of oxygen evolution during photosynthesis in higher plants. Such measurements are made possible by the development of new equipment and are of special value in the current comparative studies of contrasting ecological races and species.

In the growing field of comparative studies of photosynthesis in plants from ecologically diverse environments, recent developments have revealed that photosynthetic differentiation in higher plants is not limited only to differences in the capacities of component steps of photosynthesis, but that differences in the biochemical pathways of the process also exist. During the past few years it has been established that certain grass species, whose main distribution is in tropical regions, possess a different pathway for photosynthetic carbon dioxide fixation than do plants from temperate regions. Members of at least three dicotyledonous families have also been found to possess this newly discovered pathway. In the saltbush genus Atriplex some members have this pathway, whereas others do not.

The discovery of different CO₂-fixation pathways in photosynthesis has opened up an exciting field of investigation for those concerned with the biochemical and physiological basis of adaptation. Dr. Björkman and Mr. Gauhl have undertaken a comparative study of two Atriplex species of differing CO₂-fixation pathways. Their work is an integral part of a broad study of mechanisms of photosynthetic adaptation to environmental factors, particularly with regard to temperature. One of these Atriplex species, *A. patula*, which occurs mainly in cool coastal areas, fixes CO₂ by the normal reductive pentose phosphate pathway, whereas the other species, *A. rosea*, which grows primarily in hot, semiarid habitats, fixes CO₂ by the more recently discovered C₄-dicarboxylic acid pathway. The two species differ with regard to certain key photosynthetic enzymes, and they have profoundly different photosynthetic characteristics. For
example, the strong inhibitory effect of oxygen on photosynthetic CO\textsubscript{2} fixation in normal air, which appears to be a widespread phenomenon among higher plants from temperate climates, is present in \textit{A. patula} but absent in \textit{A. rosea}. The two species also differ markedly in their internal leaf structure. Other investigators have linked these combinations of differences to contrasts in plants of tropical and temperate climates, and these characteristics are thought to represent fundamental differences in evolutionary steps.

That these differences occur within a single genus makes possible comparative studies of functional adaptability that are more pertinent than would be the case if they occurred only in widely separated taxa. Still more important, however, the inheritance of the function, and biochemistry of the various components by which the two species differ, may now be studied. Recently, Dr. Nobs has been able to hybridize these two species of \textit{Atriplex}. First-generation hybrids are now being analyzed by Dr. Björkman with regard to their photosynthetic and biochemical characteristics. Dr. John Boynton, an Institution Research Fellow from Duke University, is making a study of cell and chloroplast fine structure of the F\textsubscript{1}-hybrid compared with that of the parental species. It is hoped that second-generation progeny can also be obtained, a development that would open up new opportunities for genetic studies of the molecular and physiological basis of natural selection and speciation.

Mr. Gauhl has also completed a two-year study on contrasting ecological races of the European bittersweet, \textit{Solanum dulcamara}, in which distinct inherited differences in photosynthetic characteristics were demonstrated.

The relationships between certain North American and European species of goldenrod (\textit{Solidago}) have long been a subject of speculation among botanists. The physiological studies on sun and shade races of European forms of \textit{Solidago virgaurea} by Drs. Björkman and Holmgren have created new interest in ascertaining these relationships. Results from hybridizations, most of which were made by Dr. Nobs, now clearly demonstrate that the European members and the North American counterparts (referable to \textit{S. multiradiata}) are forms which have evolved moderate genetic barriers to intercrossing. Within either group, highly diverse ecological races are completely interfertile.

\textit{Biochemical investigations.} The Biochemical Investigations Group continues to center most of its interest on the functional relationships between photosynthetic pigments and their associated enzymes in the two photosystems. Each of these systems contains a mixture of pigments and enzymes in the form of particles. Many laboratories are trying to improve techniques for the separation of the two photosystems of chloroplasts. Thorough resolution of the two requires both adequate disintegration methods and sharp separation procedures. The primary test for successful fractionation of chloroplasts into the two groups of particles involved in system 1 and system 2 is the ratio of the rates of two chemical reactions, which are specific for one or the other system. The pigment composition of the systems is also different. In general there is more chlorophyll \textit{b} and more of a “Ca 670” form of chlorophyll \textit{a} in system 2 than in system 1. In system 1 a “Ca 680” form of chlorophyll \textit{a} predominates, and system 1 also contains some of the forms of chlorophyll that have still longer wavelength absorption maxima.

We are trying to analyze the absorption spectra of chloroplast fractions in order to identify the specific chlorophyll \textit{a} complexes associated with each system. Different species of algae have greatly varying relative proportions of the different forms of chlorophyll. Furthermore, the system-1 and system-2 fractions of many chloroplasts show striking con-
trasts in their absorption spectra. Dr.
Brown has separated fractions of chlo-
rophyll-containing particles from a variety
of plants and has measured both their
absorption and fluorescence spectra at
low temperature. The collection and in-
terpretation of these data, and of data re-
sulting from older lines of investigation,
are still in progress. It is expected that
comparisons of a comprehensive series of
spectra resolved by digital computer
methods will show whether the spectra
for the different individual forms of
chlorophyll are alike or different in the
corresponding fractions of all species.

In determining whether all the chloro-
phyll in one fraction is actually a func-
tional part of that system, it is essential
to compare the absorption spectra of
both fractions with the action spectra for
the two chemical reactions of each frac-
tion. Action spectra, the relative effec-
tiveness of different wavelengths in caus-
ing a specific chemical effect, match the
absorption spectra of only the pho-

tochemically active pigments in the mix-
ture. Absorption spectra, however, show
all the pigments present, even though
some of them may not be functional
for the systems tested. It is therefore very
important to learn how to measure action
spectra with high precision for system-1
and system-2 activity of fractions of
disintegrated chloroplasts.

Action spectra for oxygen exchange
in whole cells have been measured for
some time with adequate accuracy, and
the results can be plotted automatically,
like absorption spectra, with moderately
satisfactory results. However, for the
partial reactions associated with the
separate steps of photosynthesis in
chloroplast fractions, the precision so far
attainable is lamentable, and the band
widths of the monochromatic light for
action spectroscopy are about ten times
the routine width for absorption spectros-
copy.

In an effort to improve this situation
Dr. Eckhard Loos, an Institution Re-
search Fellow from Munich, has made
a study of ways to improve measure-
ments of action spectra for chloroplast
fractions. So far his work has been on
photosystem 1 as determined by the pho-
tochrome reduction of the dye methyl
viologen. Initially attempts were made to
introduce a controlled oxygen leak into
the system in such a way as to balance
the reduction of the dye by the pho-
tochrome reaction. The intensities needed
at different wavelengths to maintain a
constant concentration of reduced dye
would reflect the relative photochemical
action of each wavelength. Such an ar-
angement would have made automatic
plotting possible. There were, however,
difficulties with the oxygen leak sufficient
to make this approach impractical at the
present time.

The same dye reduction system was
therefore used in a sealed vessel for
point-by-point measurements. The opti-
mum concentrations of the critical com-
ponents of the reaction mixture were
determined. The system as worked out
gives easily measurable rates at low light
intensities. With a high pressure mercury
lamp, monochromator slits giving a half-
band width of 1.5 nm can be used. The
reproducibility of rate determinations,
however, is still inadequate. The pre-
liminary results of Dr. Loos' work have
shown close agreement between the ac-
tion and absorption spectra of system-1
particles from spinach chloroplasts.

Dr. Zdenak Šesták, a Visiting In-
vestigator from Prague, followed the
changes in the relative amounts of sys-
tem-1 and system-2 pigments in develop-
ing leaves. In young radish leaves he
found about 25 percent of the chlorophyll
to be in system-1 particles while in older
leaves only 15 percent was in system 1.
Young leaves are therefore preferable
for preparation of system-1 fractions.

Several mild treatments, such as gentle
heating, ultraviolet exposure, and incuba-
tion with enzymes, disrupt the "Ca 680"
form of chlorophyll responsible for sys-
tem-1 photochemistry and change it to a
form with a shorter wavelength peak.
Similar treatments that reduce the photochemical activity of system 2 in spinach chloroplasts have been investigated by Dr. Kenneth Mantai, a Carnegie Corporation Fellow who came to us from Professor Bishop's laboratory at Oregon State University. The common basis for the effects of ultraviolet radiation and the effects of treatments by destructive enzymes is believed to be the disruption of the structural unit comprising the pigment-enzyme complex that is specific for the functioning of system 2 in the chloroplasts.

The energy of a light quantum absorbed by a chlorophyll molecule is not immediately used for making chemical changes but instead is passed on through many chlorophyll molecules until it arrives at a particular reaction center. All the chlorophyll molecules, acting together as an antenna to catch light quanta for one reaction center, constitute a photosynthetic unit. Emerson and Arnold originally determined the size of this unit by dividing the number of chlorophyll molecules in a sample of algae by the number of oxygen molecules the algae could produce from a single flash of bright light.

The same concept can be applied to groups of photosynthetic units whose products may depend on a single enzyme for further processing. Thus each pigment system can be thought of as a small group of chlorophyll molecules feeding energy to a particular reaction center, while the products from several such centers are serviced by a single enzyme molecule, which action defines a larger composite unit. The size of this larger unit can be determined by testing whether the action of a single molecule of an enzyme poison can render the enzyme molecule ineffective.

A somewhat similar experiment was done this year by Dr. Lars Olof Björn, a Visiting Investigator from Lund, who calculated a photosynthetic unit of about $10^9$ chlorophyll molecules. This result was obtained from the stimulating effect of phenazine methosulfate on the slow emission of delayed light from cells following the activation of photosystem 1 by far-red light. The size of the functional unit so measured is approximately that of the morphological unit called a thylacoid that is recognizable in electron-microscope photographs of chloroplasts.

The connections to two large computers at Stanford, described last year by Dr. David Fork, have been used extensively, and a Dataphone line to the IBM 360/67 Computer has been added. The effective use of these facilities has been made possible by a grant from the National Science Foundation (No. GB 8630) for "Pigment-Enzyme Interactions in the Electron-Transport Mechanism of Photosynthesis." This grant gives our work on the subject far greater scope than could be managed on the Department budget alone. In fact, the application of computer analysis to a wide variety of comparable chlorophyll spectra is the essential difference between one aspect of the present project and the somewhat similar, but very limited, approach to the problem that we have made in the past. Our experience in applying for this grant has, however, made clear the extreme importance of flexible funds that can be appropriated without delay. Without the interim support of the Institution to pay for computer use during the grant processing period, a severe loss of momentum and of investigators' time would have occurred. In addition to serving their computational purposes, the computers have been a means of reducing the secretarial work of manuscript revision and of handling our reprint distribution list.

The rate of publication on photosynthesis, like the rate of publication in all fields of science, has increased so much that each scientist must continually narrow and redefine the limits of his specialty. Because of the quantity of published work, the value of good review articles is now far greater than that of all but a very few original "contribu-
tions" to the subject. The important public service of the Kettering Laboratory and of a Japanese group in preparing and circulating lists of titles of papers on photosynthesis and related matters has made it possible at least to be aware of work relevant to one's current enterprises. Reading even a reasonable fraction of the important papers is already impossible. There are no longer any experts on the whole subject of photosynthesis.

In 1965 we started a cooperative card-file system to list papers of particular interest under 75 subject headings. Each card is punched for needle selection by several subject headings, by author, and by laboratory. The designation of papers for card listing requires only a few cryptic symbols on the journal or on the reprint itself made by the interested scientist. The whole operation is handled by the Department secretary. This apparently adequate and simple system has in four years produced so many cards that the selection of those in a desired category is approaching the limit of practicality.

We see no satisfactory solution to the problem of literature listing and searching that can be carried out efficiently by a small group of research workers, even with complete secretarial support. Cost and programming problems seem to make computer use for literature search-

INGAN unrealistic approach for a single laboratory. Some sort of a centralized computer selection and listing system serving the entire community of photosynthesis workers seems to be an eventual necessity. However, the practical problems of interlaboratory agreement on organization of the system and on methods for its efficient use are serious even for the literature of photosynthesis. A greater difficulty than incomplete listing will be the danger of swamping the interrogator with information only partially relevant to his immediate concerns.

The purpose of a useful literature search system is to go beyond the title or abstract in order to retrieve buried information about specific findings and experimental techniques. A central computer could perhaps answer an inquiry by searching its internally stored library and reporting only a reference and page listing of the desired information that would already be in the worker's own library. Presumably we will have to wait until such systems have been developed for other types of scientific work before it would be reasonable to attempt their use for our subject. It would, of course, be useful if some group of enterprising computerized-library specialists could be induced to use the publications on photosynthesis for developing a system to search scientific literature.

**BIOCHEMICAL INVESTIGATIONS**

**Studies on Fractions of Chlorophyll Complexes from a Variety of Plants**

**J. S. Brown**

In order to study the various forms of chlorophyll, the forms should first be separated. Detergents have been used for this purpose for several years, but in using them there is the disadvantage that the detergent may adhere to the chlorophyll-lipoprotein complexes, modify the spectra of the material, and complicate further analysis. Therefore the nondetergent, physical method of fractionation devised last year by J.-M. and M.-R. Michel is especially valuable (Year Book 67, p. 508). Briefly, the procedure consists of disintegrating the chloroplasts or algae suspended in a KCl-Tricine buffer with the French press, layering the broken material on a sucrose density-gradient, and centrifuging the layered material for 30–60 minutes to separate the two kinds of chlorophyll-containing particles.
The light fraction-1 particles are similar to the system-1 particles separated by detergent fractionation procedures. They show relatively more long wavelength absorption and fluorescence and have a lower fluorescence yield per chlorophyll than the denser particles in fraction 2. These two kinds of particles were obtained from several higher plants and algae (Year Book 67, p. 516).

This year further experiments have been performed to study some parameters of the procedure itself: to fractionate non-green algae, including the red alga *Porphyridium*, the diatom *Phaeodactylum*, and three blue-green algae, *Anacystis*, *Anabaena* and *Plectonema*; and to compare the absorption and fluorescence of the various pigmented particles. The effect of mild heating and of trypsin or porcine pancreatic lipase digestion on the absorption of spinach particles was also investigated.

The way in which the algae were grown and harvested, and the way the chloroplasts were prepared, apparently had little or no effect upon the subsequent fractionation. A buffer of 0.05 M K$_2$HPO$_4$—KH$_2$PO$_4$ at pH 8 has proved to be as suitable as the Tris or Tricine used previously.

For the experiments reported here the algae were suspended in 0.3 M KCl, 0.05 M Tricine, pH 8, and forced through the needle valve three or more times. Since the amount of breakage by the needle valve was low with certain algae, we tried the Braun “MSK” mechanical cell homogenizer. Rapid shaking of a dense algal suspension in the same buffer as above with glass beads 0.25–0.30 mm in diameter for 2 minutes was sufficient to break practically all of the cells. We have not yet standardized this breaking method completely, but if conditions such as the ratio of cells to beads and the temperature during shaking are optimal, this homogenate, after spinning in the sucrose gradient, will yield the same fractions as the material broken by the needle valve. Cells of *Scenedesmus*, *Porphyridium*, and *Anacystis* have been successfully fractionated after disintegration by the MSK homogenizer.

Whether the bands in the sucrose gradient contain different kinds of chlorophyll particles has been determined by at least one of the following spectroscopic criteria: low-temperature absorption or fluorescence-emission spectra, and relative fluorescence yields of the chlorophyll.

Table 1 shows the relative fluorescence yields of chlorophyll a in fractions of chloroplasts and algae studied since those listed in Table 9, Year Book 67, p. 518. These yields were determined by the same method as before, except that the concentration of chlorophyll a alone has been used in the current calculations in order to compare algae that lack chlorophyll b.

<table>
<thead>
<tr>
<th>Plant Material</th>
<th>Fluorescence Yield</th>
<th>Fluorescence Peak</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fraction 1</td>
<td>Fraction 2</td>
</tr>
<tr>
<td>Spinach</td>
<td>2.2</td>
<td>7.1</td>
</tr>
<tr>
<td>Atriplex semibaccata</td>
<td>0.72</td>
<td>2.2</td>
</tr>
<tr>
<td>Chlamydomonas rheinhardii</td>
<td>2.9</td>
<td>5.9</td>
</tr>
<tr>
<td>Scenedesmus obliquus</td>
<td>5.0</td>
<td>9.2</td>
</tr>
<tr>
<td>Botrydiopsis alpina</td>
<td>1.4</td>
<td>3.5</td>
</tr>
<tr>
<td>Botrydiopsis trigonum</td>
<td>1.9</td>
<td>9.6</td>
</tr>
<tr>
<td>Anacystis nidulans</td>
<td>0.54</td>
<td></td>
</tr>
<tr>
<td>Anabaena cylindrica</td>
<td>0.91</td>
<td></td>
</tr>
<tr>
<td>Plectonema boryanum</td>
<td>0.24</td>
<td></td>
</tr>
</tbody>
</table>
Low-temperature absorption and fluorescence spectra in Fig. 1 of *Chlamydomonas* and *Scenedesmus* illustrate typical separations in which fraction 2 has proportionately more chlorophyll b, absorbing at 650 nm, and less long wavelength absorption than fraction 1. Fraction 1 has greater emission at longer wavelengths than fraction 2 relative to the peak near 680 nm. The small absorption band near 700 nm was first observed in *Scenedesmus* by Butler, 1960, and called C-705. Butler, 1966, suggested that C-705 may be the same form of chlorophyll as Ca 695 in *Euglena*, and that it is also the fluorescence-excitation band seen in all the green plants that were examined. However, we have not detected this band in spectra of other algae closely related to *Scenedesmus*.

![Diagram of absorption and fluorescence spectra of fractions 1 and 2 from Chlamydomonas, Scenedesmus and Porphyridium recorded at -196°C. Excitation at 435 nm.](image-url)
The fluorescence yields of fractions from *Porphyridium* were not measured, but differences in the absorption and emission spectra can be seen in Fig. 1 and do indicate that two kinds of chlorophyll fractions were obtained. Most of the phycoerythrin remained at the top of the sucrose after centrifugation.

Attempts to fractionate the three species of blue-green algae were originally made by initially breaking the cells in the needle valve. Since disruption was incomplete, only a relatively small amount of chlorophyll-containing particles were dispersed through the sucrose gradient. Spectroscopic tests of samples from various levels in the centrifuge tube revealed no differences. More recently, *Anacystis* was thoroughly broken by shaking with glass beads in the MSK homogenizer. Centrifugation of this homogenate in sucrose produced a layering of phycoerythrin at the top of the tube and two well-separated green bands below. However, these bands had very similar absorption and emission spectra.

Since we have so far failed to find two chlorophyll fractions from the blue-green algae, we must consider the possibility that the separation of phycoerythrin from the denser fraction 2 type of chlorophyll, with which it is thought to function, may have altered the whole particle. The particles of blue-green algae all had the relatively low fluorescence yields characteristic of fraction 1.

Fig. 2 shows low-temperature absorption and emission spectra of particles from three species of blue-green algae. The differences in the relative proportions of the biological forms of chlorophyll are striking. All the spectra were measured with submicroscopic particles of about the same density and chlorophyll concentration. A positive correlation is evident between the amount of absorption at 710 nm and the height of the long-wavelength (relative to the short-wavelength) fluorescence band.

The question of which chlorophyll absorption band is the source of the long-wavelength fluorescence band, enhanced in fraction 1 at low temperature, has often been asked. Different experimenters have reported widely different peak positions for the long-wavelength emission band in different kinds of plants. Since a part of this variation might have been due to errors inherent in different spectrofluorimeters and to the measurement
of samples with too much chlorophyll, we compared the fluorescence emission spectra, measured in the same way, of very dilute fraction-1 particles from a number of species. The peak positions, listed in Table 1, varied from 711 to 735 nm and showed no apparent correlation with a particular absorption band except for the case of the blue-green algae mentioned above. The source of this emission band still remains largely unexplained.

An investigation of the stability of the chlorophyll-lipoprotein binding that may determine the characteristic absorption spectra of the biological forms of chlorophyll was attempted. With spinach, heating the homogenate to 40°C for 10 minutes, or storing it at 4°C for 2 days, had no effect upon its subsequent ability to fractionate in the sucrose gradient or upon the absorption spectra of the fractions. This is in contrast to broken cells of some algae such as Tribonema and Botrydiopsis, in which a considerable transformation of the "Ca 680" chlorophyll peak to about 670 nm occurs within a day of storage at 4°C.

Both fractions 1 and 2 from spinach were incubated at 25°C with trypsin and in separate experiments with porcine lipase for several hours. No change appeared in the low-temperature chlorophyll absorption spectra of the treated particles even though the fraction-2 particles clumped after 15 minutes.

In contrast to this lack of an enzyme effect, Michel-Wolwertz (Year Book 67, p. 505) observed that a protease (from Streptomyces griseus) caused shifts in the proportions of chlorophyll forms in particles of Euglena and Chlorella. Wheat lipase also changed the absorption of Chlorella particles. Either these enzymes from different sources act differently, or the chlorophyll complexes in spinach are more resistant to their action. However, treatment with trypsin and porcine lipase, more than sufficient to inhibit DCIP reduction completely (see Mantai, this Year Book, p. 601), need not be reflected in any detectable change in chlorophyll absorption.

References

Absorption and Fluorescence of Chlorophyllide a in vivo
J. S. Brown

The accumulation of chlorophyllide a (chlorophyll a without phytol) in a Chlorella mutant "SCA" makes it possible to measure the absorption and fluorescence spectra of that pigment in vivo. Ellsworth and Aronoff, 1965, determined that chlorophyllide a is the major porphyrin in this mutant, but that it is easily converted in part to pheophorbide a (chlorophyllide minus Mg) by exposure of the cells to strong light or during extraction by organic solvents. Dr. Ellsworth kindly supplied us with a culture of the "SCA" mutant induced by ultraviolet irradiation.

The absorption peaks of ethyl chlorophyllide and of chlorophyll a in ether are both near 660 nm. The two major biological forms of chlorophyll a absorb between 670 and 683 nm. The absorption maximum of chlorophyllide a in Chlorella is here reported at 690 nm. This long wavelength peak position of chlorophyllide in vivo shows that the wavelength shift caused by the arrangement of chlorophyll molecules on a carrier is not dependent on the presence of the phytol tail.

The cells were grown on a glucose-agar medium in darkness for 6 days. Absorption and fluorescence spectra of both the intact cells and their homogenates were measured near the temperature of liquid N2 (Fig. 3). The homogenates were prepared by passing the cells, suspended in a 0.15 M KCl, 0.05 M Tricine buffer at
pH 8, through the needle valve several times and centrifuging at 3000 g for 10 minutes to remove the larger particles.

An approximate check on the pigment content of the homogenate was made by extracting these pigments with 80% acetone in water and measuring the absorption spectrum. Characteristic absorption maxima of pheophorbide $\alpha$ at 410 and 535 nm indicated that some of this porphyrin was indeed present in the homogenates.

The absorption spectrum shows the in vivo absorption maximum of chlorophyllide $\alpha$ at 690 nm. The shoulder between 670 and 680 nm that is more prominent in the homogenate than in the cells is probably due to pheophorbide $\alpha$ because this pigment is known to be formed from chlorophyllide by extrac-
tion. The pigment causing the small band near 710 nm is unknown. A form of pheophorbide a absorbing at 710 nm has been observed in aged Euglena (Year Book 61, p. 352) and in Ginkgo leaves by Kunieda and Takamiya, 1965. However, the main peak in damaged Ochromonas (Brown, 1968) and in acid-treated chloroplasts was found at 671 nm. Whether Ochromonas contained pheophorbide or pheophytin was not determined, but the two porphyrins have very similar spectral characteristics.

The peak positions in the emission spectra are difficult to explain unless we assume that chlorophyllide a in vivo does not fluoresce, and that the emission band near 680 nm is from the pheophorbide. This is reasonable since the pheophorbide in Ochromonas fluoresced at 683 nm. The emission peak at 713 nm is from a second, unknown pigment form that is destroyed by breaking the cells. The diatom Phaeodactylum also has a similarly labile emission band at 714 nm (Year Book 65, p. 486).

References

Photosystem 1 and 2 Particles from Leaves of Diverse Ages

Z. Šestáčk

During the development of a leaf from unfolding to abscission its photosynthetic rate displays characteristic changes. The rate increases to the phase of photosynthetic maturity followed by a steady decline that may go below the compensation point. Although changes of chlorophyll content have a similar character, the slower decline of chlorophyll is reflected in a gradual lowering of assimilation numbers with the aging of leaves. This was already observed in 1918 by Willstätter and Stoll (for review see Šestáčk and Cateš, 1967). One of the reasons for these ontogenetic changes in assimilation numbers may be an interconversion of the forms of chlorophyll in vivo and/or a changed ratio of photosystems 1 and 2.

To test this possibility the fractionation method of Michel and Michel-Wolwertz (Year Book 67, pp. 508–514) was employed to separate chloroplast fractions enriched in photosystem 1 or 2. In experiments with young, middle-aged, and old spinach and radish leaves purchased at the local market, the method was used with only one minor modification: besides linear gradients, step gradients (5 ml, 12.5% sucrose solution; 20 ml, 30%; 5 ml, 50%) were used. Centrifugation times of 30 or 35 minutes were chosen for radish and 40 or 45 minutes for spinach. The standard procedure was found unsuitable for glass-house plants of Mimulus cardinalis whose chloroplasts and photosystems were probably too heavily damaged by the procedure.

With linear gradients the method affords a reasonable separation of three bands. In step gradients band 2 often appears only as an elongated tail of band 1 or in front of band 3, while band 3 is usually located at the boundary of the last two sucrose concentrations. This results in irregularities in the flow-cuvette evaluation of the results (see bands of photosystem 2 in Fig. 4).

The disadvantage of the method is that in various steps of the chloroplast isolation procedure a great deal of chlorophyll-containing material is rejected. Consequently the chloroplasts thus fractionated may represent only a specific fraction of the total plant tissue initially used, i.e., the mature chloroplasts, chloroplasts with resistant membranes, small chloroplasts, etc.

Absorption spectra of particles in bands 1 and 3 (containing particles enriched in photosystems 1 and 2, respectively) measured both at room tempera-
The distribution of chloroplast particles from young and old radish leaves fractionated by step-gradient centrifugation in sucrose. The absorbance at 678 nm is plotted against the quantity of sample withdrawn, as measured from the top of the tube.

Fig. 4. The distribution of chloroplast particles from young and old radish leaves fractionated by step-gradient centrifugation in sucrose. The absorbance at 678 nm is plotted against the quantity of sample withdrawn, as measured from the top of the tube.

TABLE 2. An Accuracy Test for the Estimation of Relative Chlorophyll Contents of Different Fractions of the Homogenate of an Old Radish Leaf*

<table>
<thead>
<tr>
<th>Amount of Sample, ml</th>
<th>Entire Sample</th>
<th>Band 1</th>
<th>Bands 2 and 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>150</td>
<td>150</td>
<td>141</td>
<td>159</td>
</tr>
<tr>
<td>200</td>
<td>207</td>
<td>211</td>
<td>210</td>
</tr>
</tbody>
</table>

*The absorbance of a sucrose step-gradient was measured in a flow-through cuvette at 678 nm. The area under the curves were computed giving that for the lowest concentration a value of 100.
systems after isolation from young leaves.

Although the results have to be confirmed on a large number of plant species by analyses either of individual leaves on a plant or of one leaf during its whole life cycle, they suggest that with the maturation and aging of leaves the amount of photosystem-1 particles in isolated chloroplasts declines in relation to photosystem-2 particles. At the same time their photoactivity relative to amount of chlorophyll falls. Because particles of the (middle) band 2 were found by Michel and Michel-Wolwertz to display photosystem-2 activity (their absorption spectra are also similar to those of photosystem-2 particles), it seems that there is a surplus of photosystem-2 particles in chloroplasts, and, therefore, the amount of photosystem-1 particles and the amount of chlorophyll in them may limit the photosynthetic rate.

Reference

An Action Spectrum for Methyl Viologen Reduction by Fractionated Spinach Chloroplasts
Eckhard Loos

In the last few years chloroplast fragments have become available which are enriched in one or the other of the two photosystems (systems 1 and 2) operating in photosynthesis of algae and higher plants. To obtain better insight into these two systems, especially with regard to their pigment composition, one should have them separated as cleanly as possible. An important criterion for the completeness of a fractionation is the degree of coincidence of the absorption spectrum of a fraction with the action spectrum for a reaction specific for system 1 or 2.

Several absorption spectra of sub-chloroplast particles containing predominantly photosystem-1 or photosystem-2 pigments have been published (Boardman and Anderson, 1964; Anderson and Boardman, 1966; Ogawa et al., 1966; Vernon et al., 1966; Briantais, 1967; Michel and Michel-Wolwertz, 1969; Bril et al., 1969). There are also a number of action spectra for system-1 and system-2 activity (Müller et al., 1963; Kelly and Sauer, 1965; Vidaver, 1966; Joliot et al., 1968; Ludlow and Park, 1969). However, they were measured with whole chloroplasts or algal cells, which are known to have flattened absorption and action spectra. In the present study an attempt was made to obtain an action spectrum with fractionated chloroplasts. It was hoped to gain in this way more exact knowledge of the pigments sensitizing a partial reaction of photosynthesis and to be able to estimate the degree of separation of the two photosystems. Because system-2 enriched particles are quite labile and tricky to experiment with, the attempt was made first with system-1 particles. Methyl viologen reduction was chosen as a system-1 reaction (Arnon, 1963; Kok et al., 1965) using an artificial electron donor and DCMU to block any activity from system 2.

Material and Methods

About 180 g spinach leaves were homogenized for 10 seconds in the Waring blender with 65 ml of buffer “A” (Jensen and Bassham, 1966) from which NaNO₃ and Na-isoascorbate were omitted. The resulting brei was filtered through 8 layers of cheesecloth and the filtrate centrifuged for 2.5 minutes at 3000 x g. The pellet was resuspended in 5-10 ml 0.05 M Tricine (pH 7.9), 0.15 M KCl and forced twice through a needle valve. Unbroken chloroplasts were then removed by centrifuging for 5 minutes at 3000 x g. From the homogenate so obtained, 2 ml (equivalent to 500-1500 μg chlorophyll) were layered on 30 ml of a linear sucrose gradient (10-50%) con-
taining 0.15 M KCl and 0.05 M Tricine pH 7.9. After 45 minutes' centrifugation at 65,000 × g, system-1 enriched particles were withdrawn from the green top band in the centrifuge tube and, henceforth in this report, are called fraction 1.

Unless otherwise indicated, the reaction mixture used contained in moles/liter: Tricine, 0.05 (pH 7.8-8.0); KCl, 0.15; NH₄Cl, 10⁻³; cysteine, 0.04; DCPIP, 2 × 10⁻⁴; DCMU, 10⁻⁴; methyl viologen 2 × 10⁻⁴. The chlorophyll concentration was 2.5 µg ml⁻¹. To make this mixture anaerobic, 1-ml plexiglas cuvettes were filled, bubbled for 1 minute with N₂, and closed with a screw cover.

Methyl viologen reduction was followed spectrophotometrically by recording the increase of absorption at 386 nm, a secondary peak of the reduced form of methyl viologen. The measuring light was isolated with an interference filter (10-nm half bandwidth) and after passage through the sample detected by an RCA photomultiplier (type IP 22), which was protected from stray actinic light by two Corning filters, No. 9782 and No. 5543. The measuring beam and the actinic light were at right angles to each other, the path-lengths being 7.9 and 4.8 mm respectively. In action spectra measurements the cuvette was backed with an aluminum foil for a more even illumination of the sample. Actinic light was obtained from a 2000-w high pressure mercury lamp used in conjunction with a monochromator, and was filtered through 4 cm water, a Balzers Calflex heat-reflecting filter and for the wavelengths 600–660 nm, through Corning filter No. 2404, for 645–680 nm, through Corning filter No. 2408 and, for 680 nm and longer, through Schott filter RG5. In experiments not dealing with action spectra, actinic light was provided by a ribbon filament lamp and a 680-nm interference filter (10-nm half bandwidth) plus a Calflex filter. Light intensity was monitored continuously by deflecting part of the beam onto a calibrated silicon cell whose output was amplified and integrated over the time interval of the exposure. Absorption spectra were measured with a spectrophotometer specially suited for light scattering samples (French and Lawrence, 1968).

Results

A. Methyl viologen reduction using cysteine-DCPIP as electron donor

The cysteine-DCPIP couple was used as electron donor because with ascorbate-DCPIP no activity was detected, confirming the results of Arnon (1963).

Kinetics. Two types of kinetics were encountered. The first one, illustrated in Fig. 5A, is characterized by a rapid increase of absorption upon the onset of illumination, after which the rate tapers off to a constant value in the course of several seconds; shutting off the light causes a sudden decrease of absorption followed by a more or less sloping back rate.

The second kind lacks the transients (Fig. 5B). The reason for the two kinetic

![Fig. 5. Kinetics of methyl viologen reduction.](image-url)
types is not yet clear. It may be a seasonal variability in the spinach leaves, for the kinetics with the transients were observed from December through March and could not be reproduced in the summer.

The rate of the back reaction increased during the course of an experiment after many alternating light and dark periods, apparently concurrently with the accumulation of reduced dye (Fig. 6). The steady-state rate of methyl viologen reduction in the following experiments was corrected for the back reaction, using the average of the steady rates before and after a light exposure.

**Optimizing the reaction conditions.**
The concentration of some components of the reaction mixture was varied to find optimum conditions, to be able to use the relatively weak light intensities available with a narrow spectral bandwidth of the actinic light.

A schedule of 5.5 minutes dark alternating with 5.7 minutes light was employed. The light intensity was about 3000 ergs cm\(^{-2}\) sec\(^{-1}\), and the wavelength, 680 nm. Each point represents the average of at least two measurements.

**The influence of DCPIP concentration.**
Table 3 shows the dependence of the rate of methyl viologen reduction on the DCPIP-concentration. The optimum is around 3 \(\times 10^{-5}\) M DCPIP, the decline in activity being sharper towards the higher concentrations than towards the lower ones. For all further experiments a DCPIP concentration of 2 \(\times 10^{-5}\) M was chosen.

**The influence of cysteine concentration.**
The concentration of cysteine, which keeps the DCPIP reduced, may affect considerably the rate of methyl viologen reduction, as is illustrated in Table 4. The optimum rates were observed only in the relatively small concentration range between 0.04 and 0.08 M.

**Fig. 6.** Accumulation of reduced methyl viologen (line through crosses) and rates of reoxidation during the dark periods (points). Periods of 7-8 minutes light alternated with 4-5 minutes dark. Nonsaturating light intensities; chlorophyll concentration 1.3 g ml\(^{-1}\). Data are taken from an action spectrum experiment.

<table>
<thead>
<tr>
<th>Concentration of DCPIP, M</th>
<th>Rate of Methyl Viologen Reduction, Rel.</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 (\times 10^{-4})</td>
<td>10.5</td>
</tr>
<tr>
<td>1 (\times 10^{-4})</td>
<td>48</td>
</tr>
<tr>
<td>3 (\times 10^{-5})</td>
<td>125</td>
</tr>
<tr>
<td>1 (\times 10^{-5})</td>
<td>100</td>
</tr>
<tr>
<td>3 (\times 10^{-6})</td>
<td>58.5</td>
</tr>
<tr>
<td>1 (\times 10^{-6})</td>
<td>25</td>
</tr>
</tbody>
</table>

*The cysteine concentration was 5 \(\times 10^{-8}\) M.

<table>
<thead>
<tr>
<th>Concentration of Cysteine, M</th>
<th>Rate of Methyl Viologen Reduction, Rel.</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 (\times 10^{-8})</td>
<td>3.7</td>
</tr>
<tr>
<td>8 (\times 10^{-8})</td>
<td>12.7</td>
</tr>
<tr>
<td>1.6 (\times 10^{-7})</td>
<td>17.2</td>
</tr>
<tr>
<td>4 (\times 10^{-7})</td>
<td>25.6</td>
</tr>
<tr>
<td>8 (\times 10^{-7})</td>
<td>25.0</td>
</tr>
</tbody>
</table>
TABLE 5. Rate of Methyl Viologen Reduction at Different Methyl Viologen Concentrations

<table>
<thead>
<tr>
<th>Concentration of Methyl Viologen, M</th>
<th>Rate of Methyl Viologen Reduction, Rel.</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-4}$</td>
<td>11.6</td>
</tr>
<tr>
<td>$5 \times 10^{-3}$</td>
<td>11.9</td>
</tr>
<tr>
<td>$2 \times 10^{-4}$</td>
<td>13.4</td>
</tr>
<tr>
<td>$5 \times 10^{-4}$</td>
<td>11.1</td>
</tr>
<tr>
<td>$1 \times 10^{-3}$</td>
<td>12.9</td>
</tr>
</tbody>
</table>

M. With the highest concentration tested (0.08 M) in some cases the rate declined with time. The reason for the relatively low rates at weaker cysteine concentrations may be a too slow re-reduction of DCPIP, which becomes oxidized in the light by the chloroplast fragments.

The influence of methyl viologen concentration. One experiment was carried out varying the concentration of methyl viologen; it did not seem to be critical under the chosen conditions (Table 5).

Dependence of the rate of methyl viologen reduction on light intensity. A four minutes light to four minutes dark schedule was used for these experiments. In general the light intensity curves were S-shaped (Fig. 7) and only in a few cases was a linear relationship found. The slopes of the curves attained their greatest steepness at rates which amounted to 10% or less of the light-saturated value. The reason for the non-linearity in the lower intensity range is not yet known. One explanation is a limited cyclic electron transport, preferentially driven at low light intensities.

B. Action spectrum for methyl viologen reduction

To minimize errors due to the non-linear intensity-versus-rate curves, two measurements were taken:

1. The light intensities at a certain wavelength and at a reference wavelength were so adjusted as to yield approximately equal rates.

2. The measurements were made in the linear portion of the light intensity curve. In order to compare also somewhat differing rates obtained with different wavelengths, all rates were corrected by adding the value of the intercept, which is produced on the rate coordinate at zero intensity by an extension of the linear part of the light curve. This correction usually amounted to 30-80% of the measured rates. Points for light intensity curves were measured at the beginning and in the middle or at the end of an experiment.

Each wavelength was given for 3-4 minutes and immediately followed or preceded by an exposure to a reference wavelength, which was 680 nm in the range 645-700 nm and 625 nm for the region between 600 and 660 nm. The dark periods lasted for 3-5 minutes.

Fig. 8 shows the points of an action spectrum obtained in six experiments involving five preparations of fraction 1. For measurements between 645 and 700 nm (points in Fig. 8) a half bandwidth of 1.5 nm was used; the chlorophyll concentration was about 1.3 µg ml⁻¹ corresponding to an optical density of about 0.05 at 680 nm. Two experiments for points between 600 and 660 nm (crosses in Fig. 8), however, were car-
ried out with 2.5 and 1.5 nm half-bandwidth and chlorophyll concentrations of approximately 6 and 2.5 µg ml⁻¹. These points were obtained using 625 nm as a reference wavelength. They were joined to the other points with 680 Dill as reference by two wavelengths (645 and 650 nm) in the overlapping region, and the average values were calculated from sets of points. The factor was determined by which the average of the 645-nm points from the one set (625 nm as reference) differed from the corresponding 645-nm average of the other set (680 nm as reference). Similarly such a factor was obtained for the 650-nm values. The average of those two factors was finally used to multiply all points of the one set (625 nm as reference) and so connect to the other set.

As can be seen in Fig. 8 the absorption spectrum (solid line) fits closely the measurements for the relative action. The divergence in the part between 670 and 680 nm is considered to be insignificant, for it was not evident in two other experiments which showed more scatter.

Discussion

The data in Fig. 8 suggest that the absorption spectrum of fraction 1 represents also the spectrum of the pigments active in light reaction 1. However, the action spectrum measurements are not accurate enough to establish firmly slight disagreements with the absorption spectrum; for instance no evaluation can be made of the degree of activity of chlorophyll b. For a more precise action spectrum, therefore, another way to measure system 1 activity must be sought.

The action spectrum from fractionated chloroplasts is less flattened than the action spectrum for methyl viologen reduction in whole spinach chloroplasts obtained by Joliot et al. (1968) (the broken line and circles in Fig. 8). This underlines the necessity to use as finely dispersed chloroplast material as possible for further action spectra.

References


The previously reported attempts to resolve the complicated absorption spectra of chlorophyll complexes into spe-
specific components representing the different natural forms of chlorophyll have been continued. This year's work represents a level of approximation that has served more to clarify the limits of usefulness of the curve analysis procedure than to define the spectra of specific chlorophyll components. Rather than reporting in detail on the extensive but still unsatisfactory curve analyses carried out this year, we will present, on identical scales, some of the spectra of different investigators that illustrate the complex nature and the range of variation within the red region of the spectrum which is caused by the presence of different forms of chlorophyll a.

The spectra of normally green cells or of whole chloroplasts cannot truly represent the sums of the spectra of the component forms because of the well-known flattening effect due to the high optical density of the particles themselves. Therefore, our recent curve analyses have been mainly restricted to spectra of small particles of broken or fractionated chloroplasts.

Methods for separating chloroplasts into two fractions corresponding roughly to the pigments of photosystem 1 and photosystem 2 are being intensively investigated in many laboratories. Previously we found the longer-wavelength form of the two major chlorophyll a components, Ca 680, to have a much narrower bandwidth in the system-2 fractions than in the system-1 fractions. Dr. Brown's recent measurements with fractions from numerous species of plants have amply confirmed the generality of this situation. Since the absorption bands near 680 nm in the spectra of the two fractions differ in half-width, it is obvious that spectra of whole chloroplasts, or of their unfractionated homogenates, are more complex than was expected from the old assumption that they were both made up of a small number of forms of chlorophyll a similar in shape and differed only in their proportions in the two fractions.

The spectra of separated chloroplast fractions, therefore, appear to offer the greatest promise for resolution into the spectra of the individual components, and most of the recent work has been on such material. Because the spectra are sharper at liquid nitrogen temperature, most of the curve analyses have been done with low-temperature data. The extreme sharpness of the Ca 680 peak in fraction 2 is consistent with the previously discussed idea that its shape may be greatly influenced by refractive index changes of the pigment near its absorption band. Devising methods for the routine measurement of such wavelength dependent scattering in a way that can lead to a calculation of the true absorbance is of great importance. Various possible methods have been considered although we have not yet attempted to make experimental tests of possible procedures. The main difficulty is to devise a measurement system that would be usable at liquid N₂ temperature as well as for suspensions at room temperature.

Theories. A basic question is whether the spectra for different chloroplast preparations are made up of identical chlorophyll components in different proportions or whether the wavelength maxima and the widths of the components themselves are different in the various preparations of corresponding chloroplast fractions. We hope to answer this question by curve analysis.

To account for the variations in observed spectra and yet to maintain the simplest realistic concept of the minimum number of chlorophyll forms that must exist, we wish to distinguish between the three alternative hypotheses illustrated in Table 6. According to the constant components concept, the basic major bands are assumed to correspond to actual forms of chlorophyll that always have the same peak position and width. Since variation in the proportions of these components can give peaks or shoulders at many different wavelength positions, the observed variation in
### TABLE 6. Three Alternative Concepts to Account for the Observed Band Positions of Chlorophyll a Types

<table>
<thead>
<tr>
<th>Concept</th>
<th>Constant Components</th>
<th>Extra Components</th>
<th>Variable Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>The components have peaks of constant wavelength and width while the observed variety of spectra is due to differences in the proportions of these forms.</td>
<td>The major components have peaks of constant position and width, extra bands may or may not be present.</td>
<td>The major components have peak positions and/or widths that vary from one sample to another within a specific range for each type.</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name of component</th>
<th>Range of possible peak position due to mixtures of these components</th>
<th>Universal forms</th>
<th>Extra forms</th>
<th>Range of variation for each type of component</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca 665</td>
<td>665-670</td>
<td>665</td>
<td>662-667</td>
<td></td>
</tr>
<tr>
<td>Ca 670</td>
<td>670-680</td>
<td>670 *</td>
<td>668-673 *</td>
<td></td>
</tr>
<tr>
<td>Ca 680</td>
<td>680-695</td>
<td>685</td>
<td>678-683 *</td>
<td></td>
</tr>
<tr>
<td>Ca 695</td>
<td>695-700</td>
<td>695</td>
<td>690-696</td>
<td></td>
</tr>
<tr>
<td>Ca 700</td>
<td>700-705</td>
<td>700</td>
<td>697-702</td>
<td></td>
</tr>
<tr>
<td>Ca 705</td>
<td>705-710</td>
<td>705</td>
<td>703-708</td>
<td></td>
</tr>
<tr>
<td>Ca 710</td>
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</tr>
<tr>
<td>Ca 715</td>
<td></td>
<td>715</td>
<td>714-718</td>
<td></td>
</tr>
</tbody>
</table>

1. The presence of other components can modify the peak position for the sum of any pair of components.
2. Predominant in system 2.
3. Predominant in system 1.

The concept of extra components, by contrast with that of constant components, posits that there are two major and universal forms in each system with constant peaks at about 670 and 680 nm. In addition, however, there may also be any of a number of other invariant forms also present in any particular sample. The probable peak positions of some of these less common extra forms are given in Table 6. The two ideas should be distinguishable by comparison of the results of many curve analyses. For the one, identical components should fit all spectra, while the other requires extra com-
ponents which also would always be at specific wavelengths.

A third hypothesis, that of variable components, unlike the other two, presumes that the chlorophyll a forms of any one type are not constant but may vary in their peak positions over a range of about ±3 nm and possibly also in their widths. If this is actually true, then an almost unlimited number of components would result from an extensive series of curve analyses.

**Curve analysis with Gaussian components.** During the past year we have attempted to resolve various absorption spectra of chloroplast fractions most of which were prepared by Dr. Brown from a variety of algae and leaves. We have also analyzed spectra of some purified chlorophyll-protein complexes prepared by Dr. J. Philip Thornber of the Brookhaven National Laboratory. One procedure used in analyzing these spectra has been to match the experimental curves by adding together simpler curves, usually Gaussian probability functions. The reason for doing so is that the Gaussian curves thus obtained may represent the individual bands of the different chlorophyll forms and, therefore, serve as a means for specifying their wavelength peaks and their widths at half-height. Neither of these parameters of a single band is directly identifiable in a composite absorption spectrum. There is no reason to expect Gaussian curves necessarily to fit even a single isolated band over its entire extent. Nevertheless, that shape has been found to be adequate for use with chlorophyll spectra except at the long wavelength tails. Lorentzian (Cauchy) curves are far less useful.

Each of the major forms of chlorophyll a that we wish to identify has a main peak in the 660–700 nm region, a wider and lower band near 620–640 nm, and a still wider and lower band with a maximum somewhere near 580 nm. So far we have not been able to distinguish between the bands of the different forms of chlorophyll a in the 570–640 nm region. The long wavelength tails of 620–640 nm bands do overlap the main peaks and hence have some small effect on the apparent shape of the main peak. This overlap of unidentifiable low and broad bands has been a source of some uncertainty in determining the height and, to a smaller extent, the half-width of the major bands.

These curve analyses made with the RESOL program have resulted in a number of adequate matches of Gaussian curves to the experimental spectra. The interpretation of the results by attributing particular Gaussian curves to the absorption bands of specific chlorophyll complexes has not yet been very successful. There are two difficulties in the use of this method. One difficulty can probably be greatly reduced by program modifications that are being explored. That trouble is the sometimes extreme modification by the program of the wavelength peaks and half-widths of the estimated input bands with which the computation starts. When this happens the resulting fit may be excellent even though the bands so determined cannot possibly be considered as representing the absorption bands of chlorophyll complexes.

Dr. Tunnicliff has recently shown us how to restrict the amount of wavelength adjustment allowed for each iteration. Mr. Lawrence is working on a modification of the program using this principle. The plan is to make a restriction for both the peak wavelength change and the width change per iteration independently specifiable for each input band. If these modifications can be made to operate successfully, the program will be much more useful for complex spectra. For instance, the whole curve for a known or suspected chlorophyll component describable as the sum of several Gaussian or Lorentzian functions can be entered and either rigidly held or allowed minor adjustments while the program determines the remaining components of the system. In this way...
the difficulties that have caused so much trouble in curve analysis this year may be avoided. With these modifications it should be possible to use enough input bands to allow for the main peaks of the minor components as well as for the side bands of the major chlorophyll forms. This has not yet been possible with the present program lacking the restraints on band adjustments.

Another and more serious difficulty is that many of the spectra do not have sharp enough characteristics to require only a single combination of Gaussian curves for a precise fit. With such spectra we have to use some other method for determining the band shapes. Characterless curves are, however, valuable for testing the reality of component spectra that have been determined from other data.

One partially successful attempt to derive the separate chlorophyll spectra in a two-component mixture is illustrated in Fig. 9. This is the room-temperature spectrum of Dr. Thornber's purified system-2 pigments from spinach. This spectrum is of particular interest because it contains the highest proportion of chlorophyll b of any fraction we have seen. In this case the RESOL program gave a very rough resolution of the 570-640 nm region but reasonable bands for the main 652 chlorophyll b and 671 chlorophyll a peaks. The larger of the minor component bands at 632 nm, however, is probably too wide, too high, and at too long a wavelength. Combining the 652-nm and 604-nm components with a portion of the 575 band to represent the spectrum of chlorophyll b in vivo gives a completely objective, although inadequate, solution to this curve resolution problem. The derived curves are given in the upper part of Fig. 9. The analogous addition of the 671-nm and 632-nm bands with part of that at 575 nm to represent the spectrum of Ca 670 of system 2 is also not satisfactory. However, this figure illustrates a method.

Fig. 8. Wavelength dependence of relative action for methyl viologen reduction by fraction 1 (points and crosses). Solid line: absorption spectrum of fraction 1. Broken line and circles: action spectrum for methyl viologen reduction in whole chloroplasts; adapted from Joliot et al. (1968).
Fig. 9. The absorption at room temperature of Dr. Thornber's purified system-2 chlorophyll protein matched with Gaussian curves. In the upper part the components have been added to approximate very roughly the spectra of the chlorophyll b and chlorophyll a components.

that may be valuable in the future after the curve analysis program is improved.

Comparison of spectra by subtraction.

One procedure occasionally useful for related pairs of characterless spectra is nearly the same except for the relative amounts of a common component. If that requirement is more or less true, then the difference spectra give a reasonable approximation to the band shape of

Page 583, following line 5 (first column): Insert to subtract one spectrum from another following multiplication by a series of scale factors. The pair of curves so used must be
one component. This procedure, described last year, has been applied to a number of curve pairs and has frequently shown that the spectra selected differ by several unexpected bands. One of the more significant of these calculations by the DSPEC program came from two spectra of a fraction from homogenized Botrydiopsis that were prepared on successive days. That from the fresh homogenate had a large Ca 680 band while that from the same homogenate stored overnight at 4°C had very little of that component. Even this pair of spectra showed an unexpected difference near 665 nm as well as the major difference peak at 680 nm (French et al., 1968).

This comparative procedure for deriving the spectra of component bands eventually should have more utility if enough appropriate pairs of spectra can be found. The method has the advantage over the approximation by Gaussian components in that it gives the entire spectrum of a component rather than only the width and position of its main band. So far, however, these difference spectra have been more useful in emphasizing the presence of unsuspected components than in finding the precise shape of the components that are obviously present.

To compare curves with each other we have found it well worth the extra expense to have all spectra plotted on the same scale with a peak height of 7.5 inches and a wavelength scale of 1 nm = ¼ inch. Such a graph made by the SPLOT program also serves to make any errors of curve digitizing strikingly apparent. An even greater convenience is the file of 4×5 positive film photographs of the standard-scale plots that are accurately aligned with each other. Many of these can be superimposed to compare all spectra of a particular type. For visual study they are held in a plastic frame with slots. When arranged in order of the increasing height of a particular band such a set of spectra gives a three-dimensional view of the interrelations of several bands.

To search for the less obvious components the spectra measured at −196°C are being compared within the following groups of preparations:

(a) Particles prepared by sucrose-gradient centrifugation of algae lacking chlorophyll b, and purified chlorophyll-protein preparations free of chlorophyll b (13 spectra).

(b) Fraction-1 particles from various sources (6 spectra).

(c) Comparable fraction-2 particles (6 spectra).

(d) Miscellaneous: unfractionated homogenates, (following centrifugation at 3000 g for 10 minutes to remove large particles), and spectra of whole cells that are pale enough to give comparatively undistorted spectra (9 spectra).

Some very revealing information about the complexity of spectra appears when two apparently similar spectra are superimposed, as shown in Fig. 10. In that figure are absorption spectra kindly supplied by Dr. Thornber for two of his purified samples of chlorophyll protein from the blue-green algae Tolypothrix and Phormidium (Thornber, 1969). Small bulges, caused by minor components, are more clearly seen by comparison with a curve that is similar but lacks these smaller bands. Figure 10 clearly shows that the Phormidium preparation absorbs relatively more at 633, 665, 688, and 708 while the Tolypothrix material has proportionately more absorption near 678 and 695 nm. Both have a major component at about 672 nm with a secondary band at about 620–635 nm. It also seems that the Phormidium may have the peak of its second largest component at 680 rather than at 678 nm, its apparent position in the Tolypothrix preparation. It is possible, however, that the apparent difference may be only in the relative proportions of identical components. If, in fact, each of the two spectra does consist of a mixture of 665, 672, 678–680, 688, 695, and
Fig. 10. Some selected spectra at -196°C showing various forms of chlorophyll. Comparisons of similar spectra suggest the presence of small amounts of extra chlorophyll forms in addition to those common forms giving the major peaks. The data was kindly provided by the following workers: M.-R. Michel-Wolwertz, C6, C18, C19, C27, C28; J. S. Brown, C34, C35, C65, C66; P. Thornber, C38, C39; L. Prager, C21, C22, C29, C30; D. C. Fork, U. Heber and M.-R. Michel-Wolwertz, C36, C37.

708 components, each with a side band in the 620-640 region, it is not surprising that the RESOL resolution of the *Tolytpothrix* spectrum into 6 Gaussian components gave the oversimplified picture shown in Fig. 11 with the indicated errors of this match.

For the *Phormidium* spectrum, C38, three widely different curve analyses were obtained, all with about the same error of fit. None of them gave interpretable components. A visual comparison of selected spectra, as in Fig. 10, gives a preference to the interpretation that differences between comparable fractions result from different propor-
Fig. 11. A partial resolution of Dr. Thornber's spectrum at -196°C for purified chlorophyll protein from *Tolypothrix*. It is not yet possible to resolve the 600-640 nm region into its components.

Considerations of similar components, provided enough separate components are considered for each curve. Smaller amounts of extra forms, however, may also exist in some of these algae.

Considered from the point of view of the constant component theory the spectra for the sharpest and for the smoothest spectra of fractionated chloroplasts of Fig. 10 show that if the *Euglena* fractions 1 and 2 each contain a Ca 680 component as sharp as those in *Stichococcus*, then there must be at least two components in the 665-675 region of *Euglena* that are low or absent in *Stichococcus*. The 695-nm and 705-nm components of *Euglena* show clearly in this comparison.

*Euglena* must have a higher proportion of components at about 665, 670, and 675 nm than does *Stichococcus*. Furthermore, *Stichococcus* has the highest chlorophyll b peak and the sharpest Ca 680 component of any alga so far investigated. These two pairs of spectra for fractions 1 and 2 of *Stichococcus* and *Euglena* are the most difficult ones to explain by the theory of constant components. A better comparison is of the same *Stichococcus* data with that for spinach fractions. Here, apparently, an increase in the amount of a 673-nm component added to the *Stichococcus* curve for either fraction could bring them up to the spinach curves in that region. For fraction 2 in addition to the other obvious differences, a component near 685 nm appears to be larger in spinach than in *Stichococcus*.

In brief, the results of the past year's work have emphasized the presence in most preparations of more forms of chlorophyll than are evident unless some sort of curve analysis is carried out.

Continuing improvements in the methods of curve analysis applied to a
large collection of precisely measured spectra from a wide variety of preparations from many diverse plant species may eventually decide between the three concepts here described. The costs of computer use for this work since March 15, 1969, have been covered by NSF Grant No. GB 8630, which has made it possible to increase greatly the number of spectra investigated. It is a pleasure to thank Mr. Mark Lawrence for programming help, Mrs. Helen Kennedy for digitizing the curves, and various colleagues for their contributions of selected spectra.

References

A COMPARATIVE STUDY OF THE LIGHT-INDUCED CAROTENOID CHANGE AND FLUORESCENCE IN THE CHLOROPHYLL-b-LESS ALGA Botrydiopsis alpina (XANTHOPHYCEAE)

David C. Fork and Yaroslav de Kouchkovsky

Introduction
A number of absorbance changes having similar kinetics were seen upon illumination of the yellow-green alga Botrydiopsis alpina that were apparently produced by a transient shift to longer wavelengths of the absorption bands of a carotenoid pigment (Year Book 66, p. 160; Year Book 67, p. 496). Action spectra measurements reported last year (Year Book 67, p. 496) demonstrated that both photochemical systems caused the shift in this particular, as yet unidentified, carotenoid. Inhibition of system 2 by DCMU permitted the observation that system 1 mediated a rapid shift of the carotenoid absorption to longer wavelengths which reversed again during illumination. The subsequent addition of the electron donor couple DAD and ascorbate produced a sustained carotenoid shift in the light, again sensitized by system 1. System 2 was also shown to be responsible for a sustained carotenoid shift that was relatively slow at the light intensities used.

We studied here the dependence of the carotenoid shift and of chlorophyll fluorescence upon treatments that would influence the primary photoreactions. A number of treatments were investigated in an attempt to obtain separately the carotenoid shift produced by each of the two photosystems. We have found both the initial rise and decay of the carotenoid shift follow first-order kinetics and an indication for another, slower first-order component.

It may be that the carotenoid pigment showing these shifts, like chlorophyll b, undergoes slight absorption changes when its supporting membrane is disturbed by electron transport. The carotenoid shift is discussed in relation to the chlorophyll-b change, electron transport and a high-energy “intermediate” produced during photophosphorylation.

Materials and Methods
Botrydiopsis alpina was cultured as previously described (Fork, 1969). Light-induced differences of absorbance at 515 nm were used to follow the carotenoid changes. These measurements were done as described earlier (de Kouchkovsky and Fork, 1964). The half-bandwidth of the measuring beam was 2 nm. The algae were diluted with culture medium so that the final transmission of the cell suspension was around 10% at 515 nm. The temperature was 20°C and the gas phase was air. Sometimes the results were also compared, using
wavelengths other than 515 nm (such as 482 and 497 nm) where absorbance changes typical of the carotenoid can be seen.

Fluorescence was measured simultaneously with absorbance changes. Actinic light to excite fluorescence and the carotenoid shift was incident on the top surface of the cell suspension, which was contained in an open cuvette. The photomultiplier, for monitoring fluorescence, was located above and to one side of this cuvette. The actinic light used in all these experiments had a wavelength of 652 nm and a half-band of about 9 nm. The light produced by using Schott RG 1, 3 mm in combination with a Balzers heat-reflecting filter Calflex C, a water filter (27 mm), and a Baird-Atomic interference filter type B-1. The intensity of this beam was about 2.9 nanoeinstein cm\(^{-2}\) sec\(^{-1}\) (5.3 \times 10^3 ergs cm\(^{-2}\) sec\(^{-1}\)). A combination of Schott RG 10 (3 nm) and Baird-Atomic interference filters (742 nm, type B-1, half-band, about 9 nm) transmitted fluorescent light of this wavelength but absorbed 652 nm actinic light. Fluorescence at 742 nm is very likely a "satellite" of the main emission band at 685 nm, and is, therefore, a reflection of the functioning of system 2 (compare the composite fluorescence band in vitro which is nearly an image of the absorption band and the experiments made in vivo that were reported by Lavorel, 1962).

A schedule of 6 seconds light and 48 seconds dark was used.

Results

The top trace of Fig. 12 shows kinetics typical for the absorbance change produced at 515 nm upon illumination of Botrydiopsis alpina (Year Book 67, p. 496). The initial increase of absorbance

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**Fig. 12.** Kinetics of light-induced absorbance changes at 515 nm produced upon illumination of Botrydiopsis alpina with red actinic light (described in the text) and the effect of DCMU, H\(_2\)O\(_2\), NH\(_2\)OH, Na\(_2\)S\(_2\)O\(_4\), and menadione. The concentrations (M) used were: DCMU, 5 \times 10^{-6} M; H\(_2\)O\(_2\), 5 \times 10^{-4} M; NH\(_2\)OH, 1 \times 10^{-5} M; Na\(_2\)S\(_2\)O\(_4\), 5 \times 10^{-2} M; and menadione, 1.6 \times 10^{-3} M. Note that a faster recording was made for the "on" than for the "off" portions of the traces.
produced upon illumination is followed by a much slower rise which was completed within about 6 seconds in the light (the length of the exposures used for Fig. 12). Darkening produced a rapid decrease of absorbance to the former dark baseline.

Fig. 13 reveals that both the initial rise and the decay of the carotenoid shift are caused by first-order reactions. The decay curve was obtained by plotting the absorbance change that still remained at intervals after darkening the cell suspension. The rise curve was obtained by plotting the carotenoid that remained unreacted at intervals after the actinic light was given. The half times \( t_{1/2} \) and the rate constants \( k \) for the rise and decay are similar in both cases. The values for the rise are \( t_{1/2} = 0.07 \) sec and \( k = 9.5 \) sec\(^{-1} \), and for the decay, \( t_{1/2} = 0.09 \) sec and \( k = 7.9 \) sec\(^{-1} \). In some cases it was possible to analyze the decay as the sum of two first-order reactions.

The absorbance change produced by carotenoids, like those caused by chlorophyll \( b \), are strongly dependent upon the dark interval given between exposures. This effect was described in *Year Book 63*, p. 441 for the chlorophyll \( b \) change. In essence, the carotenoid change also increases up to a certain maximum with increasing dark intervals between exposures. This increase is not proportional to the dark interval but varies in a complex way. Therefore, in all experiments reported here, a uniform dark interval (48 sec) was given between successive exposures until a reproducible response was obtained.

Having determined conditions needed to obtain reproducible results it was then possible to investigate the effects on the carotenoid change and on fluorescence of substances which are known (or could be expected) to exert strong effects on the early reactions of photosynthesis. Fig. 12 shows examples of the results produced on the carotenoid change and on fluorescence after addition of the inhibitors DCMU and NH\(_4\)OH, of a reductant, Na\(_2\)S\(_2\)O\(_4\), an oxidant, H\(_2\)O\(_2\), and of vitamin K\(_3\) (menadione). As noted previously (*Year Book 67*, p. 498), DCMU had only a partial inhibiting effect on the carotenoid change. The on-rate in DCMU \( V_o \) measured as shown in the insert of Fig. 14 was unaffected but the initial deflection, \( X_i \), (see insert) was increased. DCMU almost completely inhibited the steady-state part of the change, \( X_a \), and greatly depressed the off-rate, \( V_a \). Illumination in the presence of the oxidant, H\(_2\)O\(_2\), produced a large increase in the initial deflection \( (X_i) \) but a decrease in the on-rate, \( V_o \), resulting in maximum deflection being reached at longer times than in the control. The steady-state deflection, \( X_a \), and the off-rate, \( V_a \), were decreased in the presence of H\(_2\)O\(_2\). Hydroxylamine at the concentration used for Fig. 12 \( (10^{-3} \text{M}) \) had no effect on the on-rate, increased the initial deflection slightly but lowered the steady state and off-rate. Hydrosulfite \( (10^{-2} \text{M}) \) slowed down the decay of the 515-nm change and at the same time produced increased fluorescence. A transient decrease of fluorescence appeared after about 0.5 sec of illumination.

Measurements of the parameters of the 515-nm absorbance change and those
Fig. 14. The effect of concentration of DCMU, H₂O₂, NH₄OH, and menadione on various parameters of the absorbance change at 515 nm in B. alpina measured as shown in the insert.

Fig. 15. The initial, \( F_i \), and peak, \( F_p \), fluorescence (measured as shown in the insert) as a function of the concentrations of DCMU, H₂O₂, NH₄OH, and menadione. The measurements were made simultaneously with those for absorbance changes given in Fig. 14.
of fluorescence (determined as shown in the inserts) are given in Figs. 14 and 15 as a function of the concentration of H$_2$O$_2$, DCMU, NH$_2$OH, and menadione (vitamin K).

An increase in the DCMU concentration from $10^{-4}$ to $2 \times 10^{-4}$ M had little effect on the on-rate of the 515-nm change, but it gave rise to strong inhibition of the steady-state change as well as to the off-rate (Fig. 14A). By $4 \times 10^{-6}$ M the initial deflection was doubled. As customarily observed, the initial fluorescence, $F_i$, increased with increasing DCMU concentrations (Fig. 15A). The maximum peak fluorescence, $F_p$, was doubled already at a concentration somewhat below $10^{-6}$ M. (At about this concentration and above, the initial deflection of the 515-nm change was increasing strongly.) It should be noted that measurements of $F_i$ are somewhat difficult to make with certainty because the response time of the recorder makes possible an overestimation by including in this measurement part of the fast rise of variable fluorescence.

Hydrogen peroxide, at concentrations higher than $5 \times 10^{-4}$ M inhibited the on- and off-rates as well as the steady state 515-nm deflection, but it dramatically increased the initial deflection (Fig. 14, part B). Both the initial and the maximum peak fluorescence were increased by addition of up to about $5 \times 10^{-2}$ M H$_2$O$_2$ (Fig. 15, part B). Further addition of H$_2$O$_2$ did not change $F_i$ but it decreased $F_p$.

A thousandfold variation in the concentration of hydroxylamine (from $10^{-4}$ to $10^{-1}$ M) produced relatively little effect on the on-rate of the 515 change (Fig. 14, part C). At concentrations above $10^{-3}$ M, NH$_2$OH produced a progressive inhibition of the steady state and the off-rate of this change concomitantly with a large increase in the initial deflection. Figure 15, part C, shows NH$_2$OH had only a slight effect on the maximum of peak fluorescence. Initial fluorescence was increased more than $F_p$ at concentrations above $10^{-3}$ M. Since the curves are plotted as percent of the control and not as actual values of $F_i$ or $F_p$, the crossing over of the two curves does not mean that $F_i$ becomes larger than $F_p$. Below $10^{-2}$ M, however, there was little effect of NH$_2$OH on either $F_p$ or $F_i$.

Menadione (Fig. 14, part D) below $10^{-3}$ M had a slightly stimulatory effect on both the initial deflection and the on-rate of the 515-nm change, but it inhibited slightly the steady-state change and the off-rate. Above $10^{-2}$ M, all parameters measured for the 515-nm change declined, the on-rate declining most rapidly. The initial fluorescence was not effected by menadione at concentrations up to about $2 \times 10^{-3}$ M (Fig. 15, part D). Above this it produced a strong inhibition of the initial fluorescence. By contrast, the peak fluorescence began to be inhibited at concentrations above about $10^{-6}$ M. The effect of menadione on variable fluorescence (the difference between the peak and initial fluorescence) is also shown in part D of Fig. 15. The maximum effect of menadione on variable fluorescence occurred between concentrations of $10^{-4}$ and $10^{-3}$ M. At $10^{-3}$ M variable fluorescence was reduced by 50 percent.

Figure 16 shows the effect on fluorescence and on the carotenoid change of combined additions of DCMU + H$_2$O$_2$, NH$_2$OH + H$_2$O$_2$, and DCMU + NH$_2$OH.

As was seen earlier in Fig. 14, H$_2$O$_2$ alone gave a large stimulation of the initial deflection of the 515 change. Fig. 16 shows, as described above for Fig. 12, that the time needed to attain the maximum deflection $t$ was increased by addition of 14 peroxide. Variable results were sometimes obtained for fluorescence (but not for the carotenoid change) when using hydrogen peroxide. In the experiments shown in Fig. 16, H$_2$O$_2$ ($5 \times 10^{-3}$ M) decreased somewhat both $F_i$ and $F_p$, while in the experiment shown in Fig. 14 the same concentration produced increased fluorescence. However, H$_2$O$_2$ al-
ways had an effect on the kinetics of the $F_i$ to $F_p$ rise (Fig. 12).

Interestingly, the combination of both DCMU and $H_2O_2$ completely inhibited the carotenoid change while at the same time it produced the usual approximate doubling of fluorescence.

The combination of hydroxylamine plus hydrogen peroxide did not produce the dramatic inhibiting effect shown by the combination of DCMU and $H_2O_2$. Although the values could not be determined precisely, the initial deflection of the 515 change was decreased slightly and the time, $t$, needed to attain the maximum deflection was increased thus producing a decrease of the initial on-rate. Fluorescence was almost unaffected by this combination.

Hydroxylamine plus DCMU gave a 2.5-fold stimulation of the initial deflection of the 515 change in addition to increasing the on-rate and, as a consequence, the time needed to attain the maximum initial defection was shortened to about 50% of the control. The steady-state 515 absorbance change was completely inhibited. This combination also produced a 2.5-fold increase of the initial fluorescence and about half as much increase in the peak height of fluorescence.

Discussion

Since DCMU apparently acts by preventing reoxidation of reduced $Q$ (Duy-sens and Sweers, 1963), it leads to the inactivation of the traps of system 2.
The carotenoid change produced by system 2 is also inhibited with DCMU because the action spectrum for the change persisting after treatment showed system 1 sensitization (Year Book 67, p. 496). DCMU had almost no effect on the on-rate of this carotenoid change over the range of concentrations tested (10^{-7} to 2 	imes 10^{-5} M) suggesting that the change produced by system 2 is slower than that produced by system 1. Action spectra for the slow change appearing after a few seconds of illumination showed system-2 activity. In these experiments the addition of H_{2}O_{2} gave rise to a large, but slow, change (Fig. 12). Since peroxide is a strong oxidant, it may act by oxidizing a component near system 1 such as P700 (see Fig. 17). If the effect of H_{2}O_{2} is to allow only a system-2 carotenoid change to persist, then DCMU would be expected to abolish the change. This was what actually occurred when H_{2}O_{2} and DCMU were combined as shown in Fig. 16. Inactivation by H_{2}O_{2} of some component near system 1 would then allow accumulation of intermediates reduced by system 2 and produce increased fluorescence. Such a result was described in Fig. 15 for fluorescence. It is not known what conditions are required with H_{2}O_{2} to produce both a slow carotenoid change and increased fluorescence since H_{2}O_{2} sometimes did not stimulate fluorescence. In most cases, however, this is what was seen.

Hydroxylamine apparently acts on system 2 by blocking reactions near water splitting (de Kouchkovsky, 1961; Joliot, 1968). This compound at 10^{-3} M produced nearly complete inhibition of oxygen evolution but had almost no effect on the dark-decay of the 515-nm change and on the fluorescence of Chlorella (de Kouchkovsky, 1969). Apparently, hydroxylamine (NH_{2}OH) at this concentration replaces water (H-OH) as the electron donor giving rise to strong inhibition of oxygen evolution and little effect on fluorescence. Similarly, in Botrydiopsis low concentrations of hydroxylamine (10^{-4}-10^{-3} M) had almost no effect on fluorescence nor much effect on the carotenoid change. At concentrations above 10^{-3} M, however, this substance acted like DCMU by increasing the initial deflection and the on-rate but inhibiting the steady state and the off-rate of the carotenoid change and at the same time increasing fluorescence. Thus hydroxylamine at concentrations higher than a certain level may interact additionally at another site by preventing the return of Y^{+} to the Y state (Fig. 17).

Menadione has been shown (Year

![Fig. 17. Generalized scheme showing the effect of various substances on the carotenoid change in B. alpina. For details see the text.](image-url)
to be an effective quencher of the variable fluorescence originating in system 2. This quenching process is apparently caused by a direct interaction of chlorophyll and quinone molecules and not by a stimulation of electron transport. More evidence that menadione acts close to system 2 was provided by the observation that this substance inhibited the reduction of the F-type cytochrome in light absorbed mainly by system 2. In these experiments menadione inhibited the slow phase of the change (steady state) mediated by system 2. Concomitantly with this, menadione was extremely effective in quenching variable fluorescence in Botrydiopsis. The concentration for 50% quenching was 10 μM. By comparison, Ulva required a concentration of 50 μM (Fork and Amesz, 1967).

In contrast to this inhibiting effect on the steady-state 515-nm change, menadione produced a stimulation of the initial deflection over more than a hundredfold variation in concentration. Menadione produced a decline in the on-rate at concentrations greater than about 1.5 × 10⁻⁵ M and a decline of the steady state. Interestingly, DCMU, also acting close to system 2, did not have an effect on the on-rate of the 515-nm change. Quanta absorbed by system-2 traps inactivated by DCMU may become available to system 1 by a "spill-over" type of mechanism (Myers and Graham, 1963). Amesz et al. (1969) found in the blue-green alga Anacystis nidulans that light absorbed by system 2 could, in the presence of DCMU, be used for such system-1 reactions as the oxidation of cytochrome or P700. Subsequent addition of 1,4-naphthoquinone, a close relative of menadione (2-methyl-1,4-naphthoquinone) and an effective quencher, prevented this spill-over. Menadione is apparently effective in quenching fluorescence since it provides "artificial" traps for quanta absorbed by system 2 (Amesz and Fork, 1967). Thus it would prevent spill-over of quanta to system 1 and would decrease the on-rate of the 515-nm change mediated by system 1. Under these conditions the initial deflection would not be much affected (Fig. 14) but would take longer to appear.

There is no evidence to date to indicate that the carotenoid showing these light-induced shifts participates as a redox catalyst in the electron-transport chain. Rather, it appears that the change may reflect, as does the chlorophyll-b change, a disturbance of the supporting membrane as a result of electron transport. Junge and Witt (1968) recently postulated that the chlorophyll-b change (seen at 475, 515, and 650 nm in plants containing this chlorophyll) is produced by the formation of an electrical field across the thylakoid membrane, which in some unknown way, gives rise to a change in the absorption of chlorophyll b and then a translocation of H⁺. The chlorophyll-b change was found (Junge and Witt, 1968) to have a biphasic decay after a short light flash that could be decomposed into two first-order reactions. Analysis made here of the rise and decay of the carotenoid change showed that both followed first-order kinetics, and there is some indication for another, slower, component also exhibiting first-order kinetics. Junge and Witt observed a linear relationship between the rate of phosphorylation and the amplitude of the slow phase of the chlorophyll-b change in spinach chloroplasts. Conditions producing phosphorylation accelerated the decay of this change by the same amount as electron transport.

In bacteria Amesz and Vredenberg (1966) found a quantum yield of 3 for the carotenoid shift, suggesting that these changes are not produced by a chemical reaction. More likely, electron transport in the membrane causes a change in the environment of the carotenoids resulting in a small change in their absorption spectrum. Baltzehoffsky (1969) has suggested that an energy-rich intermediate of phosphorylation coupled to electron transport produced a membrane con-
formation resulting in the carotenoid shift in bacteria. Fleischman and Clayton (1968) suggested, on the basis of studies with inhibitors and uncouplers, that the carotenoid shift in *Rhodopseudomonas spheroides* depends upon the formation of an energy-rich intermediate of phosphorylation. It will be of interest to determine whether the carotenoid shift that can be seen in algae and higher plants can be related in a similar manner to a membrane change and photophosphorylation.

**References**


Fig. 18. The effect of ultraviolet irradiation (253.8-nm mercury line) on the kinetics of light-induced absorbance changes at 482 nm in Botrydiopsis alpina. A schedule of 3 seconds light and 6 seconds dark was used. Half-band width of the measuring beam was 2 nm. The red actinic light was a broad band from 620 to about 800 nm and had an intensity of $1.2 \times 10^5$ ergs cm$^{-2}$ sec$^{-1}$.

(Fork et al., 1966) and the carotenoid change (Year Book 67, p. 496; Fork, 1969). The slow phase appearing after several seconds of illumination is activated by system 2 in both cases. Earlier studies with Scenedesmus have shown (Mantai and Bishop, 1967) that the slow phase of the chlorophyll-b change is more sensitive to ultraviolet irradiation than is the rapid, initial deflection. Because of the similarities between the changes described above, it was expected that the slow phase of the carotenoid change mediated by system 2 would be more sensitive to ultraviolet irradiation than would the initial deflection. Figure 18 shows that this actually was the case. After 6 minutes of UV treatment the slow phase was strongly affected. The slow phase of the change was measured as the maximum excursion of the trace above the initial, rapid on-deflection. By contrast, the height of the initial deflection of the 482-nm change was hardly affected until longer times of irradiation had passed. After this ultraviolet treatment the cells produced a time course having a large, transient negative change upon darkening. Fig. 19A shows a semilogarithmic plot of the slow phase remaining after increasing times of irradiation with UV. Since the absorbance change observed consists of several components, a strict measurement of the fast and slow phases is difficult (see Fig. 18).

As shown in Fig. 19A, there is an initial lag period of about 5 minutes during the ultraviolet treatment before inhibition of the slow phase begins. This suggests that some reaction other than the one being inhibited by the UV irradiation is rate limiting for the absorbance change, and it is not until the process affected by the irradiation itself becomes rate limiting that a decrease in the absorbance change occurs. After the initial lag period, the decay of activity with time follows first-order kinetics; and back extrapolation of the linearly decreasing part of the curve yields a half time ($t_{1/2}$) for decay of about 5 minutes. This lag period was not always seen, however. The absorbance changes shown in Fig. 18 (obtained from a different sample than that used for Fig. 19) did not show this lag in the UV effect. Nevertheless, the slow phase, when measured as described above, also decayed by first-order kinetics and had a $t_{1/2}$ of about 6 minutes. The decay of the chlorophyll-b change in spinach chloroplasts after irradiation was also found to be first order (Mantai, unpublished).

A study was made of the effect of UV irradiation on the reaction centers of system 2. For this we measured the
Fig. 19. (A) The slow phase of the 482-nm change in \textit{B. alpina} as a function of increasing times of UV irradiation. The slow part of the change was measured as described in the text. The actinic light was the same as described in Fig. 18. The same culture of cells was used for all the measurements shown in Fig. 19. (B) Variable fluorescence in \textit{B. alpina} treated with DCMU (1 × 10^{-4} M) as a function of the time of exposure to UV. Variable fluorescence was measured as the difference in level between the fluorescence produced immediately upon illumination and the maximum level produced. Dark interval between exposures, 30 seconds. Measurements were made at 685 nm (half-band 15 nm) by using interference and colored glass filters that transmitted fluorescent light but absorbed the blue actinic light. The latter had a peak near 420 nm, a half-band of 40 nm, and an intensity of about 10^6 ergs cm^{-2} sec^{-1}. (C) Light-induced reactions of the \textit{i}-type cytochrome in \textit{B. alpina} as a function of exposure time to ultraviolet. Measurements were made at 420 nm, half-band width 2 nm. The difference spectrum matched that of cytochrome \textit{i} and had a maximum at 403 nm, with a Soret band near 419 nm. For each measurement the rate of reduction upon darkening was measured and was corrected for the endogenous cytochrome reduction remaining after the cells were poisoned with DCMU as described in (B). The initial rate of absorbance increase (reduction) divided by the steady-state level of oxidation attained in the light (in DCMU) was plotted as shown. Oxidation was taken as the light-induced steady-state absorbance decrease produced in the presence of DCMU. Red actinic light was as described for Fig. 18.
duction, the oxidation of the cytochrome (mediated by system 1) was unaffected by irradiation times up to about 20 minutes. After this, oxidation was also affected. It should be noted that all these comparative measurements on the carotenoid change, variable fluorescence, cytochrome reduction, and oxidation were made with the same sample.

The data given above supports also the hypothesis that the slow phase of the carotenoid change is produced by system 2. Since the kinetics and the effects of UV on both the carotenoid and chlorophyll-b changes are very similar, it would appear that both of these absorbance changes result from similar reactions, although the compound responsible for the change in each case may be quite different. If these changes reflect some type of disturbance produced in their supporting membrane by a process such as electron transport (for example, by generation of an electric field across a membrane as proposed by Junge and Witt, 1968), then UV may act by disrupting membranes in some way. Other evidence (Mantai, 1968) has also suggested that ultraviolet light produces a disruption of lamellar membranes.

References


Electron Transport and Degradation of Chloroplasts by Hydrolytic Enzymes and Ultraviolet Irradiation

Kenneth E. Mantai

Recent development of the chemiosmotic hypothesis for the mechanism of both oxidative and photosynthetic phosphorylation has led to increased interest in membrane structure and integrity as a vital factor in efficient operation of the energy trapping reactions. Measurement of various chloroplast reactions after treatments which disrupt the membrane structure could help to elucidate the role that the structural integrity of membranes plays in these reactions. Because chloroplast membranes consist of about 50% lipid and 50% protein, digestion of these substances with appropriate enzymes could provide information on their importance in the membrane structure. There is evidence that the inhibition of photosynthesis by ultraviolet irradiation is due to a structural disruption of the chloroplast membranes. The following report compares the effects of UV irradiation, pancreatic lipase digestion, and trypsin digestion on electron transport as measured by DCIP (2,6 dichlorophenolindophenol) reduction in spinach chloroplasts. Both UV irradiation and lipase digestion decrease the level of fluorescence in chloroplasts (Kok, et al., 1967; Okawaya, 1964), suggesting that the photochemistry itself is being affected by the treatments. Fig. 20 shows the effects of UV irradiation, pancreatic lipase, and trypsin digestion on the relative quantum yield of DCIP reduction. As seen in the figure, all three treatments decreased the quantum yield, confirming that the
Control

lipase treated

Trypsin treated

UV treated

Control

Fig. 20. Effects on relative quantum yield of DCIP reduction after UV irradiation, pancreatic lipase digestion, or trypsin digestion. Reaction mixtures consisted of: sucrose, 0.4 M; KCl, 15 mM; Tricine, 50 mM (pH 7.6); DCIP, 33 μM and chloroplasts containing about 40 μg chlorophyll, the exact amount varying slightly from experiment to experiment. The total volume was 3.0 ml. Enzyme digestions were carried out at 25°C in 0.4 M sucrose, 15 mM KCl, and 50 mM Tricine (pH 7.6). UV irradiation was performed as previously described (Maniatis and Bishop, 1967).

photochemistry was indeed being inhibited.

There is considerable evidence for two pathways of electron transport in chloroplasts, only one of which is coupled to photophosphorylation. Therefore, the possibility that there might be a difference in the sensitivity of these pathways to the action of trypsin, lipase, or UV irradiation was tested by treating the chloroplasts and then measuring the rate of DCIP reduction in the presence or absence of a chemical uncoupler (CCCP, methylamine, NH₄Cl, atebrin, or gramicidin D).

Electron transport measurements made after UV irradiation, Fig. 21, shows that there was no difference in the rates at which coupled or uncoupled electron transport was inhibited (compare irradiated and methylamine uncoupled curves). CCCP uncoupled less effectively, and finally inhibited slightly, as the period of irradiation increased (see also below). In this experiment, a high concentration of CCCP (33 μM) was used. Reducing the CCCP concentration to 3.3 μM did not qualitatively change the results. The rates shown in the figure are under light saturating conditions. It should be mentioned that in this type of experiment if the two pathways have initial steps in common (e.g., trapping centers), and one of these steps is rate limiting, any differences in sensitivity between the rest of the two pathways would not be detected.

A similar experiment measuring DCIP reduction after digestion with pancreatic lipase is shown in Fig. 22. In this case...
Fig. 22. Effect of uncoupling agents on DCIP reduction after digestion with pancreatic lipase. Reaction conditions as in Fig. 20 with un­
couplers added to give the following concen­
trations: CCCP, 3.3 μM; atebrin, 10 μM; N methyl­
ammonium, 20 mM and Gramicidin D, 0.5 g ml⁻¹. Solid line shows lipase

treated sample in absence of added uncouplers. Dashed line shows control sample without

addition of chemical uncouplers.

the lipase itself acts initially as an effi­
cient uncoupler as shown by the in­
creased electron flow. The zero-time

sample shows considerable uncoupling

because about 30 seconds elapsed be­
tween the time the enzyme was added

and a sample removed. The uncoupling

is complete after a short time, and addi­
tion of chemical uncouplers causes no

further stimulation of electron flow. After continued digestion the total rate

of light-saturated DCIP reduction de­
creases.

Trypsin digestion also uncouples

chloroplasts (Fig. 23A), although not as
effectively as lipase as seen by further
stimulation of electron transport after
addition of methylamine. Again, con­
tinued digestion led to a decrease in
photoreductive capacity. In the experi­
ment shown in Fig. 23B the uncoupler

CCCP was added to trypsin-treated

chloroplasts. Under these conditions

CCCP acted as a potent inhibitor of

electron transport. This inhibition ap­
ppears to affect only the "extra" or coupled
electron transport pathway. The same

concentration of CCCP (3.3 μM) caused
a doubling (Fig. 23B) of electron trans­

TABLE 7. The Effect of Various Concentrations of CCCP on the Rate of DCIP Reduction* in Control and Trypsin Treated Spinach Chloroplasts

<table>
<thead>
<tr>
<th>CCCP Concentration, ( \mu M )</th>
<th>Control</th>
<th>Trypsin treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>79.1</td>
<td>45.8</td>
</tr>
<tr>
<td>0.33</td>
<td>81.6</td>
<td>46.6</td>
</tr>
<tr>
<td>0.66</td>
<td>88.9</td>
<td>39.4</td>
</tr>
<tr>
<td>1.70</td>
<td>107.0</td>
<td>32.0</td>
</tr>
<tr>
<td>3.33</td>
<td>161.2</td>
<td>...</td>
</tr>
<tr>
<td>9.99</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

* Expressed as \( \mu \) moles red. mg. chl\(^{-1}\) hr\(^{-1}\). Conditions as in Fig. 20.

not stimulate DCIP reduction at concentrations as low as 0.33 \( \mu M \) in trypsin digested chloroplasts nor did it inhibit untreated chloroplasts at concentrations as high as 10 \( \mu M \). Treatment of chloroplasts with papain, another proteolytic enzyme, gave similar results.

Reports from Park’s laboratory have shown that glutaraldehyde-fixation of chloroplasts stabilized system-2 activity (Park, et al., 1966). Although the initial activity of these chloroplasts is low, the rate of DCIP reduction is stimulated by the uncoupler methylamine and inhibited by DCMU, suggesting that the activity is the result of true photosynthetic reactions and not of artifacts in the fixation procedure. It has also been shown that glutaraldehyde cross-links proteins, both inter- and intramolecularly, without greatly affecting their conformation. In view of this, experiments were performed to determine whether fixation would provide protection against the action of trypsin, lipase, or UV irradiation. In Fig. 24 the effects of UV irradiation on DCIP reduction in fixed and normal chloroplasts are shown. The fixation procedure had no effect on the inhibition by UV irradiation. Figure 25 shows the results of similar experiments measuring the effects of fixation on the inhibition by (A) pancreatic lipase and (B) trypsin. Glutaraldehyde-fixation afforded striking protection against the inhibitory effects of these two enzymes. Measurement of the fatty acids liberated after lipase digestion did not reveal any great difference between fixed and unfixed samples, indicating that the lipase was still able to attack the fixed chloroplasts. No attempt was made to assay the activity of the trypsin in fixed and unfixed samples. The possibility thus remains that the fixation procedure made the lamellar proteins unavailable to the trypsin, perhaps simply by steric hindrance. However, fixed chloroplasts exhibited partial uncoupling by trypsin, suggesting that the enzyme attacks the membrane.

Since glutaraldehyde-fixation protects against the effects of lipase, it would appear that those lipids readily hydrolyzed...
lipase digested...

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Fig. 25. Effect of pancreatic lipase or trypsin digestion on DCIP reduction in normal and glutaraldehyde-fixed chloroplasts. Conditions as in Fig. 24.

by pancreatic lipase are required only as a "glue" and not as obligate components for electron transport. The protection afforded by fixation against trypsin is somewhat less easily explained. Trypsin preferentially attacks peptide bonds in which the carboxyl group is donated by a basic amino acid residue, usually arginine or lysine. Chloroplast lamellar and structural protein is rather low in both of these amino acids and relatively few breaks in the protein molecules would be expected. Glutaraldehyde, by cross-linking both inter- and intramolecularly, may hold the protein conformation in its original state in spite of the breaks in the protein molecules themselves.

UV irradiation inhibits in a manner similar to trypsin and lipase, with some striking exceptions. The most evident is the lack of uncoupling by UV irradiation (see Fig. 21). PMS-mediated cyclic photophosphorylation is inhibited by UV irradiation at about the same rate as the loss of Hill reaction activity, at least with relatively low (although still saturating for the Hill reaction) actinic light intensities. However, lack of uncoupling implies that the site of inhibition may be in the electron transport chain rather than in the phosphorylation mechanism. As shown in Fig. 20, the relative quantum yield of DCIP reduction decreases after UV irradiation (as does the variable fluorescence) indicating that system 2 is being affected. Moreover, since cyclic photophosphorylation is at least one component removed from system 2 (DCMU blocks after the primary electron acceptor for system 2, but does not inhibit cyclic photophosphorylation), UV irradiation must either produce a general effect on the membrane system or inhibit at two separate sites. There is no strong evidence that UV irradiation destroys a specific component or components of the electron transport chain. Although plastoquinone and other quinones are in fact destroyed by UV irradiation, it does not appear that this is the major cause of the inhibition (Mantai and Bishop, 1967).

It seems reasonable to conclude that the inhibition of electron transport by pancreatic lipase, trypsin, and probably UV irradiation as well, is caused by a structural disruption of the lamellar membranes rather than inactivation of a specific component. The inhibition by UV irradiation, however, appears to be more specific and does not uncouple although phosphorylation is inhibited.
EFFECTS OF N-METHYLPHENAZONIUM METHOSULFATE AND PYOCYANINE ON DELAYED LIGHT EMISSION IN CHLORELLA CELLS AND SPINACH CHLOROPLASTS

L. O. Björn

In trying to understand the process of photosynthesis it is useful to study the reverse process, i.e., the delayed light emission (afterglow) that comes from plants even several minutes after they have been transferred from light to darkness. That the light emission is closely related to photosynthesis is indicated by the fact that many chemical agents which influence the latter also change the former. Thus the substances reported below to change the pattern of light emission are known to be efficient, although artificial, catalysts of photosynthetic phosphorylation.

One aim of biological research is to determine the minimum unit that can perform a certain function and to identify this unit structurally. It has long been known that the chlorophyll molecules do not function separately in photosynthesis, but rather cooperate in groups. The number of cooperating molecules found in a group or "photosynthetic unit" depends on the kind of experiment by which it is measured. The unit for the primary conversion of light into chemical energy contains a few hundred chlorophyll molecules, while several such units might form a larger unit capable of more complex functions. The experiments described below indicate that a specific kind of afterglow originates in units containing about the same number of chlorophyll molecules as the structural unit called a thylacoid.

In these experiments the plant sample was first illuminated for 30 seconds. The exciting light was then shut off and the afterglow measured for a few minutes. The exciting wavelength, isolated with interference filters, was either 648 or 730 nm. The sample was either Chlorella pyrenoidosa (Indiana Culture No. 252) or spinach chloroplasts ("whole chloroplasts" in 0.34 M NaCl buffered to pH 7.7-7.8 with 0.05 M Tricine). The light emission was recorded from about one second to a few minutes after the end of excitation. Air had free access to the samples, and the Chlorella were kept suspended by a continuous stream of air bubbles during irradiation and measurement.

With intact cells the decay kinetics vary with the wavelength used for excitation. With 648 nm a monotonic decay is obtained; but after excitation with 730-nm irradiation of sufficient intensity and duration the light emission drops to a minimum, rises again to a maximum and finally slowly declines. This maximum at about 1 minute in the decay curve was first described by Bertsch and Azzi (1965). This so-called component V (nomenclature of Shuvalov and Litvin, 1969) that is induced only by long-wavelength light, differs from other kinds of afterglow not only by its action spectrum and time dependence, but also by the emission spectrum (Litvin and Shuvalov, 1966). It also has a different temperature dependence (Bertsch and Azzi, 1965; Shuvalov and Litvin, 1969), and is strongly influenced by oxygen.

Among the more interesting results of the current experiments are the effects of N-methylphenazonium methosulfate (PMS) and its photooxidation product, pyocyanine, on the afterglow. Both seem to enter Chlorella rapidly. PMS accele-
rates the emission of component V. The effect can be detected with a PMS-concentration of only $10^{-9} M$ (Figures 26 and 27). The 648-nm-induced emission is affected only by much higher concentrations. Pyocyanine gives the same effects as PMS, but a ten times higher concentration is required. The same effect of pyocyanine has been described by Rubin and Venediktov (1967) for Vallisneria, but the concentration used in their investigation was very high ($10^{-2} g l^{-1} = 4.8 \times 10^{-5} M$).

The results in Figures 26 and 27 were obtained with dilute suspensions of algal cells, and in this case the effect of PMS depends on its concentration but is independent of the algal concentration. If very dense suspensions are employed, PMS is somewhat less effective on a concentration basis. However, when the amount of PMS is expressed in relation to the amount of Chlorella, the efficiency is higher in dense suspensions. In one series of experiments a suspension with the following characteristics was used: cell concentration, $1.0 \times 10^{11} l^{-1}$; chlorophyll concentration, $1.77 \text{ mM (chl a 1.34 mM, chl b 0.43 mM)}$; packed cell volume, 20% of suspension volume. Table 8 shows the effect of $1 \times 10^{-8} M$ and $3 \times 10^{-8} M$ PMS at this high cell concentration. It was found that, when a sufficiently long time had elapsed after the end of excitation, the luminescence intensity with PMS declined to a certain fraction of the intensity without PMS, and that this fraction eventually reached a fixed value. For $1 \times 10^{-8} M$ PMS, this limiting value was 0.62.

If we assume that there are "afterglow units" of approximately uniform size (perhaps related to the "photosynthetic units"), the result of the above experiment can be used to determine a maximum concentration of these units (corresponding to a minimum size). If
at any time the intensity of luminescence with PMS is only the fraction \( x \) of that without PMS, then at least the fraction \((1-x)\) of the luminescent units are in some way affected. A unit which is affected must be associated with at least one molecule of PMS. Thus the molar concentration of afterglow units cannot exceed \((\text{molarity of PMS})/(1-x)\).

In the present case with \([\text{PMS}] = 1 \times 10^{-8} \text{ M}\) and \(x=0.62\), the maximum molarity of afterglow units is \((1 \times 10^{-4})/0.38 \text{ M} = 2.63 \times 10^{-8} \text{ M}\). Since the concentration of chlorophyll is \(1.77 \times 10^{-8} \text{ M}\), there are at least \(6.7 \times 10^4\) molecules of chlorophyll per unit.

The unit estimated in this way is considerably larger than the “classical” photosynthetic unit. It may be identical with the unit estimated by Junge and Witt (1968) for the light-induced chlorophyll-\(b\) change. They estimate a size of about \(10^6\) chlorophyll molecules, and believe the unit to be one thylacoid.

This estimate of unit size applies of course only to component \(V\) afterglow. The other afterglow components may emanate from units of different sizes.

Low concentrations of PMS only increase the rate of emission but do not change the amount of light emitted. In the experiment shown in Table 8, the long-term emission was decreased to 62 and 39% by the two concentrations of PMS, but the light integrated from 5 to 370 seconds after the end of excitation was 105 and 102% of the value of the control without PMS. However, with a high concentration of PMS the total amount of light is decreased.

The acceleration of emission by PMS can also be demonstrated by injecting it into the sample in the dark period after excitation. In the experiment shown in Figure 28 a large amount of PMS was used, and it is readily seen that the total amount of light emitted was decreased by PMS.

Although leaves of many plants (including spinach) were found to give decay curves of essentially the same type as \textit{Chlorella}, isolated chloroplasts seem to lack most of component \(V\) of the delayed light. With spinach chloroplasts the decay curves appear very similar (monotonic decline) whether the emis-

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**TABLE 8. Effect of PMS on the Afterglow from a Dense Chlorella Suspension Excited for 30 sec at 730 nm, Average of Four Experiments**

<table>
<thead>
<tr>
<th>Dark Time, sec</th>
<th>Time Course: Relative Intensity without PMS</th>
<th>Ratio of Luminescence with PMS to Luminescence without PMS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(1 \times 10^{-8} \text{ M}) PMS</td>
</tr>
<tr>
<td>20</td>
<td>960</td>
<td>1.21</td>
</tr>
<tr>
<td>70</td>
<td>1009</td>
<td>1.08</td>
</tr>
<tr>
<td>120</td>
<td>611</td>
<td>0.75</td>
</tr>
<tr>
<td>170</td>
<td>319</td>
<td>0.74</td>
</tr>
<tr>
<td>220</td>
<td>176</td>
<td>0.72</td>
</tr>
<tr>
<td>270</td>
<td>106</td>
<td>0.63</td>
</tr>
<tr>
<td>320</td>
<td>59</td>
<td>0.62</td>
</tr>
<tr>
<td>370</td>
<td>35</td>
<td>0.62</td>
</tr>
</tbody>
</table>

---
sion is induced by 648 or 730 nm light. A few differences between the effects of the two wavelengths were observed:

1. Pyocyanine at a concentration of $3 \times 10^{-6} M$ slightly increases the 20-second delayed light excited by 730 nm, but decreases that excited by 648 nm (Fig. 29). The effects are not as pronounced as those found by Mayne (1967) for delayed light in the millisecond range.

2. Ascorbate ($0.01 M$) increases the long-lived afterglow excited by 730 nm (remnants of component V?), but has no effect on that excited by 648 nm (when no other additions are made, see below and Fig. 30).

3. The afterglow excited by weak 648-nm light is diminished by a previous exposure to 730-nm light, but enhanced by a previous exposure to strong 648-nm light. This holds even when the dark time after the first exposure is so long that the afterglow induced by it has become negligible. A similar effect was reported by Litvin and Shuvalov (1966).

Although ascorbate alone has no effect on the 648-nm-induced emission, it greatly enhances the effect of PMS, probably because PMS is reduced by ascorbate (Fig. 30). Ascorbate can be replaced by isoascorbate. Bertsch et al. (1969) found no effect of PMS alone on the fast delayed light (1–20 msec) from chloroplasts, while PMS ($3 \times 10^{-5} M$) plus ascorbate ($5 \times 10^{-8} M$) produced almost complete inhibition. In the present experiments PMS alone inhibits at a high concentration, but in the presence of ascorbate the same effect is produced by less than one tenth as much PMS.

In my experiments pyocyanine alone had less effect than PMS alone. In this respect the late delayed light differs from that in the millisecond range investigated by Bertsch et al. (1969). As expected, ascorbate did not radically change the effect of pyocyanine (which is not reduced by ascorbate). Ascorbate does have an effect at very high concentra-
tions of pyocyanine, possibly due to traces of PMS in the pyocyanine preparation.

References


A TEST OF FIBER OPTICS FOR FLUORESCENCE SPECTROSCOPY

C. S. French, R. W. Hart, N. Murata, and C. Wraight

The ideal geometry of a system for exciting fluorescence and for collecting the emitted light to give the minimum distortion of the emission spectrum by reabsorption of the fluorescent light within the sample is to have both optical axes perpendicular to the surface of the sample. Collection of the emitted light over a large solid angle with simultaneous perpendicular illumination is difficult with lenses or mirrors.

However, the availability of glass fiber optics in sheet form makes possible a convenient fluorescence excitation and light collection system for use in fluorescence spectrophotometers with the axes for both beams perpendicular to the sample surface. With alternate sheets of fibers for the incident and for the emitted light nearly half the sample surface may be exposed to the collector fibers.

The efficiency of light collection depends on the acceptance angle of the individual fibers and on the overlapping of the cones of illuminated spaces with the cones of the space seen by the collection fibers. The numerical aperture of the fibers, 0.5 in air, corresponds to a light cone of 60° for the ends in air and to about 83° for the ends in water. For a sample in immediate contact with the ends of the fibers the overlapping is zero at the surface but becomes high a short distance below the surface of the sample.

The original recording fluorescence spectrophotometer with automatic correction for variation in sensitivity of the detector and of the monochromator's transmission with wavelength (French, 1956) was modified to test the fiber optics system as shown in Fig. 31. Although the principle seems useful, the system we developed was not satisfactory.

The soft plastic backing of the 0.003-inch diameter fibers was removed by softening with chloroform and scraping to avoid waste space in the bundle ends. However, sheets of glass fibers parallel to each other can be bought with the backing material omitted from the ends (Fiber Optics Inc., 2557 Soquel Drive, Santa Cruz, California, 95060).

The width of each fiber optic sheet was chosen to match the length of an image of the exit slit of the monochromator.
used for the excitation light. The width of this image required the use of 12 sheets in the bundles for the incident light. At the sample position the sheets carrying the excitation light alternate with the collecting sheets, and one extra collecting sheet was added outside the last excitation sheet. The ends of each bundle of sheets were coated with epoxy cement and pressed into cavities of rectangular cross section in plastic blocks for the bundles going to the two monochromators. For the sample position an aluminum block was used. The ends of the three bundles were ground and polished.

The aluminum block has a horizontal surface so a solid sample may be laid on it. A removable plastic sleeve fitted over the round top of the block and sealed with an O-ring, holds liquid samples. A box with a light-tight cover and plastic foam insulation supports the aluminum sample block and can be filled with liquid N\textsubscript{2} or other liquid to hold the desired temperature long enough for a spectral measurement.

An aluminum cover can be used to define the thickness of a liquid sample and to act as a reflector for increasing the light collection efficiency with weakly absorbing solutions. Other aluminum inserts giving various sample depths are used to check for distortion of the emission spectrum by internal reabsorption. Spectra of identical shape for sample depths differing by a factor of two provide adequate evidence for lack of such distortion.

At the suggestion of Dr. Charles Weiss we have also made curved focusing reflectors, as shown in the upper part of Fig. 31, to increase the light collecting efficiency for dilute liquid samples. In these reflectors the space between the reflector and the sample chamber is filled with clear plastic. For highly scattering samples, such as those with ice crystals, black covers that do not reflect were made.

The system we built, although providing a convenient method for handling samples, gave 71% of the overall efficiency of our previous lens system instead of an anticipated gain. This test was made on a fragment of fluorescent Corning glass No. 3387 in a flat reflecting aluminum holder. Some of the loss may be attributed to the microscopically visible chipping of some of the ends of the glass fibers in the grinding process.

A more serious loss, however, occurs from the fact that the angle of divergence of the beam entering the monochromator exceeds the f 2.5 cone of the collector lens. An improvement of 20% was achieved by adding a small field lens inside the slit.

In testing the system, chlorophyll adsorbed on the epoxy cement in which the fibers were embedded. To remove the contaminant the fibers and plastic were cut back 0.02 inch and the space filled with epoxy cement. A \(\frac{3}{8}\) -inch lucite plate was added to keep the sample from sticking to the epoxy. These modifications led to disastrous light losses.

The fiber optics system with these modifications was compared to the old lens and mirror system. The lens and mirror system was then about 30 times more efficient than the modified fiber optics system which has been, at least temporarily, abandoned. The fiber optics arrangement could probably be made to give reasonably good performance if the sample end of the fibers were covered with a very thin layer of material to which the sample did not adhere.

Reference
putation center, that enable us to use the computer to handle real-time data. (A description of the ACME facility is given in last year's report, *Year Book* 67, p. 534).

As presently written, the programs allow us to vary the rate at which the analog signals will be sampled (up to about 1000 points per second using an IBM 1800 or up to about 20,000 points per second with the IBM 270X interface). Depending upon the needs of the experiment, a variable number of time-course curves can be analyzed. For each of these curves, measurements of up to ten differences of amplitude can be made. The data can be returned as obtained by the IBM 1800 or, to avoid a flood of unwanted numbers, averaged over a particular region of interest.

In addition, a second channel of analog input is reserved for data received from a photocell that monitors the actinic light. The program determines when the light has been turned on and off and measures its intensity. A value at any point in time, along a curve, can be obtained by entering in the program the time the measurement is to be made in relation to the time the light went on or off.

The programs are also designed to measure slopes over designated time intervals. Thus, by entering appropriate factors the quantum yields can readily be determined.

**EXPERIMENTAL TAXONOMY INVESTIGATIONS**

**The Mimulus Investigations**

*William M. Hiesey, Malcolm A. Nobs, and Olle Björkman*

A considerable part of the current effort of the Experimental Taxonomy Group has been directed towards bringing to conclusion the long-term investigations on the Erythranthe section of *Mimulus*. This group of species was selected for combined cytotaxonomic, transplant, and physiological investigation in a multiple-approach study aimed at improving our understanding of the many-sided biological question of how differentiation between species and ecological races within species is related to mechanisms of inheritance, to major external factors of contrasting natural environments, and to internal physiological functions. Plants are better suited than animals for such studies primarily because plants can be cloned and manipulated experimentally with much greater freedom in cytogenetic, transplant, and physiological investigations needed in such an integrated program.

Earlier *Year Books* (60, 1951; and 63, 1954, to 67, 1969) have reported progress on various aspects of the *Mimulus* investigations. The results from these and more recent researches are incorporated in a Carnegie Institution monograph to appear as Volume V of the series, *Experimental Studies on the Nature of Species*. It seems appropriate to review the major features of this monograph.

*Mimulus* as an experimental object. In searching for basic principles regarding mechanisms of evolution in higher plants, the choice of experimental materials is of utmost importance. Earlier studies by the Experimental Taxonomy Group on various species-complexes such as *Potentilla glandulosa*, *Achillea millefolium*, and the grass genus *Poa* pointed to characteristics lacking in these groups that would have been helpful for extending the earlier studies to include quantitative physiological investigations. The latter are needed to fill an important gap in our knowledge relating to the genetic structure of species and races, the environments in which they evolved, and their internal functioning. The Erythranthe section of *Mimulus* was chosen primarily as a vehicle to bridge this gap.
The features of the Erythranthe section that make this group particularly suitable for experimental investigations include a unique combination of essential characteristics. The most important are (1) all members are diploid with the same chromosome number \( n=8 \); (2) all of the five species that have been brought into culture can be intercrossed in any combination to produce vigorous first-generation hybrids; (3) these \( F_1 \) hybrids range from completely fertile to highly sterile, and reflect different degrees of genetic compatibility within the section that clearly evolved from a common ancestral stock; (4) the species, and often races within species, have distinct marker characters that can be followed through successive generations in genetic experiments; (5) the flower structure of all the members of the group favors easily controlled pollinations, and in interfertile combinations many seedling progeny may be obtained from a single flower; (6) species and ecological races within the Erythranthe section differ widely in their capacity to survive in contrasting climates such as are found at the Stanford, Mather, and Timberline transplant stations; (7) all members of the section can be readily propagated vegetatively as clones to obtain genetically identical plants that can be used in diverse kinds of transplant and physiological experiments and (8) all members have leaves and stems suitable for use in quantitative physiological measurements involving gas exchange on intact living plants. Few plant groups meet all of these requirements as well as this group of *Mimulus* species.

**Biosystematic relationships within the Erythranthe section.** As reported in *Year Book 64*, pp. 427–429, there are two major interfertile groups within the Erythranthe section, one composed of the two most widely distributed species, *M. lewisii* and *M. cardinalis*, and the other of the three southernmost species, *M. verbenaceus*, *M. eastwoodiae*, and *M. nelsonii*. The species of either group when intercrossed produce \( F_1 \) hybrids that are partially sterile to varying degrees. One of the most sterile combinations, *M. lewisii \times M. nelsonii*, gave rise to a fertile, vigorous, distinctive amphiploid (see *Year Book 65*, pp. 468–471), thus establishing the close ancestral relationship between the genetically most diverse members of the section. The varying degrees of interfertility among inter- and intra-specific combinations reveal in finer detail different stages of genetic isolation that have evolved within the section.

**Recombinations of morphological characters in hybrid populations.** From an experimental point of view it is a most fortunate circumstance that *M. lewisii* and *M. cardinalis*, the two species of widest geographic distribution, are interfertile and occur in distinct but complementary climates. *Mimulus lewisii* occurs in more northerly areas and at higher altitudes in contrast with *M. cardinalis* of more southern distribution, mostly at lower altitudes. First-generation hybrids between ecologically extreme forms of the two species are interfertile, and their progeny provide a means for studying the mode of inheritance of the distinctive morphological characters differentiating the two species in relation to their responses in the contrasting climates at the Stanford, Mather, and Timberline transplant stations.

The numerous morphological characters that distinguish the two species include flower color, flower structure, patterns of pigment distribution in localized areas in the corollas, and such vegetative characteristics as leaf shape and number of dentations along leaf margins. The segregation of 20 characters distinguishing *M. lewisii* and *M. cardinalis* has been studied extensively in \( F_1, F_2 \), and \( F_3 \) populations when cloned and grown at Stanford and at the Mather and Timberline transplant stations.

**Inheritance of morphological markers in relation to capacity for survival at the transplant stations.** Of the many
morphological characters studied, only one distinguishing *M. lewisii* from *M. cardinalis* has been found to be inherited in a simple Mendelian manner. The presence or absence of yellow carotenoid pigment in the upper epidermis of the corollas is determined by a single gene. Its presence in *M. cardinalis* in conjunction with other pigments causes the flowers of this species to have the bright orange-red appearance. In *M. lewisii* this pigment is absent, and the corollas are pale pink or purple, depending on the particular race. In the F₁ hybrid the expression of the yellow carotenoid pigment carried by *M. cardinalis* is suppressed by a dominant gene carried by *M. lewisii*. In F₂ and F₃ progeny this character is inherited in the ratio of 3 without carotenoid pigment to one with carotenoid pigment, its expression or lack of it being superimposed upon a wide array of independently inherited pigment characters governed in inheritance by complex gene systems. The resultant number of phenotypic expressions of flower color in F₂ and F₃ progeny is, therefore, very large.

All other characters distinguishing *M. lewisii* and *M. cardinalis* that we have studied have a complex hereditary basis that cannot be resolved in simple Mendelian terms. An example is the inheritance of leaf characteristics. *Melianthus lewisii* has relatively narrow oblanceolate leaves with nearly entire edges, as illustrated in the top row in Fig. 32 at the left. The array shown is typical of that found in seedlings obtained by self-pollinating the *M. lewisii* parent. Corresponding leaves of the *M. cardinalis* parent used in crossing experiments are shown in the same figure (top row at the right). As compared with *M. lewisii*, the leaves of *M. cardinalis* are broader, more obtuse at the tips, and have toothed margins.

The leaves of F₁ hybrids between *M. lewisii* and *M. cardinalis* are clearly intermediate, but with variations overlapping both parental populations, as shown in Fig. 32 (row next to the top). In the second generation the assortment of recombinations in leaf characters covers the entire range from one parental extreme to the other, with maximum frequencies in intermediate classes that fall within the range of the F₁ progeny.

Third-generation progenies derived by self-pollinating selected F₂ individuals possess leaf characters that may differ appreciably from one another, depending on the characteristics of the particular F₁ parent. The leaves from the series of F₂ population shown in Fig. 32 are examples. The first of this series, No. 7541, was derived from a lewisii-like F₂ individual. The leaf types segregated in this F₂ population include the range of variation of the original *M. lewisii* parent plus that found in F₁ progeny. The flowers produced by this same F₂ population ranged over various lewisii-like shades of pink, all having short styles and stamens, characters of *M. lewisii*. The correlation in the inheritance of predominantly lewisii-like characters in this population in both leaf and floral characters is clearly evident on analysis.

In contrast, the F₃ progeny derived from a cardinalis-like F₂ individual had broad, obtuse leaves with toothed margins, as shown in Fig. 32 (culture No. 7565, bottom row). In this population the range of variation in leaf characters included that of the original *M. cardinalis* parental population and the F₁ progeny. The flowers of this same F₃ population segregated into an array having predominantly cardinalis-like characters. The bias in segregation towards *M. cardinalis* in this population is as striking as in the previous F₂ progeny whose characters segregated strongly towards *M. lewisii*.

Third-generation progenies derived from F₂ individuals in classes intermediate between those of the parental types segregate over a considerably wider range than in the two examples just mentioned. Examples are shown by the arrays of leaves in Fig. 32 including the F₃
Fig. 32. Differences in leaf characters between subalpine *M. lewisii* and coastal *M. cardinalis*, their F₁ hybrid, and ranges of segregation within F₃ progenies derived from F₂ individuals of different genetic composition. See text.
populations 7545, 7566, 7543, 7530, and 7526. Some of these populations, notably 7545 and 7530, approach the diversity found in F_2 populations.

The responses of cloned transplants at the altitudinal field stations at Stanford, Mather, and Timberline of parental, F_1, F_2, and F_3 progenies which have been studied over a long period of years show definite correlations between the morphological characters they inherit and their capacity to survive in contrasting climates. The plants shown in Plate 1A, photographed in the Timberline garden in 1967, summarize the responses of *M. lewisii*, *M. cardinalis*, and their F_1 hybrid at the subalpine station. The photograph was taken in early September after the onset of early autumn frosts and the season’s first light snowfall and shows the already matured and dormant *M. lewisii* (left) with the withered remains of its flowering stems. The spot marked by the tape measure at the right is where coastal *M. cardinalis* was repeatedly planted and died from winterkill. In the center is their F_1 hybrid showing marked vigor and the ability to withstand the freezing weather to a considerably higher degree than even the *M. lewisii* parent native to the Timberline area.

Plate 1B shows a small portion of the Timberline garden in 1967 in which the responses of three F_8 progenies were being tested. The row marked (1) is a planting of the offspring of the *lewisii*-like F_2 plant 7111-16, row (2) the offspring of the F_1-like plant 7111-17, and row (3) the offspring of the *cardinalis*-like F_2 plant 7135-35. Plate 1B was taken in 1967 two years after the cloned propagules were planted in the garden. At this time the F_8 progeny of the *lewisii*-like F_2 plant shown in row 1 were nearly all well established and vigorous, and most of the plants flowered. The F_8 progeny of the F_1-like F_2 plant 7111-17 were highly variable, ranging from weak to vigorous, with a high frequency of nonsurvivors. The F_8 progeny of the *cardinalis*-like plant 7135-35 were uniform and at that time had mostly survived, but all were later in seasonal development. Plate 1C was taken in the same garden a year later (1968). Here the plants in row 1, the offspring of the *lewisii*-like F_2 individual, are quite uniformly vigorous and starting to flower, those in row 2 from the F_1-like F_2 plant show extreme variability due to segregation, with only a small proportion of survivors, and those in row 3, the offspring of the *cardinalis*-like F_8, have all succumbed to winter-killing.

**Genetic coherence.** Earlier *Year Books* (62, pp. 387–391 and 65, pp. 433–435) have reported evidence for partial genetic linkages between groups of marker characters that distinguish such species as *M. lewisii* and *M. cardinalis*. Such combinations of characters are inherited in second- and third-generation progenies in recombination frequencies that indicate that purely random assortment of such characters does not occur; parental combinations of characters tend to segregate together with greater frequency than would be predicted on the basis of free random recombination. That such characters also tend to be correlated with the responses and survival of individual plants at the transplant stations is now also fully evident. Extensive data from repeated crossings of both F_2 and F_3 progeny in *Mimulus* have been analyzed statistically with the help of an IBM 360/67 computer and clearly reveal the existence of such partial linkages. Although the observed recombinations of characters in the F_2 are always striking and spectacular, they are fewer than would be predicted on the basis of free random recombination. The expression of hybrid vigor, or heterosis, in first- and second-generation progeny in both inter- and intra-specific combinations in relation to the parental races is about as dependent on the environment in which it is observed (i.e., at the transplant stations) as upon the genetic constitution of the parents.
Physiological studies. The comparative study of species, races, and hybrids at the physiological and biochemical level as a means of probing further into mechanisms underlying natural selection requires the development of quite different techniques from those employed in the cytogenetic and transplant studies. It is, however, of enormous advantage to use the same cloned plant materials in comparative quantitative physiological studies from which a wealth of background information is available in order that the data from the various approaches can be effectively integrated. This does not, however, preclude the use of other species that may be valuable as reference points in comparative studies.

The physiological studies on *Mimulus* have centered on the study of the photosynthetic performance under a variety of controlled variables. These studies have led to ramifications that involve basic questions concerning the mechanism of some of the various steps in the photosynthetic process itself and how alterations in particular steps appear to affect the resultant performance of genetically distinct ecological races and species. Earlier Year Books have reviewed these developments, and current new findings are reported in the following pages. In the *Mimulus* monograph the physiological work will be reviewed to date, together with a report of preliminary studies on the culture of excised tissues under aseptic conditions as an aid in the comparative study of physiological and biochemical characteristics of ecological races and species.

Growth, Photosynthetic, and Biochemical Responses of Contrasting *Mimulus* Clones to Light Intensity and Temperature

Olle Björkman, Malcolm A. Nobs, and William M. Hesey

In recent months we have concentrated on a study of two contrasting clones of *Mimulus* and their F<sub>1</sub> progeny. The parental clones, as shown by the transplant and genetic investigations, are among the most contrasting members of the Erythranthe section with respect to their growth and survival at the Stanford, Mather, and Timberline transplant stations, yet are genetically compatible and differ in a large number of conspicuous morphological characters. One clone, 7635-2, is a form of *M. lewisii* from Logan Pass, Glacier National Park, at an elevation of 2100 m, and the other is a clone of *M. cardinalis*, 7211-4, originally from the hot foothills of the Sierra Nevada of California at Jacksonville at an altitude of 250 m. The environments of the two races represent temperature extremes at which members of the Erythranthe section naturally occur.

Responses when grown under different light intensities. Figure 33 shows the mean dry weight increases in growth of the two clones over a 17-day experimental period when subjected to incident light intensities of 18,000, 53,000, and 106,000 ergs cm<sup>-2</sup> sec<sup>-1</sup>. In the experiment the daylength was 16 hours, and temperature was held constant at 21°C. The CO<sub>2</sub> and O<sub>2</sub> concentrations were those of normal air. The plants were grown in Perlite in plastic pots and were watered with Hoag-
land's nutrient solution. The 17-day experimental period was considered long enough to provide a good measure of growth response to different light intensities, and short enough for growth to be exponential. As shown in Fig. 33 the growth rates of the two clones under these experimental conditions were directly proportional to the incident light intensity. The growth rate increase for M. lewisii with increasing light intensity was somewhat greater than for M. cardinalis.

The light-saturated photosynthetic rates of both clones when measured at 20°C both at concentrations of 21.0% and 1.5% of O_2 are generally higher as the light intensity during growth is increased, as shown in the upper part of Table 9. A significant difference between the two clones is evident, however, in their relative rates when grown under the intermediate intensity of 53,000 ergs cm^{-2} sec^{-1} as compared with the highest at 106,000 ergs cm^{-2} sec^{-1}. In Logan the increase in photosynthetic rate at the higher light intensity when measured in 21% O_2 is much smaller than in Jacksonville. The differences are even greater when the light-saturated photosynthetic rates are measured in 1.5% O_2. The rates measured on this Logan clone are higher than those observed on M. lewisii from Timberline, clone 7405-4 (Year Book 65, pp. 464–468). This suggests that considerable diversity exists within M. lewisii in light-saturated photosynthetic rates when measured under 21% O_2 present in normal air.

Anatomical sections of leaves of the two clones reveal parallel modifications in leaf thickness and in number of cell layers when grown under the three light intensities. Leaf thickness of both clones was approximately doubled when grown under the highest light intensity as compared with the lowest (i.e., at 106,000 versus 18,000 ergs cm^{-2} sec^{-1}). At the intermediate light intensity the leaves were of intermediate thickness. The leaves of the M. lewisii clone were about 25% thicker than those of M. cardinalis under any given intensity. The increase in leaf thickness as a result of increased light intensity is attributable to a greater number of cell layers in both the palisade and spongy parenchyma as well as to greater cell size.

It might be anticipated that the rate of light-saturated photosynthesis on the basis of leaf area should increase with increasing leaf thickness if the composition of biochemical components inside the leaves that determines the capacity

<table>
<thead>
<tr>
<th>Light Intensity for Growth, erg cm^{-2} sec^{-1} x 10^6</th>
<th>Logan 7635-2</th>
<th>Jacksonville 7211-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Photosynthesis at 20°C and 0.03% CO_2, μmole CO_2 dm^{-2} min^{-1}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>In 21% O_2</td>
<td>6.6</td>
<td>11.0</td>
</tr>
<tr>
<td>In 1.5% O_2</td>
<td>8.8</td>
<td>17.9</td>
</tr>
<tr>
<td>Carboxydismutase Activity, μmole CO_2 (g fresh wt.)^{-1} min^{-1}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>In 21% O_2</td>
<td>4.7</td>
<td>10.0</td>
</tr>
<tr>
<td>In 1.5% O_2</td>
<td>1.33</td>
<td>1.53</td>
</tr>
<tr>
<td>Chlorophyll a + b, mg (g fresh wt.)^{-1}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>In 21% O_2</td>
<td>9.3</td>
<td>20.6</td>
</tr>
</tbody>
</table>

1 Assay conditions: 0.05 M NaHCO_3; 4 x 10^{-4} M ribulose-1,5-diphosphate; pH 8.0, 30°C.
2 Protein was determined by the Folin-Lowry method.
for light-saturated photosynthesis remains constant. On the other hand, in normal air where photosynthesis is partially limited by CO₂ concentration, one might predict that light-saturated photosynthesis would not increase proportionally to leaf thickness because of the greater diffusion resistance to CO₂ in the thicker leaves. The actual experimental data reveal that photosynthesis increases faster as the leaves become thicker than would even be predicted on the basis of the first hypothesis. This result points to a third possibility, namely, that biochemical components inside the leaves that limit light-saturated photosynthesis have increased with increasing light intensity when computed on a fresh-weight or unit-volume basis.

The data presented in Table 9 support this conclusion. In both clones carboxydismutase activity is highly modified by the light intensity during growth; the higher activities occur in plants grown under the higher light intensities. The pattern of modification in the two contrasting clones differs in that Logan shows little increase in the activity of the enzyme when grown under the intermediate as compared with the highest light intensity, whereas the activity in Jacksonville in this step is approximately doubled. There is, thus, a good correlation between rate of photosynthesis at light saturation and the carboxydismutase activity. This is in agreement with the results obtained in this laboratory with other species (Björkman, 1968, a, b; Gauhl, this Year Book).

The chlorophyll content, expressed on a fresh weight basis in the leaves of both clones, generally increases with increasing light intensity during growth, but the increase is much smaller than that for carboxydismutase activity. This result is not surprising since the content of light-harvesting pigment would not be expected to have much influence on the rate of photosynthesis in the light-saturated state. The content of soluble protein, on the other hand, closely parallels the carboxydismutase activity and the photosynthetic rate in both clones. This suggests that the levels of enzymes other than carboxydismutase may also be modified in a similar manner by different light intensities during growth.

Responses to differences in temperature. The differential effects of the two temperatures, 10°C as compared with 30°C, on the growth of the M. lewisii clone 7635-2 (Logan) and the M. cardinalis clone 7211-4 (Jacksonville) are illustrated in Plate 2. The photographs shown were taken one month after comparable cuttings of each clone were placed in controlled cabinets and subjected to a light intensity of 53,000 ergs cm⁻² sec⁻¹ for 16-hour days, the temperatures being held constant at 10, 20, and 30°C day and night. The nonsurvival of the Logan clone at 30°C under these conditions, as compared with good growth and flowering of the Jacksonville clone, and, conversely, the more active growth of Logan at 10°C as compared with Jacksonville during the 30-day experimental period, is consistent with the very divergent responses of these two clones when grown at the Stanford, Mather, and Timberline transplant stations.

Propagules of the same two clones were subjected to the same experimental treatment and then harvested after a 15-day period before the Logan clone had succumbed at 30°C. This clone had then developed abortive precocious flowering stems having small green leaves. The average net increases in dry weights of the clones during this period are shown in Table 10. It can be seen that the increase in growth of the Jacksonville clone was 2.4 times greater at 30 than at 10°C, whereas in Logan the difference in increment of growth at these contrasting temperatures was approximately the same with a ratio of 0.9.

In view of the marked hybrid vigor observed at the altitudinal transplant stations in F₁ progeny between M. lewisii and M. cardinalis described in the pre-
TABLE 10. Effect of Temperature on Growth

<table>
<thead>
<tr>
<th>Clone</th>
<th>Grown at 10°C</th>
<th>Grown at 30°C</th>
<th>Ratio, Growth at 30°C to Growth at 10°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Logan 7635-2</td>
<td>139 ± 8.9*</td>
<td>126 ± 24.4</td>
<td>0.9</td>
</tr>
<tr>
<td>Jacksonville 7211-4</td>
<td>140 ± 7.0</td>
<td>327 ± 32.2</td>
<td>2.4</td>
</tr>
<tr>
<td>F1 Logan 7635-2 × Jacksonville 7211-4</td>
<td>232 ± 14.7</td>
<td>277 ± 25.0</td>
<td>1.2</td>
</tr>
</tbody>
</table>

* Standard error of the mean.

ceeding section, an experiment was conducted in which the growth of F1 seedling progeny of the M. lewisi clone Logan 7635-2 and the M. cardinalis clone Jacksonville 7211-4 was compared with the growth of rooted cuttings of the parents at 10 and 30°C. The conditions of illumination and CO₂ and O₂ concentration were the same as in the preceding temperature experiments. Although the 14 seedling F1 replicates grown at each temperature were fairly variable, their growth response at 30°C was intermediate between the parents, as shown by the mean net dry weight increases listed in Table 10. Evidence of hybrid vigor under the high temperatures is therefore lacking, but at 10°C the apparently greater growth of the hybrid over that of the parents may be significant.

Determinations of carboxydismutase activity, of the content of soluble protein, and of chlorophyll in the leaves were made on the Logan and Jacksonville clones grown at 10, 20, and 30°C. All determinations were made during the period when both clones were in active growth at all three temperatures. The results of these determinations are listed in Table 11.

In both clones the soluble protein content of the leaves was higher at 10 than at 20 or 30°C. This marked accumulation of soluble protein at low temperature is a point of interest for which no satisfactory explanation is at hand. In the Jacksonville clone where this accumulation is particularly great, there is no corresponding increase in carboxydismutase level. The activity of this enzyme is about the same regardless of whether the clone was grown at 10, 20, or 30°C.

In the Logan clone the carboxydismutase activity is about the same in leaves that have been grown at 10 and 20°C.

TABLE 11. Effect of Temperature During Growth on Subsequent Levels of Carboxydismutase, Chlorophyll and Soluble Protein in the Leaves of Two Mimulus Clones

<table>
<thead>
<tr>
<th></th>
<th>Logan 7635-2</th>
<th>Jacksonville 7211-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carboxydismutase activity,¹ mol CO₂ (g fresh wt.)⁻¹ min⁻¹</td>
<td>10.5</td>
<td>10.0</td>
</tr>
<tr>
<td>Chlorophyll a + b, mg (g fresh wt.)⁻¹</td>
<td>1.62</td>
<td>1.53</td>
</tr>
<tr>
<td>Soluble protein,² mg (g fresh wt.)⁻¹</td>
<td>26.2</td>
<td>20.0</td>
</tr>
</tbody>
</table>

¹ Assay conditions: 0.03 M NaHCO₃; 10⁻⁴ M ribulose-1,5-diphosphate; pH 8.0, 30°C.
² Protein was determined by the Folin-Lowry method.
The activity is considerably higher than in the Jacksonville clone. However, in the Logan clone growth at 30°C results in a very much reduced carboxydamutase activity. Similarly, the chlorophyll content of the leaves was much reduced at 30°C as compared with 20°C and 10°C in Logan whereas in Jacksonville the corresponding values were essentially the same at the three different temperatures. These results point to a breakdown of the photosynthetic apparatus in the Logan clone at 30°C, whereas there is no evidence of any detrimental effects of high temperature in the Jacksonville clone. We do not know whether the detrimental effects of high temperature in the Logan clone are primarily due to an intrinsically low degree of temperature stability of its photosynthetic apparatus, or whether processes responsible for the continuous synthesis of its components, such as photosynthetic pigments and enzymes, are adversely affected by high temperature so that the rate of their synthesis does not keep up with the rate of their breakdown.

Light-saturated photosynthesis in *Mimulus* and in many other higher plants as measured in air containing normal CO₂ and O₂ concentrations is often characterized by a comparatively small dependence on temperature in the range 15-30°C. This relatively weak effect of temperature on light-saturated photosynthesis has been generally interpreted to mean that photosynthesis is limited mainly by physical barriers to CO₂ diffusion since any process that is limited by enzyme activity would be expected to exhibit marked temperature dependence.

Newer evidence now indicates that the small effect of temperature on the photosynthetic rate as observed in normal air is largely due to the inhibiting effect of O₂ on photosynthetic CO₂ uptake. As shown in Fig. 34, the temperature dependence in both the Jacksonville and Logan clones is much greater in 1.5% than in 21.0%. These results are in close agreement with those obtained by Joliffe and Tregunna (1968) for wheat leaves, and by us with *Marchantia* (Year Book 67, pp. 479-482), but are at variance with those previously reported by us for *Mimulus* (Year Book 66, pp. 222-225).

In 1.5% O₂ and 0.03% CO₂ the Arrhenius equation is approximately valid for both the Logan and Jacksonville clones of *Mimulus* in the range 5-15°C. At the higher CO₂ concentration of

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**Fig. 34.** Temperature dependence of light-saturated photosynthetic rate in the Logan and Jacksonville clones of *Mimulus* under 1.5% and 21.0% O₂. Measurements were made under saturating white light of 3.4 \( \times 10^8 \) erg cm⁻² sec⁻¹ intensity (400-700 nm) from a 2.5 KW Xenon lamp and a CO₂ concentration of 0.030%.
0.07% a linear relationship between the logarithm of the photosynthetic rate and
the inverse of absolute temperature is
obtained up to at least 27°C (cf. this
Year Book, Fig. 51). Energies of activa-
tion calculated from Arrhenius plots
for the Mimulus clones yielded values
of approximately 16 to 19 Kcal mol⁻¹
equivalent to Q₁₀ values of 2.7 to 3.3.
These are comparatively high values for
biological reactions.

It is of great interest that we have
obtained very similar values for CO₂
fixation in vitro with partially purified
preparations of carboxydismutase from
both Mimulus and Marchantia. The
close agreement between the activation
energies for photosynthesis and the
carboxylation reaction in vitro might,
of course, be coincidental. On the other
hand, it could reflect a causal relation-
ship. A close agreement between mea-
surements of the two processes would
be expected if the activation energies for
the carboxydismutase-catalyzed reaction
in vitro is approximately the same as in
vivo, and if the carboxylation reaction
is a major limiting step of light-saturated
photosynthetic rates at temperatures be-
low 15°C.

No marked differences in activation
energy for light-saturated photosynthe-
sis were found between the Logan and
the Jacksonville clones when both were
previously grown at 20°C at a light in-
tensity of 55,000 erg cm⁻² sec⁻¹ (400–
700 nm). The main difference between
the two is that Logan exhibits a higher
rate of photosynthesis than Jacksonville
at all temperatures in the range 5–
30°C. Another difference is that in Logan
the rate declines at temperatures above
approximately 25°C, whereas in Jack-
sonville such a decline is not apparent
until the temperature considerably ex-
ceeds 30°C.

A question of great importance is to
what extent the temperature dependence
of photosynthesis may be affected by
the temperature under which the plants
are previously grown. This problem was
investigated on Marchantia last year
(Year Book 67, pp. 479–482). The results
of similar experiments using the Jack-
sonville clone 7211-4 of Mimulus are
shown in Fig. 35. The dependence of the
light-saturated rate of CO₂ uptake on
temperature was determined on intact
attached leaves of plants previously
grown at 10, 20, and 30°C.

As is evident in Fig. 35, the rate of
photosynthesis at 15°C and 1.5% O₂ was

![Fig. 35. Effect of temperature during growth on subsequent temperature dependence of light-
saturated photosynthetic rates under 1.5% and 21.0% O₂ in the Jacksonville clone of M. cardinalis.
Conditions for measurements were the same as in Fig. 34. The rate at 15°C in 1.5% O₂ was set to
unity for each leaf.](image-url)
little affected by the temperature under which the plants were grown. To facilitate direct comparisons of the temperature curves shown, the light-saturated photosynthetic rate as measured at 15°C and under 1.5% O₂ is plotted as equal to unity for each of the three clone-members previously grown at the three temperatures.

The temperature dependence of photosynthesis when measured under both 1.5% and 21.0% O₂ concentrations is very similar for leaves previously grown at 10 and 20°C. There is, however, a slight shift of the optimum toward lower temperatures in the clone-member grown at 10°C as compared with the one at 20°C. When grown at 30°C, a more pronounced change in the shape of the temperature curve takes place, with considerably higher light-saturated photosynthetic rates at the higher temperatures than in the clone-members grown at 10 or 20°C. This effect is evident in measurements made both in 21.0% and 1.5% O₂, but it is more pronounced at the lower O₂ concentration. This suggests that the modification in temperature dependence is not caused by changes in the rate of the processes underlying the inhibiting effect of O₂ (photorespiration). The activation energy from photosynthesis in the range 5-10°C is, nevertheless, not significantly affected by any of the three temperatures under which the Jacksonville clone of M. cardinalis was grown.

Conclusions. At this stage of our still incomplete understanding of the physiological and biochemical mechanisms that operate in higher plants, each increment of new experimental information changes our concepts of them. An example is our current interpretation of temperature dependence for light-saturated photosynthesis as a result of measurements made under low as compared with high O₂ concentration, discussed above.

It is evident that the two contrasting clones of M. lewisii and M. cardinalis differ markedly in both physiological and biochemical characteristics when studied under certain sets of controlled conditions. At the same time they possess many characteristics in common that are also shared by such unrelated plants as the liverwort Marchantia. The experimental techniques for exploring the physiological and biochemical basis of natural selection have now been developed to a point where new basic information is coming to light that needs further exploration and analysis before a satisfying understanding can be achieved.

References

Comparative Studies of Atriplex Species with and without β-Carboxylation Photosynthesis and their First-Generation Hybrid
Olle Bjorkman, Eckard Gaulth, and Malcolm A. Nobs

The recent discovery of a new CO₂ fixation pathway in photosynthetic organisms, first found to be operative in sugar-cane (Kortschak et al., 1965; Hatch and Slack, 1966), has stimulated new interest in comparative studies of photosynthesis among higher plants. Several groups of investigators, particularly in Australia, Canada, and the United States, are currently studying the biochemistry of the new carboxylation pathway and its relation to photosynthetic characteristics and leaf structure. This pathway is commonly referred to as the C₄-dicarboxylic acid pathway since oxaloacetate, malate and aspartate are the first products of CO₂ fixation, or as β-carboxylation photosynthesis since it involves β-carboxylation of phospho(enol)pyruvate. Members of at least five different families of higher plants...
belonging to both the monocotyledoneae and the dicotyledoneae (Hatch et al., 1967; Johnson and Hatch, 1968) have been shown to possess this pathway. Within each of four different genera, including *Atriplex*, some species possess the pathway while others do not.

Of particularly great interest to those of us concerned with mechanisms of adaptation and natural selection in plants are the observations that (1) the β-carboxylation pathway seems to be mainly limited to taxonomic groups distributed mostly in tropical and subtropical regions, and (2) that plants which possess this pathway apparently have profoundly different photosynthetic characteristics than do plants in which it is absent. In the plants possessing β-carboxylation the compensation point for CO₂ exchange in air approaches zero, and illuminated leaves do not release CO₂ into CO₂-free air. Moreover, the strong inhibiting effect of oxygen on the rate of photosynthetic CO₂ uptake in normal air, which is a very widespread phenomenon among plants without the β-carboxylation pathway, is absent in plants having this pathway.

The finding that differentiation in carboxylation pathways exists among species within the same genus provides a unique opportunity for studying biochemical and physiological mechanisms of adaptation. Not only are such species a more favorable material for comparative work than plants of unrelated taxonomic groups, but also they may permit studies of the inheritance of the different photosynthetic pathways if the species have sufficient genetic compatibility to allow hybridization between them. In those cases where different carboxylation pathways have been found among species within a single genus heretofore, the species belonged to different subgenera and may not be sufficiently closely related to permit intercrossing.

In our own search for suitable experimental plants, we chose two species of the family Chenopodiaceae, *Atriplex patula* ssp. *hastata* and *A. rosea* L., both of which are very common in California. In their biosystematic work on North American species of *Atriplex*, Hall and Clements (1923) wrote: "The most closely related species (to *A. patula*) seem to be the *rosea* group... There is no direct connection with any other group." Both species are annuals and are diploid with nine pairs of chromosomes.

*A. patula* L. is a common species in coastal marshes throughout North America with the possible exception of Mexico, and is widely distributed also in Europe and Asia. There is great morphological variation in this species, and many subspecies have been recognized; frequently these subspecies have morphological variation in this species. The *A. patula* material used in the present work was collected in a coastal salt marsh close to San Mateo Beach State Park, Pescadero, California, and was identified as ssp. *hastata* Hall and Clem. (= var. *hastata* Gray). It is a common plant in salt marshes along the Pacific coast of California, Oregon, and Washington.

*A. rosea* L. is a native of Eurasia with its main distribution from central Asia through southeastern Europe and the Orient. It is also common in major Mediterranean Islands as well as in Morocco and Egypt. The species is naturalized in western United States and is very abundant in semiarid places in the hot interior of the region, where it often occurs together with *A. semibaccata*, a naturalized introduction from Australia. In California *A. rosea* is a common plant in the interior valleys, but it occurs also in the southern part of the San Francisco Bay area where the present material was collected. Interestingly, both *A. patula* ssp. *hastata* and *A. rosea* are abundant in this area although also here the latter species occupies much drier locations than does the former.

Up to the present time species that have been found to possess β-carboxyla-
tion photosynthesis also possess a specialized leaf anatomy characterized by a layer of large chloroplast-containing cells which surround the vascular bundles. Whether or not this specialized leaf anatomy is essential for the functioning of \( \beta \)-carboxylation photosynthesis has not yet been established, but it is nevertheless a valuable characteristic in preliminary screening of species. Moser (1934) described almost 100 \( \textit{Atriplex} \) species, many of which, including \( \textit{A. rosea} \), possess this specialized leaf structure, whereas others such as \( \textit{A. patula} \) do not. This characterization of \( \textit{A. rosea} \) and \( \textit{A. patula} \) has recently been confirmed by Downton et al. (1969) and in this laboratory for our own material.

During the past year comparative studies of the two species' growth, as well as their biochemical and photosynthetic characteristics, have been made and are reported below. Concurrently we attempted to hybridize the two \( \textit{Atriplex} \) species. This led to success only recently when \( F_1 \) hybrids between \( \textit{A. rosea} \) and \( \textit{A. patula} \) were obtained; some early results with these hybrids are included at the end of this report.

The plant material used in the comparative studies on biochemical and photosynthetic characteristics was grown in controlled growth cabinets under a light intensity of \( 1.1 \times 10^8 \text{ erg cm}^{-2} \text{ sec}^{-1} \) (400-700 nm), a temperature of 25°C, and a photoperiod of 16h/day, except in the experiments summarized in Table 5 where the light intensity during growth was half of that given above. Young, mature leaves were used throughout the investigation. All photosynthesis measurements were made on single leaves attached to intact plants.

**Activities of carboxylation enzymes.** In plants with \( \beta \)-carboxylation photosynthesis, oxaloacetate, malate, and aspartate are the first products to be labeled when \(^{14}\text{CO}_2\) is fed to photosynthesizing leaves. There is strong evidence that phospho(enol)pyruvate (PEP) serves as substrate in the carboxylation reaction. This is further supported by the finding that plants with \( \beta \)-carboxylation photosynthesis have very high activities of PEP carboxylase whereas plants lacking this pathway have low activity of this enzyme (Hatch et al., 1967; Johnson and Hatch, 1968). As shown in Table 12, \( \textit{A. rosea} \) has about 50 times higher PEP carboxylase activity than \( \textit{A. patula} \). The values are in excellent agreement with those reported by Hatch and co-workers for other species with and without \( \beta \)-carboxylation photosynthesis.

Although the activity of carboxydismutase is considerably higher in \( \textit{A. patula} \) than in \( \textit{A. rosea} \), the values for the latter species are very much higher than those reported by Hatch and co-workers for other species with \( \beta \)-carboxylation. A comparison of carboxydismutase activity in species with \( \beta \)-carboxylation and species that lack this pathway revealed that the activity found in \( \textit{A. rosea} \) is not unusually high in comparison with other \( \beta \)-carboxylation species; neither was there consistently lower

<table>
<thead>
<tr>
<th>Species</th>
<th>PEP Carboxylase Activity, ( \mu \text{mol CO}_2 ) per min per Fresh Weight, g</th>
<th>Soluble Protein, mg</th>
<th>Chlorophyll, mg</th>
<th>Carboxydismutase Activity, ( \mu \text{mol CO}_2 ) per min per Fresh Weight, g</th>
<th>Soluble Protein, mg</th>
<th>Chlorophyll, mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \textit{A. patula} )</td>
<td>0.6</td>
<td>0.03</td>
<td>0.44</td>
<td>11.52</td>
<td>0.590</td>
<td>8.41</td>
</tr>
<tr>
<td>( \textit{A. rosea} )</td>
<td>26.8</td>
<td>1.54</td>
<td>14.41</td>
<td>4.60</td>
<td>0.265</td>
<td>2.47</td>
</tr>
</tbody>
</table>

*Enzyme activities were measured at 30°C as described by Björkman and Gauhl (1969).*
carboxydismutase activity in the species that have β-carboxylation photosynthesis as compared to those that lack it. *A. patula* is outstanding in that it has the highest activity of all species investigated. These results are summarized in Table 13 and a full account of the study is given elsewhere (Björkman and Gauhl, 1969).

Fractionation of total soluble leaf protein on Sephadex G-200 (see pp. 636–637, this Year Book) in two species with similar carboxydismutase activities and protein contents, one with β-carboxylation and one without, indicated that approximately half of the total soluble protein was located in the “fraction 1” protein peak in both species. Therefore, the presence of comparatively high carboxydismutase activities for species with β-carboxylation is apparently indicative of similarly high concentrations of this enzyme. Other experiments showed that it is unlikely that the great differences between the carboxydismutase levels obtained here and those reported by Hatch and co-workers are caused by differences in the light, or in the temperature regimes under which the plants were grown.

Experiments in which different grinding procedures were compared revealed that, while nearly complete cell breakage could readily be achieved with leaves of *A. patula* and other species without β-carboxylation, breakage of the thick-walled sheath cells surrounding the vascular bundles in *A. rosea* and other species with β-carboxylation proved quite difficult. Special measures were required to break these cells. The mesophyll cells of *A. rosea* leaves were, on the other hand, very easy to break. High carboxydismutase activity in leaf extracts of species with β-carboxylation was obtained only when a high degree of breakage of the bundle sheath cells had been achieved. Further experiments in which *A. rosea* leaves were subjected to progressively more vigorous grindings showed that carboxydismutase activity increased as an increasing fraction of the protein was released from the bundle sheath cells, indicating that the chloroplasts in these cells contain carboxydismutase levels similar to those present in the mesophyll cells of species without β-carboxylation photosynthesis. These results strongly suggest that, in *A. rosea* and other species with β-carboxylation, at least the bundle sheath cells are capable of CO₂ fixation via the reductive pentose-phosphate pathway.

PEP carboxylase activity in leaf extracts of *A. rosea* was, on the other hand, high even when only a minor fraction of the bundle sheath cells had been broken, and the activity did not increase with increased breakage of these cells. These results provide evidence that high PEP carboxylase levels are present in the mesophyll cells of *A. rosea*. Apparently, the bundle sheath cells contain little or no PEP carboxylase. This finding would indicate that the chloroplast-containing bundle sheath cells in plants with β-carboxylation photosyn-

<table>
<thead>
<tr>
<th>Species with β-carboxylation</th>
<th>Carboxydismutase Activity</th>
<th>Species without β-carboxylation</th>
<th>Carboxydismutase Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Distichlis spicata</em></td>
<td>3.6</td>
<td><em>Elymus mollis</em></td>
<td>4.1</td>
</tr>
<tr>
<td><em>Paspalum distichum</em></td>
<td>2.2</td>
<td><em>Mimulus cardinalis</em></td>
<td>5.2</td>
</tr>
<tr>
<td><em>Zea mays</em></td>
<td>2.3</td>
<td><em>Solanum dulcamara</em></td>
<td>3.7</td>
</tr>
<tr>
<td><em>Amaranthus edulis</em></td>
<td>2.0</td>
<td><em>Plantago lanceolata</em></td>
<td>3.0</td>
</tr>
<tr>
<td><em>Atriplex semibaccata</em></td>
<td>4.9</td>
<td><em>Boehlta spathulata</em></td>
<td>4.5</td>
</tr>
<tr>
<td><em>Atriplex rosea</em></td>
<td>2.5</td>
<td><em>Atriplex patula</em></td>
<td>8.4</td>
</tr>
</tbody>
</table>

*Assay conditions were as described by Björkman and Gauhl (1969). Activity at 30°C is expressed in μ mole CO₂ (mg chlorophyll)⁻¹ (min)⁻¹.
thesis are not responsible for the \( \beta \)-carboxylation of PEP. However, since PEP carboxylase seems to be rather unstable in crude enzyme preparations of \( A. \) rosea leaves, the results cannot be taken as conclusive evidence for the absence of PEP-carboxylase in bundle sheath cells.

These results appear to be consistent with the hypothesis that there are two consecutive carboxylation reactions in plants with \( \beta \)-carboxylation photosynthesis. In the first carboxylation, \( CO_2 \) reacts with PEP to form \( C_4 \)-dicarboxylic acids, the reaction being catalyzed by PEP carboxylase. This newly formed carboxyl group might then be transferred to ribulose-1,5-diphosphate to form phosphoglyceric acid either by a "transcarboxylation," or the carboxyl group may be first decarboxylated to \( CO_2 \), which is then fixed in a conventional Calvin cycle carboxylation, mediated by carboxydismutase. If PEP carboxylase is indeed absent in the bundle sheath cells, then it seems likely that the first carboxylation occurs in the mesophyll cells, and the second occurs primarily in the bundle sheath cells.

Glycolate oxidase activity. There is considerable evidence to support the hypothesis that the inhibitory effect of \( O_2 \) on photosynthetic \( CO_2 \) uptake in higher plants without \( \beta \)-carboxylation photosynthesis is caused primarily by a re-oxidation of photosynthetic products (that is, photorespiration), and that glycolate is a major component of the evolution of \( CO_2 \) by illuminated leaves in \( CO_2 \)-free air. Previous studies in which it was found that the activity of glycolate oxidase was very much lower in species with \( \beta \)-carboxylation than in species lacking this pathway suggest that this enzyme is responsible for the evolution of \( CO_2 \) in the light (Tregunna, 1966; Oeser et al., 1968). In later work where species of the dicotyledonous genera \( \text{Amaranthus} \) (Tolbert et al., 1969) and \( \text{Atriplex} \) (Osmond, 1969) were included, the differences in glycolate oxidase activity between species with and those without \( \beta \)-carboxylation were much less pronounced although still considerable.

Table 14 shows the rates of \( O_2 \) uptake in leaf homogenates of \( A. \) patula and \( A. \) rosea in the presence and in the absence of glycolate. Evidently, the activity of glycolate oxidase in \( A. \) rosea is about two-thirds as high as that found in \( A. \) patula. The true values for \( A. \) rosea may possibly be even higher than shown here since the breakage of the vascular bundle sheath cells was not quite complete. As with carboxydismutase, high activities of glycolate oxidase in \( A. \) rosea extracts were obtained only when good breakage of bundle sheath cells had been achieved.

In view of the high glycolate oxidase activity in \( A. \) rosea extracts, it appears unlikely that the absence of an effect of \( O_2 \) concentration on photosynthetic \( CO_2 \) uptake, and of other characteristics commonly attributed to photorespiration, can be explained by differences in the activity of glycolate oxidase.

\( CO_2 \) compensation point for photosynthesis. The \( CO_2 \) compensation point (the \( CO_2 \) concentration at which the rates of \( CO_2 \) fixation and production are equal) is known to be close to zero at \( O_2 \) concentrations of less than one or two percent, and to increase linearly with increased \( O_2 \) concentration in species without \( \beta \)-carboxylation photosynthesis. In species with \( \beta \)-carboxylation, however, the \( CO_2 \) compensation point remains very low regardless of the \( O_2 \) concentration. As shown in Fig. 36, the response in \( A. \) patula is typical of species without

<table>
<thead>
<tr>
<th>Species</th>
<th>Addition</th>
<th>Oxygen Uptake nmo l min(^{-1}) (mg chlorophyll)(^{-1})*</th>
</tr>
</thead>
<tbody>
<tr>
<td>( A. ) patula</td>
<td>None</td>
<td>30</td>
</tr>
<tr>
<td>( A. ) patula</td>
<td>Glycolate</td>
<td>200</td>
</tr>
<tr>
<td>( A. ) rosea</td>
<td>None</td>
<td>40</td>
</tr>
<tr>
<td>( A. ) rosea</td>
<td>Glycolate</td>
<td>150</td>
</tr>
</tbody>
</table>

* Glycolate concentration was 0.01 \( M \).
DEPARTMENT OF PLANT BIOLOGY

Fig. 36. Effect of O₂ concentration on CO₂ compensation point for photosynthesis in 21% O₂ in A. patula at three different leaf temperatures. Light intensity was 1.0 × 10⁶ erg cm⁻² sec⁻¹ (400-700 nm).

β-carboxylation. The compensation point is strongly dependent on temperature, but at any given temperature the compensation point is directly and linearly related to O₂ concentration. In A. rosea the compensation point is less than a few ppm CO₂ at all O₂ concentrations in the range 0-40%, a response characteristic of species with β-carboxylation photosynthesis.

The differential response between the two Atriplex species with regard to the CO₂ compensation point is further illustrated in Fig. 37 where the compensation point in 21% O₂ is plotted as a function of leaf temperature. In A. patula the compensation point increases exponentially with increasing leaf temperature, whereas in A. rosea it remains very low throughout the entire temperature range from 5 to 36°C. Incidentally, even though the compensation point is not in itself a rate, the data given in Fig. 37 for A. patula yield a nearly straight line in an Arrhenius plot. The “activation energy” derived from such a plot is equal to approximately 7600 calories (Q₁₀ = 1.56).

Transient changes in photosynthetic rate following changes in light intensity. Fig. 38 illustrates the time-course of photosynthetic CO₂ uptake by A. patula and A. rosea leaves in response to a sudden decrease in light intensity under 1% and 21% O₂. In A. patula the steady-state rates at both the higher and the lower light intensities are about 50% greater in 1% than in 21% O₂, whereas in A. rosea the rates are unaffected by O₂ concentration. Under 21% O₂ A. patula leaves show a pronounced “undershoot” in the rate of CO₂ uptake when light intensity is suddenly decreased, a phenomenon which is presumably closely related to the “post-illuminative burst” of CO₂ evolution that takes place in higher plants without β-carboxylation photosynthesis. Under 1% O₂ these effects are absent. In corn, a species with β-carboxylation, the transient effects are absent under both 1% and 21% O₂ (Year Book 66, pp. 224-227).

These results are consistent with the hypothesis that the effects reflect the temporary continuation of a process leading to CO₂ production which occurs in the previous steady-state, in plants...
without \(\beta\)-carboxylation photosynthesis. However, as shown in Fig. 38, a complex transient change in the rate of \(\text{CO}_2\) uptake occurs in \(A. \text{rosea}\) leaves after the light intensity is suddenly reduced. This effect is absent in corn, but a similar effect was found to be present in another species with \(\beta\)-carboxylation, namely, \(Amaranthus \text{edulis}\) (loc. cit.). The transient effect in \(A. \text{rosea}\) and \(Amaranthus\) is an interesting phenomenon for which no explanation is at hand. Its presence in both 1\% \(\text{O}_2\) and 21\% \(\text{O}_2\) suggests that it may be related to a different process than the effect observed in species without \(\beta\)-carboxylation in 21\% \(\text{O}_2\).

**Effect of \(\text{O}_2\) concentration on the light-saturated photosynthetic rate and on the resistance to gas diffusion.** At constant temperature, \(\text{CO}_2\) concentration and \(\text{CO}_2\) uptake decrease continuously in \(A. \text{patula}\) with increasing \(\text{O}_2\) concentrations in the range of 1–21\%, whereas the rate is little affected in \(A. \text{rosea}\) (Fig. 39). It is of considerable importance to know whether or not this higher rate of light-saturated photosynthesis in \(A. \text{patula}\) in low \(\text{O}_2\) concentration is accompanied by a lower resistance to \(\text{CO}_2\) diffusion from the ambient atmosphere into the leaf. If, as is often assumed, the light-saturated rate of photosynthesis in air of normal \(\text{CO}_2\) and \(\text{O}_2\) concentration

![Figure 38](image1.png)  
Fig. 38. Time-course of photosynthetic \(\text{CO}_2\) uptake in 1\% and 21\% \(\text{O}_2\) by \(A. \text{patula}\) and \(A. \text{rosea}\) leaves following a sudden decrease in light intensity. Arrows indicate the time at which light intensity was reduced from \(2 \times 10^4\) to \(5 \times 10^4\) erg cm\(^{-2}\) sec\(^{-1}\) (400–700 nm). Leaf temperature was 25°C, and \(\text{CO}_2\) concentration 0.030–0.033%.

![Figure 39](image2.png)  
Fig. 39. Effect of \(\text{O}_2\) concentration on light-saturated rate of photosynthetic \(\text{CO}_2\) uptake in \(A. \text{patula}\) and \(A. \text{rosea}\). Light intensity was \(4 \times 10^4\) and \(6 \times 10^4\) erg cm\(^{-2}\) sec\(^{-1}\) (400–700 nm) with \(A. \text{patula}\) and \(A. \text{rosea}\), respectively. Leaf temperature was 25°C, and \(\text{CO}_2\) concentration 0.030–0.032%.
is limited mainly by physical resistance to gas diffusion, then a marked increase in the photosynthetic rate due to a decreased O₂ concentration could not occur without a concomitant decrease in diffusion resistance.

Of the various component physical resistances to gas diffusion, only the one imposed by the stomata can be expected to be influenced by changes in the gaseous composition of the atmosphere. Since any changes in stomatal resistance that would affect the diffusion of CO₂ would also affect the rate of the diffusion of water vapor from the leaf, the influence of O₂ concentration on resistance to CO₂ diffusion can be followed by measuring the rate of transpiration. Simultaneous measurement of CO₂ uptake and transpiration were therefore made at different O₂ concentrations on A. patula and A. rosea leaves. Some of the results of these measurements are presented in Table 15. A full account of this work is given elsewhere (Gauhl and Björkman, 1969).

The results clearly show that resistance to CO₂ diffusion in the gas phase is not significantly influenced by O₂ concentration in leaves of either A. patula or A. rosea in spite of the fact that the rate of light-saturated CO₂ uptake in the former species is enhanced by approximately 50% when the O₂ concentration is reduced from the normal 21% to 1.5%. It can be concluded, therefore, that at least in A. patula the rate of light-saturated photosynthesis in normal air is not limited primarily by physical resistance to gas diffusion even at the comparatively high temperature of 25°C (see p. 634, this Year Book).

Other experiments in which the rate of transpiration in the two Atriplex species was measured at 6 different O₂ concentrations gave no evidence of any effect of O₂ concentration on stomatal diffusion resistance. The resistance of the stomata to water vapor transfer varied from 1.0 to 1.4 sec cm⁻¹ with A. patula and from 1.1 to 1.3 sec cm⁻¹ for A. rosea. Thus, there is no significant difference in resistance to water loss between the two species when they are grown and kept under conditions of ample water supply. This does not, of course, preclude the possibility that the two species may differ in their transpiration rates under water stress. Since under low O₂ concentration the light-saturated rate of photosynthesis is similar in the two Atriplex species, there is no intrinsic difference in the ratio of photosynthesis to transpiration. However, because of the strong inhibiting effect of 21% O₂ on photosynthesis in A. patula, but not in A. rosea, the efficiency of photosynthesis in normal air in terms of water loss is greater in the latter species.

Temperature dependence of light-saturated photosynthesis. There are several reports that species which possess β-carboxylation photosynthesis have higher optimum temperatures for photosynthesis in normal air than plants that lack this pathway. Figs. 40 and 41 show the temperature dependence for photosynthetic CO₂ uptake at a high light intensity of 3.5 × 10⁵ erg cm⁻² sec⁻¹ (400–700 nm) for A. patula and A. rosea, respectively. This light is not fully saturating for A. rosea at high temperatures (Fig. 43). The curve for A. rosea was determined in 21% O₂, but since the rate of photosynthesis in this species is unaffected by O₂ concentration in the range from 6 to 38°C, the curve is also valid for low O₂ concentrations.

The temperature dependence of light-saturated photosynthesis in A. patula is markedly affected by O₂. As was also

<table>
<thead>
<tr>
<th>Species</th>
<th>CO₂ Uptake, μmole cm⁻² min⁻¹</th>
<th>Water Vapor Release, mg dm⁻² min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>21% O₂, 1.5% O₂</td>
<td>21% O₂, 1.5% O₂</td>
<td></td>
</tr>
<tr>
<td>A. patula</td>
<td>12.2</td>
<td>51.6</td>
</tr>
<tr>
<td>A. rosea</td>
<td>21.2</td>
<td>48.0</td>
</tr>
</tbody>
</table>

* Measurements were made under saturating white light, and 0.031–0.032% CO₂ at 25°C.
found with *M. cardinalis* (see p. 618, this *Year Book*), the inhibitory effect of \( \text{O}_2 \) increases with increasing temperature. (It should be noted, however, that the *light-limited* rate of \( \text{CO}_2 \) uptake in both *A. patula* and *Mimulus* is markedly inhibited by 21% \( \text{O}_2 \) at both high and low temperatures; see *Year Book* 67, p. 482). A comparison of the rates of \( \text{CO}_2 \) uptake by the two *Atriplex* species in strong light and normal air shows that in *A. rosea* the rate is more temperature dependent in the upper range. This is consistent with previously reported differences between unrelated species with and without \( \beta \)-carboxylation photosynthesis. However, the temperature dependence is remarkably similar in the two *Atriplex* species when the \( \text{O}_2 \) concentration is kept at a low level, particularly in the low temperature range where the Arrhenius equation is approximately valid. In this range the activation energy is high, and no significant differences in activation energy are apparent between the two species.

These results fail to support the hypothesis that the differences in photosynthetic response to temperature between species with and without \( \beta \)-carboxylation are indicative of different temperature characteristics of the carboxylation enzymes. They rather suggest that the rate-limiting step is either the same in the two species, or, if different steps are limiting, these have approximately the same energies of activation. It is tempting to speculate that carboxydismutase may be a rate-limiting enzyme in both species at low temperatures (cf. p. 619, this *Year Book*).

CO\(_2\) dependence of light-saturated photosynthesis. The response of light-saturated photosynthesis to \( \text{CO}_2 \) concentrations in the two *Atriplex* species is of special interest in view of the presence of high PEP carboxylase activities in *A. rosea* but not in *A. patula*. PEP carboxylase has been reported to have a much higher affinity for \( \text{CO}_2 \) \( (K_m \approx 10^{-4} \text{ M}) \) than carboxydismutase \( (K_m \approx 10^{-2} \text{ M}) \). If these different affinities in vitro result in differences in \( \text{CO}_2 \) depend-
ence of photosynthesis between the two species, then such differences should be apparent under low O₂. Comparisons of the CO₂ dependence of photosynthesis in the two species when in 21% O₂ are complicated by the inhibiting effect of O₂, particularly since the degree of the inhibition by O₂ increased with decreasing CO₂ concentration.

Figure 42 depicts the CO₂ dependence of photosynthetic O₂ evolution under 0.15% O₂ in the two species. The measurements were made as described on pages 637–638. The response to CO₂ concentration is very similar in A. patula and A. rosea, with half of the maximum rate being reached at approximately 0.02% CO₂ in both species. The somewhat earlier saturation in A. rosea in comparison with A. patula may possibly be significant. However, at the light intensity used (4 × 10⁵ erg cm⁻² sec⁻¹; 400–700 nm) photosynthesis in A. patula is light saturated under all CO₂ concentrations, but it is not fully saturated at the highest CO₂ concentrations in A. rosea. This may have resulted in a somewhat lower CO₂ saturation in the latter species. The results thus indicate that the two Atriplex species do not differ markedly in their photosynthetic response to CO₂ concentration when reoxidation of photosynthetic products in the light is suppressed by keeping the leaves at a low O₂ concentration.

The saturation of photosynthesis at low CO₂ concentrations in A. patula appears to be inconsistent with the high Kₘ-values that have been reported for carboxydismutase. However, recent work (Cooper et al., 1969) provides evidence that carboxydismutase cannot utilize bicarbonate, and that only CO₂ itself can serve as substrate for the enzyme. At the pH used for the determination of the Kₘ, the concentration of CO₂ is only on the order of one percent of the total “CO₂” added to the reaction mixture. If the Kₘ is recalculated for CO₂, it becomes 100 times lower than the value based on total “CO₂”. The differences in the Kₘ-values between PEP carboxylase and carboxydismutase based on total “CO₂” may, therefore, not be relevant to photosynthetic CO₂ fixation.

Dependence of photosynthesis on light intensity. It is well known that unusually high light intensities are required to saturate photosynthesis in corn and certain other species recently found to possess β-carboxylation photosynthesis. As shown in Fig. 43 this is also true for A. rosea. Complete light saturation is not reached even at light intensities equal to full sunlight (approximately 5 × 10⁶ erg cm⁻² sec⁻¹, 400–700 nm) in this species whereas in A. patula light saturation is essentially reached at about half this intensity. In normal air (21% O₂, 0.033% CO₂) the rate at high light intensities is considerably higher in A. rosea than in A. patula, but at low light intensities the rates are approximately equal.

Since the degree of enhancement of photosynthesis that takes place in A. patula when O₂ concentration is lowered is approximately the same at all light intensities, and since no enhancement
at all occurs in A. rosea, the former species becomes more efficient in utilizing light of low intensities than A. rosea under 1.5% O₂. These results suggest that the quantum yield for CO₂ uptake under low O₂ is higher in A. patula than in A. rosea.

Experiments in which light-limited rates of CO₂ uptake of the two species were measured in monochromatic light (at 665 nm) provided further evidence that this is indeed the case. As shown in Fig. 44 the quantum requirement for CO₂ uptake under low O₂ concentration is 35-40% higher in A. rosea than in A. patula. This indicates that β-carboxylation photosynthesis may be intrinsically less efficient than conventional photosynthesis in terms of the amount of CO₂ reduced to carbohydrate per amount of energy expended. This would be the case if β-carboxylation photosynthesis requires more ATP or NADPH₂ for each CO₂ fixed.

If this is true the main advantage of
\(\beta\)-carboxylation photosynthesis appears to be the conservation of carbon under high levels of irradiance and high temperatures. Under saturating light intensities the supply of chemical energy would not be expected to be limiting and, therefore, a higher ATP or NADPH \(2\) requirement than in conventional photosynthesis would have no effect on the light-saturated rate of \(\text{CO}_2\) uptake. It should be remembered, however, that photosynthesis in species with \(\beta\)-carboxylation is not completely light-saturated even in full sunlight, and consequently the rate of \(\text{CO}_2\) uptake in such plants in natural habitats will always be partially dependent on the supply of chemical energy. Under low light intensities the benefits gained by eliminating the inhibitory effect of \(\text{O}_2\) in plants with \(\beta\)-carboxylation photosynthesis would be counteracted by the greater requirement for chemical energy. This is consistent with the finding that the quantum requirement for \(\text{CO}_2\) uptake is approximately the same for the two \(Atriplex\) species in normal air (Fig. 44). Comparative measurements of light-limited rates of photosynthesis in other species should reveal whether or not the higher quantum requirement of \(A.\) rosea in comparison with \(A.\) patula under low \(\text{O}_2\) concentration is indeed attributable to \(\beta\)-carboxylation photosynthesis per se.

**Growth responses.** Our comparative studies on the photosynthetic characteristics of the two \(Atriplex\) species demonstrate that in normal air under conditions of high light intensity and high temperature \(A.\) rosea is capable of considerably higher rates of photosynthetic \(\text{CO}_2\) uptake than \(A.\) patula. The differences between the two species decreased with decreasing light intensity and decreasing temperature, and disappeared at rate-limiting light intensities. A question of considerable interest is whether or not these differences in photosynthetic performance are reflected in growth.

Preliminary experiments in which the two \(Atriplex\) species were grown at different light intensities indicate that \(A.\) rosea requires higher light intensities for maximum growth than \(A.\) patula. Nevertheless, both species grow slowly at low light intensity \((1.5 \times 10^4 \text{ erg cm}^{-2} \text{ sec}^{-1}, 400-700 \text{ nm})\) and must be considered to be sun species.

When the two \(Atriplex\) species were grown under a light intensity of \(1.1 \times 10^5 \text{ erg cm}^{-2} \text{ sec}^{-1}\), the optimum temperature for growth was in the range 20-25°C for \(A.\) patula and 25-30°C for \(A.\) rosea. The growth rate of \(A.\) rosea was much greater at 30 than at 15°C, whereas there was no marked difference in growth at these two temperatures with \(A.\) patula. At 38°C \(A.\) patula grew poorly, whereas \(A.\) rosea grew considerably better at this temperature than at 15°C.

The differences that exist between the two \(Atriplex\) species in response of growth to different light intensities and temperatures thus seem consistent with differences in photosynthetic characteristics. Comparative growth experiments at high temperatures and different oxygen concentrations might provide further information on the causal relationship that appears to exist between growth response and photosynthetic characteristics that distinguish plants with and without \(\beta\)-carboxylation photosynthesis. Such growth experiments are planned.

**Characteristics of \(F_1\) hybrids: \(A.\) rosea \(\times A.\) patula.** As mentioned earlier, attempts to hybridize the two \(Atriplex\) species were successful only very recently, and only by using \(A.\) rosea as the female parent. The hybrid material is currently being subjected to intensive investigation with regard to biochemistry, leaf anatomy, and cytogenetics. Some early results are reported here.

Like both of the parents, the \(F_1\) hybrids are diploid, and are highly uniform in appearance, having intermediate morphology and growth habit. As shown in Fig. 45, leaf shape and size are clearly intermediate. This is also true of the
betacyanin content in the lower leaf epidermis; in *A. rosea* the betacyanin is very prominent, whereas it is absent in *A. patula*.

Examination of leaf sections with the light microscope revealed that the general leaf anatomy of the F₁ hybrid is also roughly intermediate between the parents. The mesophyll of the hybrid leaves resembles that of *A. patula*, but the palisade cells are less densely packed. There is also a greater density of chloroplasts in cells around vascular bundles in the hybrid than in *A. patula*, but the large thick-walled bundle sheath cells, characteristic of *A. rosea* and many other species with β-carboxylation photosynthesis, are absent. Cell and chloroplast ultrastructure of the hybrid in comparison with the parental species is now being studied in this laboratory by Dr. John Boynton.

The data presented in Table 16 show clearly that the F₁ hybrid is definitely not intermediate between the parental species with regard to photosynthetic characteristics. Photosynthesis is strongly affected by O₂ concentration in the F₁ hybrid, apparently even more so than in *A. patula*, the male parent. The rate of photosynthesis in the F₁ hybrid is lower than in either parent under 21% O₂.

The activity of carboxydismutase in the F₁ hybrid is only about half of that in *A. patula*, but higher than in *A. rosea*. PEP carboxylase activity in the F₁ hybrid is only about one-tenth of the activity present in *A. rosea*, the female parent, even though it may be slightly higher than in *A. patula*. The chlorophyll content of the leaves, which is about the same in both parental species, is about one-third lower in the hybrid.

These results demonstrate that, in

### TABLE 16. Some Photosynthetic and Biochemical Characteristics of the F₁-Hybrid Between *A. rosea* and *A. patula* in Comparison with the Parental Species*

<table>
<thead>
<tr>
<th>Species</th>
<th>Photosynthesis Rate</th>
<th>Enhancement in 1.5% O₂</th>
<th>Carboxylase Activity</th>
<th>Carboxydismutase Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.5% O₂</td>
<td>21% O₂</td>
<td>%</td>
<td>Activity</td>
</tr>
<tr>
<td><em>A. rosea</em></td>
<td>3.8</td>
<td>3.8</td>
<td>0.0</td>
<td>14.7</td>
</tr>
<tr>
<td><em>A. rosea</em> × <em>A. patula</em></td>
<td>4.1</td>
<td>2.4</td>
<td>0.0</td>
<td>14.4</td>
</tr>
<tr>
<td><em>A. patula</em></td>
<td>6.0</td>
<td>4.1</td>
<td>0.8</td>
<td>0.8</td>
</tr>
</tbody>
</table>

*Photosynthetic rates as well as enzyme activities are expressed in μmole CO₂ min⁻¹ (g fresh wt.)⁻¹. Photosynthesis was measured in white light of an intensity of 1 × 10⁶ erg cm⁻² sec⁻¹ (400-700 nm), at a leaf temperature of 25°C, and a CO₂ concentration of 0.032-0.034%. Enzyme assays were made at 30°C; other conditions were as described by Björkman and Gauhl (1969). The plants were grown at a light intensity of 5.5 × 10⁶ erg cm⁻² sec⁻¹ (400-700 nm).*
Atriplex, photosynthetic and biochemical characteristics associated with β-carboxylation photosynthesis are not transmitted simply by the plastids from the female parent to the progeny. Instead, inheritance of these characteristics appears to be predominantly under nuclear control. The results further suggest that the number of genes that govern the processes underlying β-carboxylation photosynthesis, and the absence of an inhibitory effect of oxygen on CO₂ uptake, may be small.

Attempts are currently being made to obtain second-generation progeny from the F₁ hybrids. If these attempts are successful, it may be possible to find out which of the several correlated characteristics, physiological, biochemical, and anatomical, are essential to photosynthetic function in plants with β-carboxylation photosynthesis, as well as to analyze genetically the inheritance of these characteristics. This could greatly enlighten our understanding of the physiological and molecular mechanisms of adaptive differentiation and natural selection in plants.

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Cooper, T. G., D. Filmer, Marcia Wishnick, and M. D. Lane, J. Biol. Chem., 244, 1081–1083, 1969.

Leaf Factors Affecting the Rate of Light-Saturated Photosynthesis in Ecotypes of Solanum dulcamara

Bokard Gauhl

As reported last year (Year Book 67, pp. 482–488), clones of Solanum dulcamara L. from sunny and shaded habitats show marked differences in their response to light intensity during growth. When propagules of clone Mb 1, originally from a densely shaded habitat in a reed-grass marsh near Mönchbruch, Germany, were grown under a low light intensity of 24 × 10³ erg cm⁻² sec⁻¹ (400–700 nm) and subsequently exposed to a high intensity of 11 × 10⁴ erg cm⁻² sec⁻¹, the leaves showed evidence of photoinhibition. After a few days under the higher light intensity the quantum efficiency of photosynthesis was markedly reduced. No such detrimental effect was detected in leaves of clone Fe 2, which originated from a sunny habitat on an open sand dune on Fehmarn Island in the Baltic Sea. Fully mature leaves of this clone show a considerable increase in light-saturated rate of photosynthesis after transfer to a high light intensity. More recent work revealed that when leaves of the clone from the shaded habitat are subjected to prolonged exposure to high light intensities, not only light-limited rates but also the light-saturated rate of photosynthesis is reduced, as shown in Fig. 46.
The light-saturated rate of apparent photosynthesis in normal air may be limited by physical resistances to gas diffusion as well as by the capacities of enzymatic reactions. It is also affected by the rate of reoxidation of photosynthetic products with O₂ (photorespiration). An attempt was made to determine which of these factors are of greatest importance in determining (1) the decline in photosynthesis when leaves of the shaded habitat clone are transferred from a low to a high light intensity and, conversely, (2) the increase in photosynthesis that takes place when leaves of the sunny habitat clone are transferred from a low to a high light intensity.

**Diffusion resistance and O₂ effect.** To determine whether the changes in the rates of photosynthesis of the *Solanum* ecotypes are caused simply by changes in physical diffusion resistances, simultaneous measurements of CO₂ uptake and transpiration were made with the method described by Gauhl and Björkman (1969). When the transpiration rate is known, the resistance against the transfer of water vapor from the surface of the mesophyll cells to the ambient atmosphere (Rᵥ) can be calculated. This resistance includes the resistance to gas diffusion through the stomatal openings and that of the external boundary layer. Photosynthetic CO₂ uptake involves these same resistances and an additional resistance due to the diffusion of CO₂ in the liquid phase from the mesophyll cell walls to the carboxylation sites in the chloroplasts.

Of these three resistances, only the stomatal diffusion resistance can be expected to be influenced by changes in the gaseous composition of the ambient atmosphere. Since it is well established that the rate of CO₂ uptake of many plants, including *S. dulcamara*, is enhanced when the oxygen concentration surrounding the leaf is reduced from 21% to a lower level, photosynthesis and transpiration were measured simultaneously under alternate O₂ concentrations of 21% and 1.5%, and a constant CO₂ concentration of 300 ppm. If physical barriers to the diffusion of CO₂ into the leaf are mainly limiting the rate of light-saturated CO₂ uptake, enhancement of photosynthesis due to the lower O₂ concentration could not take place without a decrease in the resistance Rᵥ. The results summarized in Table 17 show that the enhancement of the photosynthetic rate in low O₂ found in the leaves investigated is not accompanied by a decrease in Rᵥ. The degree of enhancement was about 50% in all leaves.

Carboxydismutase activity and protein content. There is strong evidence that the activity of certain photosynthetic enzymes, particularly carboxydismutase, may be a major limiting factor determining the light-saturated rate of photosynthesis in leaves (Björkman, 1968; Waring et al., 1968). Parallel increases in protein content and light-saturated photosynthesis found in the leaves of clone Fe 2 originally from a sunny habitat after exposure to strong light for 1 to 6 days suggested that synthesis of one or several photosynthetic enzymes could, at least in part, account for the increased rate of CO₂ uptake. The activity of carboxydismutase was,
TABLE 17. Resistance to Water Vapor Transfer, \( R_w \) and Rate of \( \text{CO}_2 \) Uptake in 21% and 1.5% Oxygen

<table>
<thead>
<tr>
<th></th>
<th>21% ( \text{O}_2 )</th>
<th></th>
<th>1.5% ( \text{O}_2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( R_w, \text{sec cm}^{-1} )</td>
<td>( \text{CO}_2 ) Uptake, ( \mu \text{mole dm}^{-2} \text{min}^{-1} )</td>
<td>( R_w, \text{sec cm}^{-1} )</td>
</tr>
<tr>
<td>Clone Mb 1 grown</td>
<td>1.74</td>
<td>1.78</td>
<td>10.35</td>
</tr>
<tr>
<td>in weak light</td>
<td>1.95</td>
<td>1.23</td>
<td>5.77</td>
</tr>
<tr>
<td>After 12 days in</td>
<td></td>
<td></td>
<td>18.32</td>
</tr>
<tr>
<td>strong light</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clone Fe 2 grown</td>
<td>2.22</td>
<td>2.25</td>
<td>5.77</td>
</tr>
<tr>
<td>in weak light</td>
<td>1.95</td>
<td>7.35</td>
<td></td>
</tr>
<tr>
<td>After 6 days in</td>
<td>1.75</td>
<td>12.55</td>
<td></td>
</tr>
<tr>
<td>strong light</td>
<td></td>
<td>18.32</td>
<td></td>
</tr>
</tbody>
</table>

Note: The measurements were made under saturating light and a \( \text{CO}_2 \) concentration of 300 ppm.

therefore, determined in leaves of this clone grown in weak light and again after the leaves had been exposed to strong light for 2, 4, and 6 days. Procedures used for the preparation of the leaf extracts and for the enzyme assays were as described by Björkman (1968). The results of these determinations are summarized in Table 18. The activity of carboxydismutase increased when computed on the basis of fresh weight and chlorophyll, but the specific activity remained constant. These data strongly indicate that the increased protein synthesis that takes place upon exposure to strong light includes de novo synthesis of carboxydismutase.

There is much evidence that carboxydismutase and fraction-1 protein of leaves are the same protein entity. Fraction-1 protein, which comprises a major portion of the total soluble protein in leaves, can readily be separated by gel-filtration on Sephadex G-200. This technique was used in the present work to follow changes in the amount of fraction-1 protein in leaves of the clone Fe 2 grown in weak light and then transferred to strong light for 2, 4, and 6 days. Leaves with the major veins excluded were homogenized in a buffer containing 0.1 \( M \) Tris-HCl, 0.01 \( M \) \( \text{MgCl}_2 \), \( 2.5 \times 10^{-4} M \) EDTA, 1 \( g \) \( l^{-1} \) isoadorbate and 5 \( mM \) DTT (dithiothreitol). The final pH was 7.95. The homogenate was spun at 25,000 rpm for 20 minutes, the supernatant treated on a Sephadex G-50 column (0.9 \( 	imes \) 15 cm), and the protein-containing portion of the eluate subsequently applied to a Sephadex G-200 column (2.5 \( 	imes \) 40 cm). Both columns were equilibrated with the same buffer used for homogenization except that isoadorbate and DTT were omitted. All operations were carried out at 2°C. The eluate from the Sephadex G-200 column was collected in 2.5-ml fractions, and the protein in each fraction precipitated with \( \text{CCl}_3\text{COOH} \) and determined with the Folin-Lowry method. Fig. 47 shows the elution pattern of the protein from leaves grown in weak light and after being placed for 4 days in strong light. The prominent peak in these curves represents fraction-1 protein. Table 19 lists the content of total soluble protein and fraction-1 protein in leaves grown

---

TABLE 18. Carboxydismutase Activity in Leaf Extracts from \textit{Solanum dulcamara}, Clone Fe 2, Grown in Weak Light and Transferred to Strong Light for 2, 4, and 6 Days

<table>
<thead>
<tr>
<th>Days in Strong Light</th>
<th>Enzyme Activity, ( \mu \text{mole} \text{CO}_2 ) per min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Per dm(^2) Leaf Area</td>
</tr>
<tr>
<td>0</td>
<td>6.21</td>
</tr>
<tr>
<td>2</td>
<td>8.41</td>
</tr>
<tr>
<td>4</td>
<td>11.44</td>
</tr>
<tr>
<td>6</td>
<td>13.92</td>
</tr>
</tbody>
</table>
in weak light and after exposure to strong light for 2, 4, and 6 days. Both total protein and fraction-1 protein increase in a roughly parallel way during the exposure to strong light.

Conclusions. A change in the O₂ concentration surrounding the leaf from 21% to 1.5% did not have any effect on the degree of stomatal opening. Photosynthesis was enhanced almost 50% in all leaves tested regardless of origin or preconditioning. This indicates that the change in light-saturated photosynthesis that takes place in the different ecotypes as a result of short-term exposure to high light intensities cannot be due to changes in physical barriers to diffusions of CO₂, or in the rate of reoxidation of photosynthesis in the light (photorespiration). The causes of the reduction in the light-saturated rate of CO₂ uptake in the shaded habitat clone upon exposure to strong light are still unknown. The parallel increase in photosynthesis, protein content and carboxydismutase activity in leaves of the sunny habitat clone as a result of exposure to strong light suggests strongly that synthesis of key photosynthetic enzymes may be the major factor increasing the rate of photosynthesis after transfer to a higher light intensity.

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APPLICATION OF A NEW O₂ SENSING DEVICE TO MEASUREMENTS OF HIGHER PLANT PHOTOSYNTHESIS

Olle Björkman and Eckard Gauhl

Until recently no technique for measurements of photosynthetic O₂ exchange by leaves that combines the accuracy, simplicity, and convenience in operation of the infrared analyzer for measurements of CO₂ was available. Almost all information on photosynthetic O₂ exchange by higher plants has been obtained from mass spectrometric measurements.

Polarographic measurements of O₂ exchange have been widely used in studies of algal photosynthesis for many years. Last year we successfully adapted this technique for measurements of photosynthesis by thalli of the liverwort Marchantia polymorpha. Unfortunately, however, the technique is unsuited for use with higher plant leaves.

Very recently, a greatly improved version of the paramagnetic O₂ analyzer...
was tested for use with higher plants in Professor Egle's laboratory in Frankfurt, Germany, with promising results (Schaub et al., 1968). In addition to this improved paramagnetic analyzer, an entirely new O₂ sensing device, exploiting the high ionic conductivity to oxygen of a newly developed ceramic, has become commercially available. Because of its very high sensitivity, it appeared to be potentially useful for measurement of photosynthetic O₂ evolution and the testing of the device for this purpose was therefore undertaken in this laboratory. The sensor is manufactured by the Westinghouse Electric Corporation, New Products Division, Pittsburgh, Pa., and is incorporated in Model 209 O₂ Monitor of this company. Scientific Products, Menlo Park, California, kindly made one such instrument available for testing.

The sensor consists of a nonporous tube of a calcium stabilized zirconium oxide ceramic to which porous electrodes are attached. It is also equipped with a furnace operating at 850°C. This temperature is kept constant with a solid-state proportional controller.

The gas to be analyzed is admitted to one side of the ceramic tube and the reference gas to the other side. Oxygen molecules on the side with the higher O₂ pressure gain electrons to become ions, while simultaneously on the other side, oxygen molecules are formed by reverse action. The potential of the cell is then given by

$$V = (RT/nF) \ln \left( \frac{P_s}{P_r} \right)$$  \hspace{1cm} (1)

where $R$ is gas constant; $T$, absolute temperature; $F$, Faraday; $P_s$, partial pressure of O₂ in the sample gas; and $P_r$, partial pressure reference gas (Burke, 1969). With the cell used in the present study the open circuit potential (mV) is given by

$$V = 55 \log \left( \frac{P_s}{P_r} \right)$$  \hspace{1cm} (2)

The cell voltage is unaffected by the presence of noncombustible gases such as water vapor and carbon dioxide, but care has to be taken not to introduce combustible gases into the cell since at the high operating temperature these will react with oxygen and reduce its concentration.

In measurements of photosynthesis with an open flow system under rate-limiting CO₂ concentrations it is usually desirable that the uptake of CO₂ by the leaf does not result in an excessive reduction of the CO₂ concentration in the leaf chamber. In most cases a reduction exceeding 25% would be undesirable. Under a CO₂ concentration of normal air this would amount to about 80 ppm. Since the uptake of one mole of CO₂ can be expected to correspond to a roughly equal amount of O₂ being evolved, the change in O₂ concentration also should not exceed this value. It is therefore desirable that a concentration change of about 1 ppm be resolved by the device.

Figure 48 shows the calculated rela-

![Fig. 48. The open circuit voltage produced by the zirconium-oxide ceramic cell as a function of the difference in O₂ concentration between the sample and the reference gas, at different O₂ concentrations in the reference gas.](image-url)
tionship between the cell voltage and the difference in O_2 concentration between the sample and the reference gas at various O_2 concentrations. From these data it may be predicted that sufficient sensitivity can be obtained at O_2 concentrations of less than 2000-10,000 ppm O_2.

In the photosynthesis measurements reported here the O_2 concentration of the gas entering the leaf chamber was kept at 1500 ppm. At this concentration the sensitivity of the device was sufficiently high and there was no evidence of adverse metabolic effects that might be caused by anaerobic conditions. The O_2 concentration in the leaf chamber was kept in the range of 1500-1560 ppm. As shown in Fig. 48, the relationship between the O_2 concentration and the cell voltage is very close to linear in this narrow range.

The output voltage of the O_2 cell was measured with a Keithley Model 150 B Microvoltmeter and the amplified signal displayed with a dual channel Hewlett-Packard 7100 BM potentiometric recorder. The indicating circuitry and other accessory components that are integral parts of the Westinghouse Model 209 Monitor were either inadequate or unnecessary for the present application. With the exception of the O_2 cell assembly and the temperature controller, all circuits in the Model 209 O_2 Monitor were disconnected. An open system similar to that described by Björkman (1966) was employed. All measurements were made on single leaves, attached to the plants.

With the O_2 concentrations used in our photosynthesis measurements the noise level of the output voltage from the O_2 cell was very low. The estimated signal-to-noise ratio of the amplified and recorded signal was about 500 to 1. A change in concentration of 0.2 ppm could easily be detected when the O_2 concentration was kept at about 1500 ppm. Under these same conditions the baseline drift was estimated to be 1% of full scale deflection over a 10-hour period. These data suggest that a considerably higher amplification factor could be used while a tolerable noise level could still be maintained. As far as we are aware, there is no other O_2 sensing device presently available with as high a sensitivity in this concentration range.

The instrument was completely unaffected by vibrations from pumps and other equipment that were mounted on the same bench. It was also unaffected by considerable changes (some ±5°C) in the ambient temperature. Another attractive feature is that no elaborate calibrations are needed. The response of the cell to changes in O_2 concentration can readily be predicted from equation 2. Experimental values obtained by diluting pure O_2 with N_2 agreed with these predicted values within the experimental error of the calibration procedure. This error was approximately ±3% and that of the analyzer is presumably smaller.

Figures 49–51 show typical results of experiments in which the dependence of photosynthesis on light intensity and temperature was determined. Tracings from the recorded charts presented in Fig. 49 illustrate the time course of photosynthesis when the leaves were subjected to changes in light intensity. The rate of O_2 evolution, calculated from the predicted response of the O_2 cell, was in very close agreement with the rate of CO_2 uptake in all cases where both rates were measured. Results of determinations of the dependence of photosynthetic O_2 evolution on CO_2 concentration in leaves of *Atriplex patula* and *A. rosea*, using this new O_2 sensing device, are shown in another section of this *Year Book* (p. 629, Fig. 42).

The response time of the O_2 measuring system is determined by the volume of the leaf chamber and the gas connections between it and the O_2 cell, and by the flow rate. The response time of the O_2 cell itself is, according to the manufacturer, only one millisecond.

Because of its insufficient sensitivity
Fig. 49. Recorder traces of the time courses of O₂ evolution and CO₂ uptake in a *Mimulus verbenaceus* leaf subjected to decreases in light intensity. The leaf temperature was 25°C and the CO₂ concentration in the leaf chamber was approx. 0.08%. White light was provided by a 2.5 kW high pressure Xenon lamp.

at high O₂ concentrations, the device is of limited usefulness in photosynthesis measurements under field conditions and in studies on the inhibitory effect of O₂ on net photosynthesis (photorespiration). It is, however, probably the best instrument currently available for kinetic studies of higher plant photosynthesis under low O₂ concentrations where reoxidation of photosynthetic products in the light is inhibited.

A particularly valuable feature of the O₂ cell is its complete insensitivity to CO₂. This greatly simplifies measurements of the dependence of photosynthesis on CO₂ concentration, and of photosynthetic responses under saturating CO₂ concentrations where the infrared CO₂ analyzer has a relatively poor resolution. The O₂ analyzer, described here, provides an excellent supplement to the infrared CO₂ analyzer in comparative studies of photosynthetic characteristics in higher plants, and it has
Fig. 51. Arrhenius plots of the effect of leaf temperature in the range 6-24°C on the rates of \( \text{O}_2 \) evolution and \( \text{CO}_2 \) uptake in an \textit{M. verbenaeeus} leaf. Saturating white light of an intensity of \( 2.5 \times 10^6 \) erg cm\(^{-2}\) sec\(^{-1}\) (400-700 nm) was from a 2.5 kW high-pressure Xenon lamp.

now been incorporated into our photosynthesis measuring system.

References

INTERCONTINENTAL CROSSES IN \textit{Solidago}

Malcolm A. Nobs

The goldenrods of the sunflower family (the genus \textit{Solidago}) comprise about 100 species that are distributed mostly in the Northern Hemisphere with only a few representatives in South America. The greatest diversity occurs in Eastern North America with about 75 species. On the Pacific Slope only about 12 species are found, while in the vast continent of Eurasia only a single widespread species, \textit{Solidago virgaurea} L., is considered to be native.

Dr. Jean Beaudry and his co-workers at the University of Montreal (Beaudry, 1963; Kapoor and Beaudry, 1966) are making a comprehensive biosystematic study of the North American members of the genus. Results from comparative physiological studies on contrasting ecotypes of \textit{Solidago virgaurea} from northern Europe by Björkman and Holmgren interested us in the long-standing question regarding the degree of relationship between the Old World \textit{S. virgaurea} and Pacific Slope forms of the genus that appeared to be ecological counterparts. Crossings begun by us in 1965 (\textit{Year Book 66}, p. 471) and continued during the last two years have yielded hybrids that now provide information relating to our original question.

Table 20 lists the species used in the crossings, their origins, the pollen fertilities and chromosomal pairing of both parents, and of their F\(_1\) hybrids. The crossings included, as far as possible, combinations of pairs of races from similar ecological habitats in western Europe and western North America. One pair consists of arctic forms, a member of \textit{S. virgaurea} from northern Norway, and a form of \textit{S. multiradiata} from Umiat, Alaska. Both are continental interior forms from approximately 70° N. latitude. Another pair consists of an alpine form of \textit{S. virgaurea} from the Sierra Nevada of Spain at 3000 m elevation and 38° N. latitude and a form of \textit{S. multiradiata} from the Sierra Nevada of California at the same altitude and latitude. A third pair of lowland counterparts were crossed. These were a tall, branched form of \textit{S. virgaurea} from near Barcelona, Spain, and a coastal form of \textit{S. spathulata} D.C. from central California, both from approximately 38° N. latitude. Another North American representative, \textit{S. spectabilis} (D.C. Eat.) Gray from Mono Lake, California, at
TABLE 20. Pollen Fertilities and Chromosome Pairing in Parental and Hybrid Combinations of *Solidago*

<table>
<thead>
<tr>
<th>Species or Hybrid Combination</th>
<th>Culture No.</th>
<th>Origins (Elevations and Latitudes)</th>
<th>Percent Normal Pollen*</th>
<th>Meiotic Pairing (Metaphase I)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Parental Species:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. multiradiata</em> Ait.</td>
<td>7654</td>
<td>Timberline, Sierra Nevada of California, 3100 m, 38° N.</td>
<td>90 Regular, 9 pairs</td>
<td></td>
</tr>
<tr>
<td><em>S. spectabilis</em> (D.C. Eat.) Gray</td>
<td>7338</td>
<td>Umiat, Alaska, 100 m, 70° N.</td>
<td>78 Regular, 9 pairs</td>
<td></td>
</tr>
<tr>
<td><em>S. spathulata</em> D.C.</td>
<td>7657</td>
<td>Mono Lake, California, 2000 m, 38° N.</td>
<td>92 Regular, 9 pairs</td>
<td></td>
</tr>
<tr>
<td><em>S. spathulata</em> D.C.</td>
<td>7659</td>
<td>Coastal Central California, San Mateo Co., 50 m, 38° N.</td>
<td>87 Regular, 9 pairs</td>
<td></td>
</tr>
<tr>
<td><em>S. virgaurea</em> L.</td>
<td>7335</td>
<td>Coastal Central California Ft. Ross, 20 m, 38° N.</td>
<td>90 Regular, 9 pairs</td>
<td></td>
</tr>
<tr>
<td><em>S. virgaurea</em> L.</td>
<td>B 039</td>
<td>Beskades, Norway, 600 m, 70° N.</td>
<td>80 Regular, 9 pairs</td>
<td></td>
</tr>
<tr>
<td><em>S. virgaurea</em> L.</td>
<td>HV 124</td>
<td>Hallands Vadero, S. Sweden, 50 m, 56° N.</td>
<td>90 Regular, 9 pairs</td>
<td></td>
</tr>
<tr>
<td><em>S. virgaurea</em> L.</td>
<td>7612</td>
<td>Barcelona, Spain, 600 m, 41° N.</td>
<td>89 Regular, 9 pairs</td>
<td></td>
</tr>
<tr>
<td><em>S. virgaurea</em> L.</td>
<td>7613</td>
<td>Sierra Nevada, Spain, 3000 m, 38° N.</td>
<td>80 Regular, 9 pairs</td>
<td></td>
</tr>
<tr>
<td><strong>Hybrid Combinations:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>multiradiata</em> × <em>multiradiata</em></td>
<td>7592</td>
<td>Umiat × Timberline</td>
<td>82 Regular, 9 pairs</td>
<td></td>
</tr>
<tr>
<td><em>multiradiata</em> × <em>spectabilis</em></td>
<td>7608</td>
<td>Timberline × Mono Lake</td>
<td>54 Moderately regular, 15% with univalents</td>
<td></td>
</tr>
<tr>
<td><em>multiradiata</em> × <em>spathulata</em></td>
<td>7672</td>
<td>Timberline × Coastal San Mateo</td>
<td>51 Moderately regular, 12% with univalents</td>
<td>Nearly regular, 6% with univalents</td>
</tr>
<tr>
<td><em>multiradiata</em> × <em>spathulata</em></td>
<td>7590</td>
<td>Timberline × Coastal Ft. Ross</td>
<td>60 Nearly regular, 6% with univalents</td>
<td>Nearly regular, 5% with univalents</td>
</tr>
<tr>
<td><em>virgaurea</em> × <em>virgaurea</em></td>
<td>7692,7693</td>
<td>South Sweden × Barcelona and Reciprocal</td>
<td>78 Nearly regular, 6% with univalents</td>
<td></td>
</tr>
<tr>
<td><em>virgaurea</em> × <em>multiradiata</em></td>
<td>7573</td>
<td>South Sweden × Timberline</td>
<td>15 Irregular, 7 pairs + 2 tetravalents</td>
<td></td>
</tr>
<tr>
<td><em>virgaurea</em> × <em>multiradiata</em></td>
<td>7678</td>
<td>Sierra Nevada (Spain) × Timberline (California)</td>
<td>19 Irregular, 7 pairs + 2 tetravalents or univalents</td>
<td></td>
</tr>
<tr>
<td><em>virgaurea</em> × <em>spectabilis</em></td>
<td>7679,7680</td>
<td>Barcelona (Spain) × Mono Lake (California)</td>
<td>23 Irregular, 7 pairs + 2 tetravalents</td>
<td></td>
</tr>
<tr>
<td><em>virgaurea</em> × <em>spathulata</em> (2n)</td>
<td>7682-102</td>
<td>Sierra Nevada (Spain) × Coastal California (San Mateo)</td>
<td>13 Irregular, univalents plus chains</td>
<td></td>
</tr>
<tr>
<td><em>virgaurea</em> × <em>spathulata</em> (4n)†</td>
<td>7682-111</td>
<td>Ditto</td>
<td>50 Multivalents plus univalents</td>
<td></td>
</tr>
<tr>
<td><em>virgaurea</em> × <em>spathulata</em></td>
<td>7684</td>
<td>Barcelona (Spain) × Coastal California (San Mateo)</td>
<td>18 Irregular, 7 pairs + 2 tetravalents and univalents</td>
<td></td>
</tr>
<tr>
<td><em>virgaurea</em> × <em>spathulata</em></td>
<td>7690</td>
<td>Beskades (Norway) × Coastal Central California (San Mateo)</td>
<td>20 Irregular, 7 pairs + 2 tetravalents</td>
<td></td>
</tr>
<tr>
<td><em>virgaurea</em> × <em>spectabilis</em></td>
<td>7691</td>
<td>Beskades (Norway) × Mono Lake (California)</td>
<td>12 Irregular, 6 pairs + 2 tetravalents and univalents</td>
<td></td>
</tr>
</tbody>
</table>

* Pollen stainable with lacto-phenol and cotton blue.
† Spontaneous tetraploid F₁ hybrid.
2000 m elevation has also been included.

As indicated in Table 20, the F₁ hybrids of all the combinations indicate that there is a high degree of homology between their chromosomes and those of the parental species. Even the most highly irregular combinations as, for example, F₁ hybrids between different forms of *S. virgaurea* and *S. multiradiata*, or between *S. virgaurea* and *S. spathulata*, have about 80% chromosomal pairing. That some structural repatterning of the chromosomes has taken place is, however, very evident. These appear to be primarily due to segmental interchange. The strong sterility barriers, as indicated by the high percentage of aborted pollen, also suggests that accumulated gene differences as well as other small undetectable structural rearrangements may have taken place in the differentiation of the North American and European counterparts. The combination 7683 (Table 20) between *Solidago virgaurea* from the Sierra Nevada in Spain and *S. spathulata* from coastal Central California yielded a spontaneous tetraploid with \( n = 18 \) chromosomes. The pollen of the tetraploid is 50% normal as compared with only 13% for the diploid hybrid plants, suggesting that the tetraploid may have considerably higher fertility than the diploids. The extreme vigor of the tetraploid in the Stanford garden as contrasted with the diploids further indicates that it is an amphiploid, and implies that a fairly high degree of genetic divergence has taken place between the parental species.

The very close homology between the chromosomes of even the most contrast-
ing ecological races of the European *S. virgaurea*, and also between corresponding North American forms of *S. multiradiata*, is evident from Table 20. That parallel differentiation in the two species has taken place in the two continents is now clear. It is equally clear that the two species have been derived from the same ancestral stock and are closely enough related to have preserved most of their chromosomal homology.

Figure 52 summarizes the relationships between the North American and European forms of *Solidago* in graphic form and illustrates the major morphological differences between the parental forms used in the crossings. The drawings of the parental plants are made to scale as the plants are observed in the Stanford garden. It should be remembered that such vegetative characters as stem height may be strongly modified in contrasting environments.

References

Vegetation of the Harvey Monroe Hall Natural Area

*Jens Clausen*

Occasioned by the XIth International Botanical Congress meeting at the University of Washington, Seattle, August 24 to September 3, 1969, two major field excursions are planned that include the Institution's Department of Plant Biology field stations and central laboratory. The Mather and Timberline transplant stations will be featured, the latter situated in the Harvey Monroe Hall Natural Area. A list of plant species native to this unique area that has been compiled over a period of years is being printed by the Institution in booklet form and will be available to the visiting botanists.

In view of the wide biological interest in the Harvey Monroe Hall Natural Area a brief review of its history appears to be appropriate. In his annual report (*Year Book 32*, pp. 20–21) President John C. Merriam discussed the establishment of this Natural Reserve Area in connection with future research on fundamental problems in biology, such as environment and heredity. Dr. Herman A. Spoehr, then Chairman of the Division of Plant Biology, in his report the same year (p. 180) discussed the significance of the Area "combining an unusual complex of environmental conditions and biological materials."

Dr. Harvey M. Hall proposed the establishment of such a "natural area." He emphasized that "natural conditions will be preserved virtually free from disturbance excepting those necessary for the conduct of scientific research." Hall's proposal was unique in that it would permit scientific research consistent with the conservation of the native vegetation, animal life and other natural features in essentially undisturbed form. Hall's very extensive first-hand field knowledge of the entire flora of California enabled him to perceive the special value of having available for basic experimental work such a strategically located preserve. After Hall's death, in 1932, a mutual contract between the U. S. Forest Service and the Carnegie Institution was drawn setting up the area as Hall had proposed, to be named in his honor.

The approximately 20-square-kilometer area (about 7 square miles) includes diverse topographic features that are truly representative of the high Sierra Nevada of California. Included are three valleys lying in an east-west direction that touch the east rim of the Sierra Nevada at 38° N. latitude. The valleys lie at altitudes between 3000 and 4000 m (about 10,000–13,000 ft.), and each has steep north- and south-facing slopes that provide unusual ranges of temperature, with cold night air collect-
ing at the bottom of the valleys (Year Book 64, pp. 431-435).

The present-day vegetation includes a total of 347 species-complexes and 4 interspecific hybrids, an unusually high number of species for an area above 3000 m altitude. Within the Hall Area are local edaphic niches that range from bogs and moist meadows to dry screes, rough, rocky talus and glacial moraines that extend up to the still active Connex Glacier. It appears the Sierra Nevada was uplifted more than 1000 m during the 1-2 million years since the midglacial period. During the shifts in altitude and climate the species, possessing genetic flexibility, evolved new ecological races as they were raised in altitude with the mountains.

Within the Hall Area the present-day vegetation consists of what appear to be 72 species that are members of circum-polar to circumboreal complexes that must have immigrated from the north through the Cascade-Sierran mountain ranges. About 68 species belong to complexes that have related races and species along the Pacific slope, some spilling over to the east of the Sierras; related to this group are 32 high-alpine, endemic Sierra Nevada species that could have occupied ice-free refuges during the period of heaviest glaciation. The remaining 175 species appear to have their relatives in the Great Basin–Rocky Mountain region, and must have entered the Hall Area over the dry mountain ridges from the east, although many spill over to the higher altitudes on to the Sierran west slope. Of these, 14 reach the Atlantic, and 31 occur in Alaska.

As the Sierras gradually rose, the aridity of the Great Basin increased, providing habitats for the multiple forms of sagebrushes and rabbitbrushes. The Hall Area itself is geologically recent, probably having been free of ice only within the last 2000-6000 years, although forms of harder species may have occupied refuges for longer periods.

STAFF ACTIVITIES

Dr. William M. Hiesey retired on June 30, 1969. He joined the Institution in 1926 and has led the Experimental Taxonomy Group since 1956. Dr. Hiesey plans to remain active for the completion of an Institution monograph, Experimental Studies on the Nature of Species, Vol. V., Biosystematics, genetics, and ecological physiology of the Erythranthe section of Mimulus, by William M. Hiesey, Malcolm A. Nobs, and Olle Björkman.

Because of overlapping interests of the Experimental Taxonomy Group and of several Stanford professors, the ties between the Institution and the Department of Biological Sciences of Stanford University have become closer. In addition to direct experimental collaboration with Professors Harold Mooney, Peter Ray, and Peter Raven, and their graduate students, a Stanford Seminar on Plant Physiology and Ecology has met weekly at our Department under Professor Ray’s direction. Plans are in preparation for expansion of the collaborative work of Drs. Björkman and Nobs with the Stanford group at the mountain stations and at the laboratory.

Dr. Jens Clausen was invited to attend the Vth Congress of EUCARPIA, the European Association for Research in Plant Breeding, meeting at Milan, an international symposium in Denmark of the Scandinavian Association of Geneticists in honor of Professor C. Syrach Larsen’s retirement, and a meeting of the Danish Botanical Society. His talk on “Genecology and Breeding” will be published in English and in Russian. Dr. Clausen has prepared a description of the Harvey Monroe Hall Natural Area, with a check list of its plants showing also their relatives in other
regions. This Institution publication which developed from his talk at the Danish Botanical Society is available to two groups of botanists visiting the mountain stations in connection with the XI International Botanical Congress held in Seattle in August, 1969.

Dr. Fork and Dr. Jan Amesz of Leiden, a former Carnegie Corporation Fellow, have collaborated on an article, "Action spectra and energy transfer in photosynthesis," for Annual Reviews of Plant Physiology and have also contributed a chapter, "Spectrophotometric studies on photosynthesis," for a comprehensive text on photophysiology, edited by Professor Giese of Stanford.

Dr. Norio Murata, an Institution Research Fellow has been awarded the prize for promotion of research by the Japanese Biochemical Society for his accomplishments in the study of fluorescent pigments of photosynthesis.

Mr. Jan Kowalik was awarded the Jurzykowski Award by the Alfred Jurzykowski Foundation on January 24, 1969, in recognition of his outstanding achievements in the field of bibliography.


During the year the Institution's Bush Cabin at Inverness, California was built in a heavily wooded area close to the Tomales Bay State Park and the Point Reyes National Seashore. This was made possible by Dr. Bush's gift to the Institution for staff recreation. The cabin shell was built by a contractor while completion of the roof, deck, interior finish, wiring, and finish plumbing is providing an abundance of recreational challenge for the Department's Staff and Fellows.

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454 Björkman, Olle, see Gauhl, Eckard.


442 Brown, Jeanette S., see French, C. S.


and their functions in photosynthesis. 


Gautl, Eckard, see Björkman, Olle.

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Clausen, Jens, Geneecology and breeding, Vth Congress of Eucarpia, European Association for Research in Plant Breeding, Milan, Italy, October 2, 1968.

Clausen, Jens, Geneecology and breeding. Scandinavian Association of Geneticists Symposium on Seed Orchards, in honor of Dr. C. Syrach Larsen, Scandinavian Seminar College, Holte, Denmark, October 7, 1968.


Clausen, Jens, Populationstudier over træer i en alpin-subalpin dal i Sierra Nevada, California (Population studies on trees in an alpine-subalpine valley in Sierra Nevada, California). Danish Botanical Society, Botanical Laboratory, University of Copenhagen, Copenhagen, Denmark, October 23, 1968.


French, C. S., The forms of chlorophyll \( a \) in plants. American Society of Plant Physiologists, Amherst, Massachusetts, August 31, 1968.


Hiesey, William M., Biosystematic and comparative physiological studies in Mimulus. Stanford University Plant Physiology Graduate Seminar, Carnegie Institution, Stanford, California, February 19, 1969.

Hiesey, William M., Experimental studies on comparative plant physiology at the Carnegie Institution Laboratory. Biology Department Seminar, San Jose State College, San Jose, California, May 7, 1969.


Nobs, Malcolm, Genetic diversity in Mimulus species and races and their responses to contrasting climates. Biology Department Seminar, University of Iowa, Iowa City, Iowa, January 9, 1969.
PERSONNEL

Biochemical Investigations

Staff: C. Stacy French, Director; Jeanette S. Brown, David C. Fork; James H. C. Smith, Emeritus
Carnegie Corporation Fellow: Kenneth E. Mantai
Institution Research Fellows: Eckhard E. Loos, Lars Olof Björn, Norio Murata, Colin Wraith
Visiting Investigators: Zdenek Šesták, Marcel Andre, Yaroslav de Kouchkovsky
Guest Investigator: Teruyo Murata
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Clerical Assistant: Maryke Eldredge
Administrative Secretary-Accountant: Clara K. Baker
General Department Secretary: Wilma M. Stewart
Mechanical Engineer: Richard W. Hart
Electrical Engineer: Mark C. Lawrence
Custodian: Jan Kowalik
Custodian Helper: Dietrich G. Seaman

1 From September 5, 1968. From Oregon State University, Corvallis, Oregon.
2 From January 6, 1968. From Institut für Angewandte Botanik, Technische Hochschule, Munich, Germany.
4 From June 3, 1969. From University of Tokyo, Tokyo, Japan.
6 From September 11, 1968, through October 20, 1968. From Czechoslovak Academy of Sciences, Prague, Czechoslovakia.
7 From October 14, 1968, through November 21, 1968. From Commissariat à l'Energie Atomique, Centre d'Études Nucléaires de Cadarache, France.
8 From November 27, 1968, through December 22, 1968, from CNSR, Gif-sur-Yvette, France.
9 From June 3, 1969. From University of Tokyo, Tokyo, Japan.
10 From July 1, 1967, through August 2, 1968.
11 From July 1, 1968, through February 7, 1969.
12 From February 3, 1969.
13 From June 9, 1969.
15 From June 9, 1969. From Botanisches Institut der Johann Wolfgang Goethe-Universität, Frankfurt, Germany.
16 From June 8, 1969. From Duke University, Durham, North Carolina.
17 From June 18, 1968, through September 3, 1968.
18 From June 15, 1969.
20 From May 13, 1969.
21 From October 15, 1956, through February 15, 1969.
23 From March 24, 1969.
24 From January 22, 1969, through February 15, 1969.
PIATES
Plate 1(A). Subalpine *M. lewisii* (left), the *F*₁ hybrid between *M. lewisii* × *M. cardinalis* (center), and the vacant position of the nonsurviving coastal *M. cardinalis* parent (right, marked by tape) in the Timberline garden, September 1967, after an early autumn snowfall.

Plate 1(B). Three third-generation progenies from the above cross in the Timberline garden, summer of 1967; row 1, *F*₂ plants from the *M. lewisii*-like *F*₁ plant, 7111-16; row 2, *F*₂ plants from the *F*₁-like *F*₂ plant 7111-17; and row 3, plants from the *M. cardinalis*-like *F*₂ plant 7135-35.
Plate 1(C). The same scene in the summer of 1968, all plants in row 3 having been eliminated by winter-kill.
Plate 2. The Logan and Jacksonville clones of *Mimulus* after a 30-day period at 10 and 30°C. The light intensity was maintained at $5.3 \times 10^4$ erg cm$^{-2}$ sec$^{-1}$ (400-700 nm) during a 16-hour photoperiod, and the CO$_2$ concentration at 0.03%.