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*Carnegie Institute Year Book 66, 1966–1967*
INTRODUCTION

As an introduction to reports of the photosynthesis group during the past year it may be useful to mention first a few of the critical discoveries that have had a particularly strong influence on the Department’s line of interest. During the past twenty-five years many people from very different fields of science have discovered the fascination of studying the photosynthetic process, and their efforts have greatly changed the basic concepts of the subject. Warburg’s introduction of *Chlorella* as experimental material for quantitative investigations had been widely taken up before that time. At the beginning of this period the evolution of oxygen by isolated chloroplasts had recently been discovered by Hill; the long series of investigations with isolated chloroplasts had begun, and today continues with increasing understanding and complexity. Some of the other subjects of concern at the start of this period were the chemistry of the intermediates in the path of carbon, the maximum efficiency of light conversion by photosynthesis, and the possibility of deriving from rate measurements a comparatively simple physicochemical mechanism for the process. Another problem of that time, which is still with us, is the clarification of the chemical nature and mode of action of the pigment complexes that capture the energy of sunlight for driving photosynthesis in plants. Some of the forms in which chlorophyll *a* occurs have been identified and their complementary functions in photosynthesis have become evident. This problem remains of major concern to the Department.

Studies on the participation of various pigments in photosynthesis have changed radically because two separate photochemical steps rather than a single light-driven reaction are now recognized. This discovery of Emerson and of Blinks provided an entirely new concept about the mechanism of the process. Furthermore, this led to thinking about the efficiency of the overall process as a resultant of the quantum yields of two separate photochemical reactions and of the losses in the electron transport system through which the light-activated steps are correlated.

The discovery by Duysens—that it was possible to measure changes induced by light in the absorption spectra of components of the photosynthetic mechanism—initiated many detailed investigations of the nature and sequence of individual steps in the electron transport chain. The steps of this chain are now being arranged into a sequence that is logical both thermodynamically and kinetically, an enterprise to which Dr. Fork and his collaborators at the Department are making numerous basic contributions. Absorption change measurements have not only been used for following various steps in photosynthesis but, in the hands of Kok, have also led to the discovery of a new form of chlorophyll into which the energy absorbed by one of the photochemical systems is concentrated. This pigment called “P700” provides a reasonable interpretation of the so-called photosynthetic unit, the existence of which was deduced long ago by Gaffron and by Emerson and Arnold from studies of photosynthesis rates in flashing light.

Another of the striking discoveries of this period was the finding of Krasnovsky that, under appropriate conditions, chlorophyll can be reduced by light to a pink substance. Just
what the functional significance of this pink chlorophyll may be in photosynthesis still remains to be clarified. Why this simple reaction of a substance, so widely and thoroughly studied for a hundred years by innumerable chemists, was not found long ago is astonishing. From this example we must suspect that there may well be equally simple, basic phenomena that have not yet been stumbled upon.

Another highlight in the understanding of chlorophyll chemistry was Dr. Smith's elucidation of the nature of the precursor of chlorophyll, protochlorophyll, and its transformation into chlorophyll by light, a reaction which takes place only when the protochlorophyll is attached to its particular protein.

During this twenty-five-year period the path of carbon was described in considerable detail by Calvin and his collaborators. Another significant discovery was made by Frenkel with purple bacteria and by Arnon with green plants: the formation of adenosine triphosphate through photochemical action. Just how this production of a high-energy storage compound is linked with the electron transport chain is now one of the more vigorously pursued aspects of our subject. These and other important findings have built a framework within which the new discoveries may, at least temporarily, be placed.

When plants are exposed to light a dynamic equilibrium is eventually reached between the reduced and the oxidized forms of each electron-transferring component. For any one component the ratio of the oxidized to the reduced form at equilibrium may vary with the light intensity. This variation with the intensity of the proportion of the oxidized forms of cytochrome \( f \) and of the pigment \( P_{700} \) has been used this year by Dr. Fork and Dr. Amesz to study the energy-transfer system within and between the submicroscopic functional packages of pigments and enzymes that constitute the photosynthetic units.

Within a single unit the light energy absorbed by any of the hundreds of chlorophyll molecules migrates around among them until it is finally caught by the reduced form of a particular trapping molecule. There has been some discussion as to whether cytochrome \( f \) or \( P_{700} \) acts as this energy trap for system 1. These investigators found that photosynthesis can proceed at its maximum rate when a part of the cytochrome is oxidized but not when any appreciable fraction of the \( P_{700} \) is oxidized. This means that \( P_{700} \) but not cytochrome \( f \) is the active trapping center of system 1. They compared the rates of photosynthesis with the equilibrium ratios of the oxidized and reduced forms of both cytochrome \( f \) and of \( P_{700} \). From the relation between the oxidation ratio of \( P_{700} \) and the rate of photosynthesis they concluded furthermore that in certain red algae there was transfer of migrating exciton energy from one photosynthetic unit to another. The probability of such inter-unit transfer from a unit where \( P_{700} \) is nonoperational to a unit containing an active reduced \( P_{700} \) molecule was about 0.5.

Spectral absorption changes caused by light can be used to follow the participation of certain substances in photosynthesis only when the substances responsible for the changes at particular wavelengths are known. Not all of these measurable changes can be definitely attributed to known substances. This year Dr. Fork and Dr. Amesz found that increased absorption at certain wavelengths and corresponding decreases at other wavelengths, in a variety of algae, and in a leaf lacking chlorophyll \( b \), were due to carotenoids. The changes
show that the spectra of some carotenoids shift to longer wavelengths when the live plants are given light that is absorbed by chlorophylls.

Some quinones found in plants are believed to be an integral part of the photosynthetic system. Furthermore, "unnatural" quinones added to chloroplast suspensions can be reduced by light acting on the chloroplasts. Various quinones quench the fluorescence of chlorophyll in algae or chloroplasts and also in pure solutions. The quenching effect of added quinones, in algae or in chloroplasts, has been attributed to the resulting oxidation of natural quenchers that are in close contact with the chlorophyll. Furthermore, quenching of fluorescence has been taken as an indication of the influence of quinones on the photosynthetic electron-transport system. Recently, however, Dr. Amesz and Dr. Fork have found that many quinones added to live algae act directly on the chlorophyll rather than quench the fluorescence indirectly through oxidation of components in the electron-transport chain. The effect of a given quinone concentration on chlorophyll fluorescence is greater in live algae than it would be in solution. This result is attributed to the accumulation of the quinone in fats surrounding the chlorophyll.

For detecting the presence of the different forms of chlorophyll, we have been using for many years a specialized kind of spectrophotometer, which gives the first derivative of absorbance with respect to wavelength. The curves it produces are excellent for the detection of chlorophyll components but are not easy to analyze in quantitative terms. We therefore modified the instrument to plot also conventional absorbance spectra. We have modified the sample holder, which is now particularly suitable for studying highly scattering material such as intact algae, and usable at liquid-nitrogen temperature. At this low temperature the absorption bands of the forms of chlorophyll are appreciably more distinct than at room temperature.

With the new spectrophotometer we have investigated the absorption spectra of particular mutants of Chlorella that, when grown in the dark, contain very small amounts of chlorophyll a and are completely free of chlorophyll b. One of these mutants also lacks carotenoid pigments and has a preponderance of one form of chlorophyll a. With this alga we have come closer than ever before to determining the absorption spectrum of a single in vivo form of chlorophyll a over the entire visible spectrum.

By curve analysis of spectra for these mutants, and for a variety of different algae grown under appropriate conditions, we hope to be able to establish the shapes of the absorption spectra of the natural chlorophyll a complexes. Such information is particularly important because the different forms of chlorophyll a are known to be associated with two different photochemical reactions in photosynthesis. At present we can give only a very rough estimate of the proportions of light of a particular wavelength that are absorbed by the different forms of chlorophyll. However, by knowing the shapes of the spectra of the different forms, it should be possible to calculate the distribution between the different chlorophyll forms of absorbed light at any wavelength.

A basic unanswered question is whether there are only a few specific forms of chlorophyll a, identical in their spectra, occurring in various proportions in different plants, or each of the recognized "forms" is actually a class of pigments within which the individual components may have appreciable variation of wavelength peak position.
This question is being investigated by comparison of absorption curves of intact algae and by attempts to isolate the different forms of chlorophyll from each other by using disintegrated chloroplast material from appropriate algae. In this isolation work the experimental difficulty is to find effective methods for disintegration and separation that do not themselves modify the spectral characteristics of the chlorophyll complexes.

Most of the past attempts to separate the forms of chlorophyll from each other have depended mainly on differential centrifugation. Mr. Jean-Marie Michel has been using electric fields in polyacrylamide gels to separate particles of disintegrated chloroplasts that differ in their electric charges. The particles used for these electrophoretic separations, already selected by differential centrifugation, gave preparations differing in their absorption spectra only after treatment with a detergent. A particularly significant preparation from *Euglena* was free from carotenoids. Various methods of further chloroplast disintegration are being tried to avoid the need for detergents while other separation procedures are being studied by Mr. Michel and Dr. Michel-Wolwertz.

Plants grown in the dark, then exposed to light, form chlorophyll $a$ rapidly. The newly formed chlorophyll changes its absorption peak from 684 m$\mu$ to 672 m$\mu$ in a short time. During this transition period chlorophyll acquires its phytol tail. It was therefore thought that the difference between the forms of chlorophyll in fully greened plants might correspond to differences in the chlorophyll extractable by organic solvents. Such extracts had been found by Dr. Sironval and Dr. Michel-Wolwertz in Belgium to give several modified chlorophylls separable by paper chromatography, in addition to the common chlorophylls $a$ and $b$. It was thought that these "satellite chlorophylls" might be related to the forms of chlorophyll in live plants. This year, however, Dr. Michel-Wolwertz found that they were formed by oxidation during the extraction and chromatographic separation.

The detection of certain pigments in the presence of others can often be done by fluorescence spectroscopy, since the wavelength distribution of light from a fluorescing pigment is as characteristic a property as is its absorption spectrum. Dr. Brown has investigated the fluorescence spectra of various algae to correlate, insofar as possible, the absorption and fluorescence spectra of the forms of chlorophyll in living algae.

The relative contribution of each pigment to the observed total may be very different in absorption and in fluorescence spectra. This is because the fluorescent efficiency of the individual pigments can be very different and, furthermore, the efficiency of energy transfer from one pigment to another may be greatly influenced by the spatial relations between them.

Many investigations have been made of the variations in rate of photosynthesis with time when plants are illuminated after a dark period. The time-course curves from such experiments show all sorts of complex induction and outburst effects that have been widely used to deduce the nature of various steps in the mechanism of photosynthesis. Not only the rates of oxygen and of carbon dioxide exchange resulting from illumination, but also the converse effect, the readjustment of the rate of gas exchange in the beginning of a dark period following a light exposure, have been the subject of many studies.

Such research has usually shown big differences in the results when blue light is compared with other
parts of the spectrum. Blue light also
has other specific effects, such as con­
trolling the shape of higher plants,
and influencing the types of chemical
compounds formed by photosynthesis.
For blue light to do these things there
must be a particular pigment, or sev­
eral of them, that absorbs blue light
preferentially. This year Dr. James
M. Pickett measured the influence of
short exposures of blue light on the
oxygen uptake of *Chlorella* in the suc­
ceeding dark period. The results show
that some sort of a flavin type of com­
pound is the pigment responsible for
this blue light effect. Simultaneous
work by Dr. Kowallik in Professor
Gaffron's laboratory in Florida and
by Professor Ried of Frankfurt, a
former Visiting Investigator at the
Department, gave identical results.
Because of the different plants and
procedures used in the three labora­
tories the agreement greatly strength­
en the theory that this pigment may
be of basic significance and may be
widely distributed in various plants.

Phytochrome is another pigment
that is probably not directly a part
of the mechanism of photosynthesis,
but is very important in controlling
the pathways of biochemical metabo­
lism. By contrast with the flavin re­
sponsible for blue light effects, phyto­
chrome has been isolated in pure form
and is far better known as a chemical
entity. When phytochrome is illumi­
nated it changes from one form to
another. The direction of the change
depends on the color of the light to
which it is exposed. This year Pro­
fessor Briggs of Stanford and Dr.
Fork continued the collaborative
work on phytochrome, discussed in
last year's report, in which some in­
termediate substances in the trans­
formation reaction of the purified
pigment were described. The recent
results show that similar intermedi­
ate products are formed when phyto­
chrome is transformed by light in
live plants, as well as when it is in
pure form. The rate constants for the
opposing reactions, whose balance
determines the relative concentration
of the two forms for an exposure to
a particular wavelength, were, how­
ever, found to differ in the plant and
in solution. They also determined the
absorption spectra for some of the
intermediate forms in the photo­
chemical transformation of phyto­
chrome.

In recent years many of the investi­
gators of photosynthesis have come
together annually or more often from
all over the world. Furthermore, sev­
eral comprehensive books on the sub­
ject appear each year and a new
international journal, *Photosynthe­
tica*, has been started. Such close com­
munication between different workers
is essential in planning experiments
and in modifying theories so that the
current work is relevant to present
thought on the subject. There is,
however, a danger inherent in too
much unity of thought in science, as
well as in politics and religion. When
most of the people in any field think
along the same lines, there may be
little likelihood that they will accept
new concepts. No doubt there have
been occasions when the investigators
of photosynthesis have all been simul­
taneously following major misconcep­
tions. However, the volume of special­
ized journal articles has become so
great that no one person can keep in
mind the detailed findings of various
laboratories even within a narrow
part of the field of photosynthesis.
This fact itself may help to diversify
thinking on the subject, thus increas­
ing the probability of hitting upon
important new interpretations.

In spite of all the intense research
efforts on different aspects of the
mechanism of photosynthesis, many
basic questions are still far from
clarification and some significant
areas are practically neglected in the
rush to make new contributions to the detailed understanding of some of the more popular aspects of the subject.

**Experimental taxonomy.** Much of the current year's effort of the Experimental Taxonomy group has been directed toward continued researches on the comparative physiology and biochemistry of plants originating from contrasting environments. These studies are aimed at discovering basic physiological mechanisms that operate in natural selection and evolution of higher plants.

The discovery last year that the amount of oxygen present in normal air depresses the photosynthetic rate in most higher plants to about 30% below that in oxygen-free air has led to experiments to determine whether or not growth of such plants is also inhibited in normal air. Initial results from two unrelated plants, monkey flower and beans (*Mimulus cardinalis* and *Phaseolus vulgaris*), do show that dry weight yield may be twice as great during a less than 2-week period in an atmosphere containing only 2.5% or 5% O₂ as compared with 21%. In contrast, a third plant, corn (*Zea mays*), whose photosynthetic CO₂ uptake is unaffected by O₂ concentration in the range 0%-21%, failed to show a significant weight increase under the same conditions. These results open a new field of inquiry into basic differences that have evolved in the photosynthetic mechanism of higher plants.

Using improved quantitative techniques, Dr. and Mrs. Björkman have demonstrated that the light-saturated photosynthetic rate in goldenrod (*Solidago virgaurea*) is closely correlated with the activity of the enzyme carboxydismutase in the same leaves of these plants. Furthermore, genetically determined differences in the light-saturated photosynthetic rates in sun and shade races of *Solidago* are closely linked with their capacity to produce this enzyme.

A race of *Solidago* originally from a sunny habitat in northern Norway attains a high photosynthetic rate under saturating light intensities when the plants are previously grown at high light intensities. The amount of the enzyme carboxydismutase of the leaves likewise is high. Another race of the same species but from a shaded habitat in southern Sweden is capable of producing only about half as much of the enzyme. It also lacks the ability to attain a high light-saturated photosynthetic rate.

The marked differences in the capacity of lowland and alpine races of the *Mimulus cardinalis-M. lewisii* complex to fix CO₂ under light-saturating conditions reported last year by Dr. Hiesey, Dr. Björkman, and Dr. Nobs have been followed in first- and second-generation progeny of crosses between alpine and lowland forms. The differences in photosynthetic capacity under light-saturating conditions are inherited in much the same way as morphological differences such as flower color and leaf structure. The differences in photosynthetic capacity are partially linked through genetic coherence with the morphological characters. The chlorophyll content of leaves also differs markedly in contrasting races, but is inherited independently of photosynthetic capacity, and bears no relation to light-saturated photosynthetic rates.

This year Dr. Nobs and Dr. Hiesey completed an extensive five-year study of the performance of first-generation hybrids between ecological races of *Mimulus* of various degrees of relationship. The study was carried out at the Stanford, Mather, and Timberline transplant stations. There are marked differences in the degree to which hybrid vigor is expressed in these three contrasting environments. The expression of these differences is
highly dependent both upon the genetic composition of the parental races used in the crosses and upon the environment in which they are being observed. An important finding is that the survival capacity of first-generation hybrids between distinct ecological races within this species-complex in widely different climates is inherited in an intricate and largely unpredictable way. The results are being incorporated in a monographic study of the Erythranthe section of *Mimulus* that includes the results of biosystematic, transplant, and physiological investigations.

Dr. Jens Clausen, on field excursions in conjunction with attendance at the Eleventh Pacific Science Congress at Tokyo, made a study of altitudinal vegetational belts in Japan in comparison with comparable altitudinal transects in western North America, with particular emphasis on tree species. The belts of tree vegetation are closely related on both sides of the Pacific. This concept is achieved when one studies clusters of morphologically closely related species that circle the earth in various latitudinal belts.

Modern tendencies to split species and genera into smaller and smaller entities, naming them as species, have obscured our perception of the relationships that exist between gross morphological characters and broad evolutionary clusters of species occupying different continents.

### BIOCHEMICAL INVESTIGATIONS

**ROLE OF P700 AND CYTOCHROME f IN THE REACTION CENTER OF PHOTOSYSTEM 1**

*Jan Amesz and David C. Fork*

In spite of extensive studies relatively little is known of the nature of the primary photochemical reactions that bring about photosynthesis. Although it may be stated that more is known about photosystem 1 than about photosystem 2, our knowledge even of the photochemistry of system 1 is limited. For this reason we studied light-induced reactions of P700, the presumed primary reactions of photosystem 1 and cytochrome f in the red algae *Iridaea splendens*, *Schizymenia pacifica*, and *Porphyra perforata*.

Illumination of *Iridaea*, which had been in the dark for a few seconds, with red or far-red (>680 mµ) or blue light mainly absorbed by system 1, gave absorbancy changes in the regions of P700 and cytochrome absorption. One light-induced signal showed a delay upon onset of illumination and a relatively fast (~0.5 sec) decay upon darkening. The negative maxima in the difference spectrum of this signal were at 435 and 705 mµ, indicating an oxidation of P700 (Kok and Hoch, 1961) in the light. The second signal showed an immediate response to illumination and a slow (~6 – 10 sec) decay in the dark. The difference spectrum with minima at 553 and 420 mµ and a maximum at about 402 mµ indicated the oxidation of an f-type cytochrome, probably similar to that isolated by Katoh (1960) from *Porphyra tenera*. Figure 1 shows some typical kinetics of light-induced absorbancy changes. Except for a lowering of the rate of dark decay upon lowering the temperature, the absorbancy changes in response to light were essentially the same at room temperature and 1°C, as well as in the presence of DCMU [3-(3, 4-dichlorophenyl)-1, 1-dimethylurea]. No evidence was found for
Fig. 1. Kinetics of light-induced absorbance changes $\Delta A$ in *Iridaea splendens* at 420, 435, and 705 m$\mu$ due to oxidation of the f-type cytochrome and P700. For traces a and b the wavelength of the actinic light was 708 m$\mu$ (6.1 nanoeinstein cm$^{-2}$ sec$^{-1}$). For traces c and d the actinic light was a band around 414 m$\mu$, 0.7 nanoeinstein cm$^{-2}$ sec$^{-1}$. Trace d shows the absorbance change, superimposed on a fluorescence signal, which caused the rapid deflections upon illumination and darkening; trace c shows the fluorescence signal alone. The preceding dark time was 6 seconds for all traces and the temperature was 20°C.

reactions of b-type cytochromes under the conditions applied.

The amounts of oxidizable P700 and cytochrome f relative to chlorophyll were calculated from the maximum light-induced absorbancy changes to be $1/4.1/310$ in *Iridaea*. Calculations were based on estimated specific extinction coefficients of 70 mM$^{-1}$ cm$^{-1}$ at 420 m$\mu$ for the oxidation of cytochrome and of 73.3 mM$^{-1}$ cm$^{-1}$ for bleaching of P700 at 705 m$\mu$, which is the same as that of chlorophyll a in 80% acetone at the red maximum. Comparison of the size of the absorbancy changes at 435 and 705 m$\mu$ indicated a specific extinction coefficient of 25.5 mM$^{-1}$ cm$^{-2}$ at 435 m$\mu$ for P700. The amount of cytochrome is about the same as found by Nishimura (1967) in *Porphyra*, but high compared to that present in blue-green algae, where cytochrome and P700 appear to be present in about equal amounts of one per several hundred chlorophyll molecules.

Quantum efficiency for cytochrome oxidation. As illustrated in Table 1,
### TABLE 1. Quantum Yields for Cytochrome Oxidation

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Actinic Wavelength, m(\mu)</th>
<th>Intensity [nE cm(^{-2})sec(^{-1})]</th>
<th>Temperature, (^\circ)C</th>
<th>DCMU Concentration, M</th>
<th>Quantum Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 692</td>
<td>5.0</td>
<td>21</td>
<td>(5 \times 10^{-5})</td>
<td>0.54</td>
<td></td>
</tr>
<tr>
<td>1 692</td>
<td>2.6</td>
<td>21</td>
<td>(5 \times 10^{-5})</td>
<td>0.54</td>
<td></td>
</tr>
<tr>
<td>1 692</td>
<td>1.1</td>
<td>21</td>
<td>(5 \times 10^{-5})</td>
<td>0.54</td>
<td></td>
</tr>
<tr>
<td>1 692</td>
<td>0.47</td>
<td>21</td>
<td>(5 \times 10^{-5})</td>
<td>0.64</td>
<td></td>
</tr>
<tr>
<td>2 692</td>
<td>2.8</td>
<td>21</td>
<td>none</td>
<td>0.47</td>
<td></td>
</tr>
<tr>
<td>2 692</td>
<td>2.8</td>
<td>21</td>
<td>(5 \times 10^{-5})</td>
<td>0.49</td>
<td></td>
</tr>
<tr>
<td>3 708</td>
<td>6.0</td>
<td>20</td>
<td>none</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>3 708</td>
<td>6.0</td>
<td>20</td>
<td>none</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>4 708</td>
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</tr>
<tr>
<td>1 692</td>
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<td>20</td>
<td>none</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>1 708</td>
<td>6.5</td>
<td>20</td>
<td>none</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td>1 684</td>
<td>5.0</td>
<td>20</td>
<td>none</td>
<td>0.12</td>
<td></td>
</tr>
</tbody>
</table>

Quantum yields were measured from the initial decrease in absorbance at 420 m\(\mu\) upon illumination. Preceding dark time was 6 seconds for *Iridaea*, 5 seconds for *Schizymenia* and 3 seconds for *Porphyra*. Thallus absorption was about 60%-70% at 692 m\(\mu\) and 20%-30% at 708 m\(\mu\).

Quantum efficiencies for the oxidation of the f-type cytochrome in *Iridaea* and *Schizymenia* were about 0.5-0.65 molecules of cytochrome oxidized per quantum of red or far-red light absorbed. These quantum efficiencies were calculated from the initial rate of the absorbancy decrease at 420 m\(\mu\) after a preceding dark time of a few seconds. Calculation of the efficiency for oxidation of P700 from the steepest part of the absorbancy curves at 435 m\(\mu\) gave considerably lower yields of about 0.2. In *Porphyra perforata* the highest yield for cytochrome oxidation observed was about 0.15. Similarly low or lower values have been reported by Nishimura (1967) for other *Porphyra* species.

Relation between the rates and oxidation levels of P700 and cytochrome f. An explanation for the delay in P700 oxidation upon illumination could be that photooxidized P700 reacts rapidly with reduced cytochrome f and that accumulation of oxidized P700 can occur only when all cytochromes in the same reaction center have been oxidized. It has been shown (Beugeling and Duysens, 1966; Duysens 1966) that this hypothesis can explain satisfactorily the kinetics of P890 (the presumed analogue of P700) and of a cytochrome in the purple bacterium *Chromatium*. Figure 2 illustrates an experiment with *Iridaea*, in which the alga was illuminated for 3 seconds with an intensity of light sufficient to oxidize P700 and cytochrome nearly completely. After various dark periods a second illumination of short duration was given and the oxidation of these compounds caused by the second illumination was recorded. At the beginning of the second illumination P700 was almost completely reduced in all experiments, but the level of oxidation of cytochrome varied widely for different dark times applied. If it is assumed that the oxidation of cytochrome by oxidized P700 proceeds very rapidly, then the relative initial rate of P700 oxidation in the light will be proportional to the fraction of reaction centers that contain only oxidized cytochrome molecules. When it is further assumed that the chance
of being reduced in a given time in the dark is the same for every cytochrome molecule, then

$$X = x^n$$  \hspace{1cm} (1)

where $X$ is the fraction of reaction centers in which all cytochromes are oxidized, $x$ is the fraction of cytochromes that is oxidized, and $n$ is the number of cytochromes per reaction center. Thus

$$\phi_e = kx^n$$  \hspace{1cm} (2)

where $\phi_e$ is the quantum efficiency for P700 oxidation; $k$ is a constant, equal to $\phi_e = 1$. As Fig. 3 shows, the experimental values for the initial rate of P700 oxidation fit the calculated relation for $n = 4$ rather well, in good agreement with the earlier mentioned finding that the amount of cytochrome in *Iridaea* is 4.1 times larger than that of P700.

As Fig. 3 also shows, the sum of the rates of P700 and cytochrome oxidation, which might be taken as the rate of the primary photochemical reaction, is independent of the oxidation level of cytochrome. Experiments in which we measured the sum of these rates as a function of the oxidation-reduction level of P700 gave the results shown in Fig. 4.

As discussed in this year’s report (pp. 155–160), if P700 is a primary reactant, photochemistry should become less efficient when part of P700 is in the oxidized state, because part of the reaction centers then are not operative and light quanta will be wasted, unless they are transferred to another reaction center. The results of Fig. 4, in agreement with those obtained on photosynthesis of *Cryptopleura violacea* (pp. 155–160, this volume), suggest that P700 is a primary reactant. However, the decline in photosynthetic efficiency appears to be less than proportional to the fraction of P700 that
Fig. 3. Quantum yield for the initial rate of P700 oxidation in *Iridaea* (circles) and for the sum of the initial rates of P700 and cytochrome oxidation (squares) as a function of the level of oxidation of cytochromes. The oxidation rates were measured from the rate of absorbance change at 435 and 420 mμ in experiments as shown in Fig. 2. The solid lines give calculated curves for P700 oxidation for reaction centers containing one P700 molecule and up to 5 cytochrome molecules (as described in the text).

is oxidized. This suggests that energy transfer between the reaction centers occurs. A calculated curve (A. and P. Joliot, 1964) for 50% probability of energy transfer to a second reaction center when the first one is inoperative seems to give the best fit for the experimental data.

**Conclusion.** The results are in quantitative agreement with the hypothesis that a reaction center in *Iridaea* contains one P700 molecule and four cytochrome f molecules. The observation that the efficiency of the photochemical reaction is dependent on the oxidation level of P700 but not on that of cytochrome indicates that P700 but not cytochrome f is a primary reactant. The kinetics and efficiencies of cytochrome and P700 oxidation in the light can be explained by the hypothesis that both P700 and cytochrome are in the main path of photosynthetic electron transport and that P700, when oxidized photochemically, oxidizes cytochrome f in a rapid reaction. Accumulation of oxidized P700 occurred only when all cytochromes in a reaction center were oxidized.

The results do not prove that cytochrome f is oxidized via P700,
although this is the simplest hypothesis that explains the results quantitatively. Direct information about this could possibly be obtained by the use of intense flashes of short duration to oxidize P700. In this respect it is interesting to note a recent experiment of Nishimura (1967) who reported the oxidation of a relatively large amount of cytochrome f in *Porphyridium cruentum* by a single laser flash. This could argue against the hypothesis that the cytochrome is oxidized via P700, which is probably present in smaller amounts than the amount of cytochrome that was oxidized.

References


Transfer of Energy between Reaction Centers of Photosystem 1 in Algae

David C. Fork and Jan Amesz

The concept of the photosynthetic unit stemming from the early work of Emerson and Arnold (1932, a, b) has been substantiated considerably during recent years. A photosynthetic unit can be defined as a primary reaction center with associated pigment molecules (chlorophylls, carotenoids, biliproteins) having a higher probability of transferring their excitation energy to this reaction center than to another center.

Experiments with purple bacteria (Vredenberg and Duysens, 1963; and Clayton, 1966) and with the green alga Chlorella pyrenoidosa (A. and P. Joliot, 1964) indicate that the photosynthetic units in purple bacteria, as well as the photosynthetic units of system 2 (the $O_2$ evolving system) in algae are not separated, but that excitation energy can move more or less freely from one unit to another. When one reaction center is inoperative, by just having trapped an exciton, a second exciton may be transferred to a different reaction center.

The extent of energy transfer from one photosynthetic unit to another is an important factor in determining the amount of light energy that eventually reaches a functional reaction center when some of the reaction centers are inoperative. Therefore, it is possible in principle to obtain information about this transfer by measuring the efficiency of conversion of light energy into photochemical products as a function of the fraction of reaction centers that is operational. To obtain information about energy transfer between system 1 units in algae we therefore compared, in a number of algal species, the relative efficiency of $O_2$ evolution with the oxidation level of P700 and of cytochrome $f$ as a function of light intensity.

At various intensities of light we measured the fraction of P700 and cytochrome $f$ that was in the reduced state. Under the same conditions the relative rate of $O_2$ evolution was measured as a function of light intensity with a Teflon-covered electrode (Year Book 61, p. 343). We used adjacent parts of the thalli or, with Schizothrix, samples of the same culture. In all experiments discussed below, the actinic light was of a wavelength band such that absorption by system 2 exceeded that by system 1 (blue light for the green alga Ulva lobata; green light for the red algae). Therefore, we can assume that photochemistry in system 1 occurred at maximum efficiency in the linear part of the light curve of photosynthesis.

Figure 5 shows the rate of $O_2$ evolution and the absorption changes corresponding to the oxidation level of P700 and of cytochrome as a function of light intensity in the red alga Cryptopleura violacea. The rate of $O_2$ evolution was the steady-state rate after several minutes of illumination. The oxidation levels of P700 and cytochrome were measured with the apparatus described earlier (Year Book 63, p. 435) by the increase of absorption at 706 for P700 and 420 mp for cytochrome, upon turning off the actinic light. The exposure was long enough to give a steady-state rate of $O_2$ evolution. To minimize signals caused by chlorophyll fluorescence the photomultiplier was placed about 30 cm from the sample cuvette. The measurements were corrected for fluorescence by subtraction of the signal obtained under the same conditions but without the measuring beam.
the rate of electron transport in system 1 to a level higher than computed from $O_2$ evolution, as discussed below. The same explanation could apply to the red alga Porphyra perforata, also shown in Fig. 7. Results with Ulva lobata were similar to those obtained for Porphyra.

Discussion. The results obtained with Cryptopleura support the hypothesis that P700, but not cytochrome $f$, acts as a primary reactant of light reaction 1 in photosynthesis. Accumulation of oxidized P700 was accompanied by a decline in photosynthetic efficiency, but the cytochrome was already partly oxidized at light intensities where no such decline was observed. The results with the other algae also agree with the assumption that P700 is a primary reactant. In no instance did oxidized P700 accumulate when $O_2$ evolution proceeded at optimum efficiency. From these experiments alone it might be argued that the oxidation of P700 is a side reaction that only occurs when photosynthesis is saturated or inhibited. However, this would be in disagreement with the observation of Kok et al. (1963) that the turnover rate of P700 in spinach chloroplasts is strongly stimulated by the addition of NADP and ferredoxin and is inhibited by DCMU.

The results with Cryptopleura and Schizothrix indicate that energy transfer occurs between photosynthetic units of system 1. In Cryptopleura photosynthesis proceeded with 50% relative efficiency even though P700 was about 70% oxidized. A sim-
ilar phenomenon was seen in *Schizothrix* at high light intensities.

No evidence for energy transfer was obtained by the experiments with *Porphyra*. However, these experiments could prove the absence of energy transfer in this alga only if two conditions were fulfilled:

1. The rate-limiting step in photosynthesis is not in the Calvin cycle or in one of the reactions leading to the reoxidation of the hypothetical primary-reduced product of system 1. If it were, accumulation of primary reductant could limit the efficiency of light utilization in system 1. It is unlikely that this effect occurred in our experiments, since the concentration of CO$_2$ was not rate limiting; moreover, there is other evidence that the rate-limiting step is in the electron-transport chain between the two systems.

2. The second condition is that the rate of O$_2$ evolution is equal to the rate of electron transfer by system 1. This is not true when a "cyclic" reaction occurs between the reduced and oxidized products of system 1, because then the rate of photochemistry in system 1 would be higher than estimated from measurements of O$_2$ evolution. There is evidence that cyclic reactions occur in intact algae and in isolated chloroplasts, especially in the presence of redox catalysts. This would explain the results with *Porphyra* and other divergent results as, for example, those with *Schizothrix* at low light intensity.

It must be noted that the probability of energy transfer $p$ of 50% to 60% as derived from the data of Fig. 6 probably gives only an approximate measure of the extent of energy transfer between the photosynthetic units in system 1. The number $p$ gives the probability for transfer to a second reaction center when the first one visited is inoperative, but gives no information about transfer between photosynthetic units prior to the first visit. A formally different model, applied to bacteria, by Vredenberg and Duyssens (1963) implies that the reaction centers are homogeneously dispersed between the light-harvesting pigment molecules, and that the chance of finding a second reaction center depends only on the number of random walks involved. This model can probably not be applied to algae, for reasons discussed below.

In *Schizothrix* no correlation has been found (Vredenberg and Duyssens, 1965) between fluorescence of system 1 and the oxidation level of P700. For this reason it has been assumed (Duyssens, 1965) that energy from the bulk chlorophyll is transferred to the reaction center via a special, weakly fluorescent chlorophyll that is present in small amounts and located near the reaction center. Back transfer of energy from this chlorophyll to the bulk chlorophyll would be negligible, so that bleaching of P700 would not affect the fluorescence yield of the bulk chlorophyll. A consequence of this hypothesis is that energy transfer between reaction centers would occur via the special chlorophyll which should form a more or less continuous structure containing the reaction centers.

**References**


Kok, B., B. Cooper, and L. Yang, in *Studies on Microalgae and Photosynthetic Bacteria*, Japanese Soc. of Plant

LIGHT-INDUCED SHIFTS IN THE ABSORPTION SPECTRUM OF CAROTENOIDS IN RED, BROWN, AND YELLOW-GREEN ALGAE AND IN A BARLEY MUTANT

David C. Fork and Jan Amesz

It has been known for a number of years that certain photosynthetic bacteria exhibit light-induced changes of absorption in the region 450 to 540 m\(\mu\), which are apparently produced by a shift toward longer wavelengths of the absorption of a carotenoid pigment. Characteristic difference spectra are observed which show maxima and minima separated by about 30 m\(\mu\). There is evidence (Amesz and Vredenberg, 1966) that these changes are not caused by oxidation-reduction reactions of carotenoid pigments but rather that they may be produced by a change in the environment of the pigment molecules resulting from electron transport.

We have found in a number of variously colored algae and a barley mutant light-induced absorbance changes which appear to be caused by a shift toward longer wavelengths of the absorption bands of a carotenoid.

Kinetics and absorption difference spectra. Figure 8 shows absorbance changes at 495 m\(\mu\) seen in the red alga *Iridaea splendens*, which we have attributed to a shift of absorption of a carotenoid. The absorbance change produced by low-intensity orange light (622 m\(\mu\), absorbed mainly by system 2), were relatively simple (trace d). Illumination produced a slow decrease of absorbance followed by a steady state and a reversal upon darkening. Red light (684 m\(\mu\), absorbed mainly by system 1) produced more complicated kinetics. A rapid negative transient occurred, followed by a slower increase to a steady state.

![Fig. 8. Light-induced changes of absorbance \(\Delta A\) at 495 m\(\mu\) in the red alga *Iridaea splendens* induced by actinic light of 684 and 622 m\(\mu\). The intensities, \(I\), are expressed in nanoeinstein cm\(^{-2}\) sec\(^{-1}\).](image-url)
above the dark level (traces a, b).

The response induced by light of 622 

\text{m}_{\mu}\text{ of high intensity was rather vari-

able. Here the first decrease was fol-

lowed by a second, slower one (trace c), but sometimes it was followed by a slow and small increase in absorbance. Kinetics at 515 m\_\mu were similar but opposite in sign, suggesting that they were at least partly caused by the same compound. Kinetics at these wavelengths indicated that the changes were not caused by P700 or cytochrome f; moreover, these compounds have little absorption in this spectral region.

Light-induced absorption difference spectra were measured in the region 440 to 540 m\_\mu in \textit{Iridaea}. The difference spectrum of the steady-state change produced by orange light is shown in Fig. 9. A very similar spectrum resulted from plotting the rate of change instead of the steady state. Figure 10 shows difference spectra obtained with red actinic light and the same conditions as for trace a of Fig. 8. The open circles give the deflection after 20 msec of illumination, which

is the time needed to achieve the maximum negative deflection. The curve with the solid circles gives the difference between the maximum deflection and the steady state, corresponding to the slow reversal of the signal at 495 m\_\mu.  

The spectra of Figs. 9 and 10 are all similar and show maxima and minima at about 465, 480, and 495 m\_\mu, and a maximum (or shoulder) at 515 m\_\mu, which suggests that they are due to the same compound. The shape of the spectrum of Fig. 9, which seems uncomplicated by other changes in the region above 510 m\_\mu, suggests that it is caused by a shift toward longer wavelength of a compound with three maxima at or somewhat below 445, 473, and 504 m\_\mu. The location and distance of the maxima (30 m\_\mu) suggest that this compound is a carotenoid.

The difference spectrum of the red alga \textit{Schizymenia pacifica} induced by orange light, shown in Fig. 11, was very similar to that of \textit{Iridaea}, although the changes were much smaller, and showed maxima and

\begin{center}
\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure9.png}
\caption{Light-minus-dark difference spectrum of \textit{Iridaea} produced by 1 second of illumination with actinic light of 622 m\_\mu (1.1 nanoeinstein cm\^2 sec\(^{-1}\)).}
\end{figure}
\end{center}
Fig. 10. Difference spectra of *Iridaea* produced by light of 684 mμ (17.7 nanoeinstein cm⁻² sec⁻¹). Open circles: absorbance change, ΔA, produced during the first 20 msec of illumination. Solid circles: spectrum for the reversal of the absorbance change during illumination (difference between maximum deflection produced initially and the steady state; see Fig. 1, trace a).

minima at about 452, 463, 480, 495, and 511 mμ. The difference spectrum of *Porphyra perforata*, also shown in Fig. 11, had maxima and minima at 470, 483, 497, and 515 mμ, which suggests a similar shift in carotenoid absorption as in the other red algae, but the spectrum is apparently more strongly distorted by other absorption changes. This was also true of the

Fig. 11. Difference spectra of the red algae *Schizymenia pacifica* and *Porphyra perforata* produced by light of 622 mμ (1.5 and 8.7 nanoeinstein cm⁻² sec⁻¹, respectively).
brown alga *Phaeostrophion irregulare* (Fig. 12).

The shape of the difference spectrum (Fig. 13) for *Botrydiopsis alpina* (Xanthophyceae) was very similar to that of *Iridaea* and had maxima at 450, 482, and 520 and minima at 468 and 498 m\(\mu\).

We were unable to distinguish a carotenoid shift in green algae (Chlorophyceae), since the presence of chlorophyll b—which produces the large negative change at 475 and the positive change at 515 m\(\mu\) (Year Book 65, p. 473)—obscured other changes in this region. However, we were fortunate to have a mutant strain (Chlorina 2) of barley (*Hordeum vulgare*), which lacks chlorophyll b (Highkin and Frenkel, 1962) but is capable of growing to maturity. This mutant, which was obtained by Robertson in 1938, was made available to the Department through the courtesy of Dr. Harry R. Highkin. The plant produced a difference spectrum (Fig. 14) which was still complicated by other absorbance changes but nevertheless had maxima and minima suggesting a shift in absorption of a carotenoid.

We did not invariably observe the carotenoid absorbance changes. In *Iridaea*, at times, we were unable to observe them. We have thus far not seen them in the blue-green algae examined (*Synechococcus cedrorum* and *Schizothrix calcicola*). In barley we previously saw (Year Book 65, p. 474) only another change with a peak near 525 m\(\mu\) and no negative changes below 500 m\(\mu\). This change may distort the spectrum of Fig. 14.

**Pigment system responsible for the absorption shift.** The kinetics of the absorption changes in light mainly absorbed by system 1 or by system 2 illustrated in Fig. 8, suggest that the shifts in carotenoid absorption were driven by both pigment systems; excitation of system 1 causes a rapid change, followed by a slow reversal, and system 2 causes only a slow change of absorption. Additional evidence that the rapid spike (as in trace a of Fig. 8) is brought about by system 1 was obtained by experiments with DCMU which showed no inhibition of the spike by this compound. For *Iridaea* the relative efficiency of 684- or 622-m\(\mu\) light for bringing about the carotenoid absorption shift
was measured, and the results (Table 2) indicated that the relative activities were the same as for cytochrome oxidation, a well-known system 1 reaction.

The shifts in carotenoid absorption are remarkably similar in properties to those seen at 475, 515, and 650 m\(\mu\) in the Chlorophyceae and in other green organisms containing chlorophyll b (Year Book 63, p. 441 and Year Book 65, p. 473). In both cases excitation of system 1 gives rise to a rapid transitory change upon illumination, followed by a reversal upon darkening. The initial, fast carotenoid shift, like the 515-m\(\mu\) change, is dependent upon the preceding dark time, and increases up to a certain maximum with increasing dark time.

It has been argued (Fork, Amesz, and Anderson, 1967; Witt et al., 1967) that the 515-m\(\mu\) change in green algae is not produced by an oxidation-reduction reaction but rather by a change in the environment of a compound, probably chlorophyll b, which gives rise to a relatively small change in its absorption spectrum. The change in environment would then be caused in some way by electron transport in system 1 or 2. The same might be true for the carotenoid shift. There is evidence (Amesz and Vredenberg, 1966) that the shift in carotenoid absorption in the purple bacterium *Rhodopseudomonas spheroides* is not caused by a chemical reaction, because the absorption of one quantum of light by this organism resulted in a shift of the absorption of about 3 molecules of carotenoid. A similar analysis of the changes observed here has, so far, not been possible because the location of the absorption bands of the carotenoid is not known.
Fig. 14. Difference spectrum for the initial change of absorbance in attached mutant barley leaves (lacking chlorophyll b) produced by excitation with a broad band of red light, from about 620 to 800 μm (3.6 × 10^5 ergs cm^-2 sec^-1). A schedule of 6 seconds light and 12 seconds dark was used.

References


\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{Reaction} & \textbf{Flash Interval} & \textbf{Inhibitor} & \textbf{Activity Ratio, 684/622 μm} \\
\hline
Carotenoid shift & 1 min & none & 1.47 \\
& 3 sec & none & 1.34 \\
Cytochrome oxidation & 1 min & DCMU & 1.33 \\
& 3 sec & DCMU & 1.34 \\
& 6 sec & DCMU & 1.46 \\
\hline
\end{tabular}
\caption{Relative Activity of Light of 684 and 622 μm in Causing the Shift in Carotenoid Absorption and Cytochrome Oxidation in Iridaea splendens}
\end{table}

The measurements were done at 495 μm for the carotenoid shift and at 420 μm for the cytochrome oxidation. Actinic light was given in 10-msec flashes with dark intervals as indicated in the second column. The last column gives the activity of absorbed quanta of 684 μm relative to those at 622 μm. The concentration of DCMU, where used, was 5 × 10^-5 M.


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\section*{Quenching by Quinones of Chlorophyll Fluorescence in Vivo}

\textit{Jan Amesz and David C. Fork}

During the past years quinones have attracted interest as possible intermediates in photosynthesis. There is evidence that plastoquinone is an intermediate in the photosynthetic chain, and as early as 1954 Wessels made the hypothesis that a quinone functions as the primary photooxidant of photosystem 2. Quinones have been found to act as Hill oxidants, as cofactors of photosynthetic phosphorylation, and as inhibitors of photosynthesis.

Livingston and Ke (1950) reported that benzoquinone and substituted benzoquinones quench the fluorescence of chlorophyll in organic solution, and more recently some observations have been published about quenching of fluorescence of chloro-
phyll in isolated chloroplasts by naphthoquinones. We have studied quenching of chlorophyll fluorescence in Swiss chard chloroplasts and algae. The results indicate that the quenching process is caused by direct interaction of chlorophyll and quinone molecules and not by stimulation of photosynthetic electron transport, as has been postulated.

The effect of DCMU and quinones on fluorescence kinetics. Figure 15 shows recordings of the time course of fluorescence for Swiss chard chloroplasts in the absence and in the presence of $10^{-5}$ $M$ DCMU. The initial level of fluorescence upon illumination is about the same under both conditions but with DCMU the fluorescence rises much faster and to a higher level than in its absence. According to Duysens and Sweers (1963) this increase is caused by the reduction of $Q$, the primary photooxidant of system 2. The slower rise in the absence of DCMU probably reflects the reduction of a larger pool, which may give rise to the oxygen burst reported earlier (Year Book 61, p. 334), and which tends to keep $Q$ in the reduced state initially. In the following we will call the final level of fluorescence the "total fluorescence" and the difference between the total and the initial fluorescence will be called the "variable fluorescence."

Figure 15 also shows the effect of 2-methyl-1,4-naphthoquinone (menadione, vitamin K₃). Menadione strongly quenched chlorophyll fluorescence, especially the variable fluorescence, which was already strongly quenched at $1.8 \times 10^{-5}$ $M$, a concentration that hardly affected the initial fluorescence. The quenching effect as a function of concentration is shown in Fig. 16.

Fig. 15. The effect of DCMU ($10^{-5}$ $M$) and of DCMU with three different menadione concentrations on the kinetics of fluorescence in Swiss chard chloroplasts. The excitation light was a band centered at 545 $\mu$m for the traces on the left and at 420 $\mu$m for the traces on the right. The intensities and vertical scales for the traces on the left and right, which were made with separate samples, are different. A 24-second dark period preceded each illumination.
Fig. 16. Quenching of the initial, variable, and total fluorescence of Swiss chard chloroplasts as a function of menadione concentration in $10^{-5}$ M DCMU: $\phi$ is the relative fluorescence yield in the absence of quencher and $\phi'$ in the presence of quencher. The excitation light was green (545 m$\mu$).

A number of other quinones were tested for quenching activity. Table 3 summarizes the results. Like menadione, most quenchers acted much more strongly on the variable than on the initial fluorescence. Some quinones such as phthiocol and lawsone showed little quenching activity but others were equal to or more active than menadione. Reduced menadione and 2,3,5,6-tetramethyl-benzoquinone had little or no quenching.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Initial Fluorescence Concentration (mM)</th>
<th>Variable Fluorescence 50% Quenching</th>
</tr>
</thead>
<tbody>
<tr>
<td>$p$-benzoquinone</td>
<td>1200</td>
<td>1500</td>
</tr>
<tr>
<td>2, 3-dimethyl-p-benzoquinone</td>
<td>800</td>
<td>120</td>
</tr>
<tr>
<td>2, 3, 5, 6-tetramethylbenzoquinone</td>
<td>450</td>
<td>83</td>
</tr>
<tr>
<td>1, 4-naphthoquinone</td>
<td>200</td>
<td>63</td>
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<tr>
<td>2-methyl-1, 4-naphthoquinone</td>
<td>160</td>
<td>28</td>
</tr>
<tr>
<td>(menadione)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-hydroxy-1, 4-naphthoquinone</td>
<td>$&gt;2000$</td>
<td>$&gt;2000$</td>
</tr>
<tr>
<td>(lawson)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-hydroxy-3-methyl-1, 4-naphthoquinone</td>
<td>$&gt;1000$</td>
<td>500</td>
</tr>
<tr>
<td>(phthiocol)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-hydroxy-1, 4-naphthoquinone</td>
<td>70</td>
<td>13</td>
</tr>
<tr>
<td>(juglone)</td>
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<td></td>
</tr>
<tr>
<td>1, 2-naphthoquinone</td>
<td>580</td>
<td>340</td>
</tr>
<tr>
<td>1, 2-naphthoquinone-4-sulfonic acid</td>
<td>$&gt;2000$</td>
<td>$&gt;2000$</td>
</tr>
<tr>
<td>Phenanthrenequinone</td>
<td>68</td>
<td>16</td>
</tr>
<tr>
<td>1, 2-dihydroxyanthraquinone</td>
<td>66</td>
<td>21</td>
</tr>
<tr>
<td>(alizarin)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$m$-dinitrobenzene</td>
<td>930</td>
<td>130</td>
</tr>
</tbody>
</table>

* Fluorescence was excited by green light (545 m$\mu$, 2 to $6 \times 10^3$ ergs cm$^{-2}$ sec$^{-1}$), after a preceding 24-second dark period. The DCMU concentration was $10^{-5}$M.
activity. Several quinones were also tested for their effect on fluorescence of intact algae. Menadione and 1,4-naphthoquinone, which were active with chloroplasts, were found to be active with algae too, although generally at a somewhat higher concentration. Lawsone and phthiocol, inactive with chloroplasts, were inactive with algae also. Figure 17 illustrates the effect of 1,4-naphthoquinone on the red alga *Porphyra perforata*.

**Mechanism of the quenching effect.**

There are several indications that the quenching, which occurred both with DCMU and without it, is not caused by an oxidation of reduced Q (QH) by quinones:

1. The $E_0'$ of the quencher varied widely: between +0.18V (2,3-dimethylbenzoquinone) and about -0.40V (alizarin); and many of these compounds should be unable to oxidize QH.

2. The quenching effect was independent of the intensity of the exciting light, and the regeneration of Q in the dark after an illumination period was found not to be affected by menadione.

3. With relatively high concentrations of quencher the fluorescence yield was considerably lower than that observed (in the absence of quencher) under conditions where Q could be assumed to be fully oxidized (e.g., during efficient photosynthesis, or shortly after onset of illumination).

4. In several experiments both with chloroplasts and with algae we did not see any reversion by quinones of the inhibition of oxygen evolution by DCMU.

The above observations indicate that the quenching effect is not caused indirectly by stimulation of the rate of electron transport, but rather by a direct interaction of quinone and chlorophyll molecules, which then act as traps for the excitation energy.

![Fig. 17. Fluorescence kinetics of Porphyra perforata in the presence of DCMU (5 X 10^-5 M) and 1,4-naphthoquinone. Fluorescence was excited by green light. The preceding dark intervals were 18 seconds for the traces on the left, and 30 seconds for the traces on the right. The traces on the right were recorded at about 20 times higher light intensity and lower sensitivity of the apparatus than those on the left.](image-url)
Since the fluorescence lifetime of chlorophyll is longer when \( Q \) is in the reduced state than when it is oxidized (Müller and Lumry, 1965), a stronger quenching can be expected for the total (and thus for the variable) than for the initial chlorophyll fluorescence, even if the quencher acts only on the bulk of the pigment. It is doubtful, however, if this could explain the large difference in the extent of quenching of the initial and variable fluorescence, as observed at low concentrations of menadione (Fig. 16).

Additional evidence that the quinones act at a site close to system 2 was given by experiments shown in Fig. 18. Measurements of absorption changes at 420 m\( \mu \) in Porphyra indicated that menadione and other quinones inhibited the reduction of the \( f \)-type cytochrome by light absorbed mainly by system 2. Quinones thus exhibited the same effect as well-known system 2 inhibitors such as DCMU.

In agreement with the results of earlier experiments of Wessels (1954) we found that a number of quinones inhibited the Hill reaction of chloroplasts with 2,6-dichlorophenol-indophenol (DCPIP). In general, strong quenchers were more powerful inhibitors of cytochrome reduction and of the Hill reaction than were weak quenchers. However, a quantitative relation was not found. Menadione gave 50% inhibition of DCPIP reduction at \( 2 \times 10^{-4} \text{ M} \), about the same concentration as needed for 50% quenching of the initial fluorescence, but with 1,4-naphthoquinone, lawsone, and phthio-

![Fig. 18. Effect of menadione on the cytochrome absorbance changes in Porphyra perforata. Red actinic light, to excite mainly system 1, had a half band between 670 and 780 m\( \mu \) and green actinic light to excite system 2 from 550 to 570 m\( \mu \). A downward trace corresponds to the oxidation of cytochrome.](image-url)
col the effect on cytochrome and DCPIP reduction was stronger than that on fluorescence.

**Emission spectra.** We measured the emission spectra of *Porphyra* with green exciting light in the presence and in the absence of 1,4-naphthoquinone and DCMU, using the apparatus for automatic recording of fluorescence emission spectra constructed by French (*Year Book 65*, p. 493). The emission spectra (Fig. 19) showed three maxima at 658, 684, and 731 m\(\mu\). The first one is produced by phycocyanin and the second by chlorophyll \(a\). The maximum at 731 m\(\mu\) was first observed by Duysens (1951). As Fig. 19 shows, naphthoquinone quenched the chlorophyll band at 684 m\(\mu\) and, somewhat less strongly, that at 731 m\(\mu\), but had no effect on the intensity of phycocyanin fluorescence. This indicates that the quinone does not interact with the biliprotein chromophore, and that the transfer of energy between phycobilins and chlorophyll is not affected.

DCMU alone caused about a three-fold increase of the 684-m\(\mu\) band and a 2.5-fold increase of the 731-m\(\mu\) band, but had no effect on the phycocyanin emission.

If it is assumed, according to current theories, that DCMU stimulates only the fluorescence of pigment system 2, then these data indicate that the pigment fluorescing at 731 m\(\mu\) belongs not only to system 1 (as indicated by experiments of Duysens, 1951) but also to system 2. Naphthoquinone then quenches system 2 fluorescence, possibly by shortening the lifetime of the excited state of chlorophyll \(a\) fluorescing at 684 m\(\mu\), and thus decreasing both the 684-m\(\mu\) fluorescence and the extent of the transfer of energy to the pigment fluorescing at 731 m\(\mu\).

**Conclusion.** Our experiments demonstrate that a number of substi-
tuted quinones strongly quench the fluorescence of chlorophyll in vivo. The quenching in vivo occurs at a much lower concentration of quinone than when the chlorophyll is dissolved in organic solvent (Livingston and Ke, 1950). The quenching is probably due to direct interaction between chlorophyll and quinone molecules, rather than to stimulation of electron transport. It is possible that the actual quenching mechanism (see Seely, 1966, for a discussion) is the same in vivo as in chlorophyll solution. The stronger quenching in vivo may be caused by a concentration of the quinone in the lipoid part of the chloroplast lamellae, and also may be explained by the fact that quenching of the same fraction of chlorophyll molecules, by formation of traps, could produce a stronger quenching in vivo than in a dilute chlorophyll solution. The low activity of phthiocol and 1,2-naphthoquinone-4-sulfonic acid may be due to salt formation in the water phase.

There is no direct spectrophotometric evidence (Amesz, 1964) for the hypothesis that a quinone is the primary photooxidant for system 2. However, the strong quenching of chlorophyll fluorescence in vivo indicates that association of a quinone with a chlorophyll molecule can produce an efficient trap for the excitation energy. It is possible that a photosynthetic reaction center of system 2 consists of a similar trap, supplemented by the enzymatic and structural arrangement necessary for the formation of stable photochemical products.

References

Some Essential Considerations in the Measurement and Interpretation of Absorption Spectra of Heterogeneous Samples

James M. Pickett and C. S. French

The following discussion of well-known but, of necessity, often neglected factors is given here primarily as a review of basic principles essential to the study of absorption spectra of photosynthetic cells. These considerations are particularly important for spectra that are to be analyzed in terms of their component forms of chlorophyll.

Sieve effect. The principal source of error in measuring the shape of absorbance curves with thin suspensions is that some light gets through spaces between the cells. This makes the value of transmitted light greater than it would be if the pigment complexes were uniformly dispersed in solution. The sieve effect makes the peaks of the absorbance curve lower than they should be, relative to regions of lower absorbance.

The error introduced by the sieve effect becomes smaller as the number of particles increases because the absorbance of a given wavelength for various light paths then becomes more uniform throughout the suspension. However, at high suspension concentrations the effective path length is reduced for wavelengths of high ab-
sorbance relative to that for wavelengths of low absorbance (Butler, 1964). Thus peaks in absorbance spectra of dense algal suspensions are usually flattened because scattering (Latimer, Year Book 56, p. 259) and reflectance (Rabideau et al., 1946) are not independent of wavelength, while the sieve effect is negligible.

The absorbance of a suspension relative to that of the same pigment in solution can be calculated for a single layer of absorbing particles. The calculations are complex for suspensions of more than one layer. The case of oriented cubic particles has been described in detail by Duysens (1956). The analysis in the case of spherical particles is more complex because the absorbance is not uniform over the projected area of the spherical particles.

**Sieve effect for a single layer of uniform particles.** Let \( f \) be the fraction of the light beam that encounters a particle of transmission \( T_p \). If mutual shading of the particles is negligible, the measured absorbance \( E_s \) of a dilute suspension of uniform particles is

\[
E_s = \log \left( \frac{1}{(1-f) + fT_p} \right)
\]

But the apparent absorbance of a single particle is

\[
E_p = \log \left( \frac{1}{T_p} \right)
\]

The important conclusion is that the measured absorbance of dilute suspensions is not proportional to the apparent absorbance of one particle. Moreover, if all light paths through the particle are not equal, \( E_p \) is not proportional to the extinction coefficient of the pigment present in the particle. Therefore, we propose to review the types of errors generally introduced by absorbance measurements of suspensions relative to the absorbance \( E \) that the same pigments would have in the same volume of solution with a uniform path length.

**Spherical particles.** Consider a sphere of radius \( r \) suspended in a medium of equal refractive index illuminated by a circular beam of light whose radius is equal to that of the sphere. Assume that each spherical particle contains a concentration \( c \) of pigment whose absorption coefficient is \( \varepsilon \). The absorbance along a light path of length \( x \) through the sphere is

\[
E_s = \varepsilon cx
\]

The transmission along the same path is

\[
T_s = 10^{-E_s}
\]

All paths of length \( x \) through the sphere are in a circle of radius \( y \) perpendicular to the incident beam of light where

\[
y^2 = r^2 - \left( \frac{x}{2} \right)^2
\]

The absorbance \( E \), which is proportional to the extinction coefficient of the pigment, and transmission \( T_s \) of a single sphere are obtained by integrating the weighted values of \( E_s \) and \( T_s \) over all light paths (Duysens, 1956, and Pickett, 1965)

\[
E = \int_{y=0}^{r} E_s \frac{2\pi y \, dy}{\pi r^2}
\]

\[
= \frac{4}{3} \varepsilon cr
\]

\[
T_s = \int_{y=0}^{r} T_s \frac{2\pi y \, dy}{\pi r^2}
\]

\[
= \frac{10^{2\varepsilon cr} - 4.606 \varepsilon cr - 1}{10.61 (\varepsilon cr)^2 10^{2\varepsilon cr}}
\]

from which

\[
E_p = \log \frac{10.61 (\varepsilon cr)^2 10^{2\varepsilon cr}}{10^{2\varepsilon cr} - 4.606 \varepsilon cr - 1}
\]

It is clear from equation 8 that \( E_p \) is not proportional to \( \varepsilon \), the absorb-
tion coefficient of the pigment within the sphere. The ratio of the apparent absorbance of a single sphere, $E_p$, to the absorbance of the uniformly dispersed pigment $E$ is given in Fig. 20, for various values of $2e_{cr}$, the absorbance through the center of a single sphere.

The transmission $(10^{-2e_{cr}})$ for 675 m$\mu$ of single Euglena chloroplasts as measured by Wolken et al. (Strother and Wolken, 1959; Wolken and Strother, 1963) falls in the range of 0.4 to 0.6 ($2e_{cr} = 0.6$ to 0.8) and for Chlorella, about 0.6 ($2e_{cr} = 0.8$). The corresponding ratio $E_p/E$ is 0.94 to 0.92. Flattening of the absorption peak may be much less than 6% to 8% in these microspectrophotometric measurements because the absorption spectrum was determined by measuring the transmission of a single chloroplast with a beam of light whose cross section was small compared to that of the chloroplast.

In practice it is generally more convenient to measure the transmission of a relatively dilute suspension. The ratio of the measured absorbance $E_s$ of a suspension with negligible overlap of particles to the absorbance $E$ of the uniformly dispersed pigment is plotted as a function of the absorbance through the center of the sphere, $2e_{cr}$, in Fig. 21. For particles with a maximum absorbance ($2e_{cr}$) of greater than 0.5, the absorbance maxima are reduced by more than 10%. The error becomes greater, the smaller the fraction $f$ of the light beam intercepted by particles. Thus accurate absorbance spectra of dilute suspensions can only be obtained for particles of very low maximum absorbance.

**Dense suspensions.** The sieve effect is relatively insignificant in very turbid suspensions, since the effective path length is much greater than the thickness of the suspension. However, the path length is not increased to the same extent at all wavelengths. The principal variable influencing effective optical path length is particle reflectance (Butler, 1964). Anomalous dispersion, which is maximum on the long-wavelength side of absorption bands (Latimer, *Year Book 56*, p. 259), is relatively insignificant except when a very small solid angle of light is collected by the spectrophotometer (Butler, 1964).

![Fig. 20. The apparent absorbance $E_p$ of a single spherical particle divided by the absorbance $E$ of the pigment in the same cylindrical volume of solution with path length 1.33 $e_{cr}$, is plotted as a function of the absorbance through the center of the sphere $2e_{cr}$. The spherical particle of radius $r$ contains a uniform concentration $c$ of pigment with absorption coefficient $e$.](image)
The height ratio \( H \). We need a rough test for the likelihood of serious distortion in an experimentally measured curve. For this purpose with green plants we have used the height ratio \( H \), defined as the absorbance at the secondary chlorophyll \( a \) maximum at about 625 \( \mu \text{m} \) divided by the height of the red absorbance peak at about 675 \( \mu \text{m} \).

As a standard of comparison we have taken the value of \( H \) for the alga showing the smallest ratio. This is Claes Chlorella mutant 871 grown in the dark, which appears to have nearly all its chlorophyll as \( \text{Ca}672 \) and lacks chlorophyll \( b \). For that alga \( H = 0.28 \) at \(-196^\circ\text{C}\).

In a green alga containing two forms of chlorophyll with absorption spectra of similar shape but shifted in wavelength, the value of \( H \) would be larger and would depend on the wavelength separation interval. This variation of \( H \) was obtained graphically for a hypothetical system having two identical spectra but shifted in wavelength by various amounts. The basic curve shape was taken as the sum of the 625 and 671 components that fit the absorption curve of Fig. 26 for dark-grown Chlorella mutant 871. This basic curve is the closest approximation to the actual shape of the spectrum of \( \text{Ca}670 \) that we have. In calculating \( H \) for the hypothetical two-component spectrum, curves of the same height were used. For any other proportion the change of \( H \) with the separation interval would be smaller.

The results in Table 4 show that the ratio in question would be only 0.36 for the maximum separation interval of 13 \( \mu \text{m} \) expected between “\( \text{Ca} 670 \)” and “\( \text{Ca} 680 \)” Ratios for experimental spectra of algae containing only chlorophyll \( a \) that are larger than 0.36 can therefore be taken as an indication of distortion by the flattening effects.

According to Butler (1964) accurate absorption spectra of dense suspensions can be obtained by correcting the measured spectrum for variation of effective path length with wavelength. The flattening may also be greatly reduced by suspending the pigmented particles in a much greater concentration of white powder.

In summary, there are three principal means of measuring in vivo absorption spectra which may closely approximate the absorption spectra of the pigments: (1) The absorbance may be measured along a fixed light path with a light beam whose cross section is small compared to that of the pigment complex (microspectrophotometry). (2) The absorption spectra of very weakly absorbing cells such as pale-green mutants may be measured by means of thin suspensions in opal glass containers. (3) According to Butler (1964) the absorption spectra of very dense suspensions can be measured in the presence of large amounts of white powder or corrected for variation of effective path length with wavelength.
TABLE 4. The ratio of the Orange, "625"-mμ, Peak to the Red, "675"-mμ, Peak of a Hypothetical in vivo Chlorophyll a Spectrum Composed of Equal Parts of Two Identical Components at Several Wavelength Separation Intervals

<table>
<thead>
<tr>
<th>Separation Interval (mμ)</th>
<th>Height Ratio of Orange to Red Maximum, H</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.29</td>
</tr>
<tr>
<td>5</td>
<td>0.30</td>
</tr>
<tr>
<td>10</td>
<td>0.32</td>
</tr>
<tr>
<td>13</td>
<td>0.36</td>
</tr>
<tr>
<td>15</td>
<td>0.39</td>
</tr>
</tbody>
</table>

References

A SPECTROPHOTOMETER PRIMARILY FOR LIGHT-SCATTERING SAMPLES AT LOW TEMPERATURE
C. S. French and Mark Lawrence

In Year Book 65 (p. 498) a plan was discussed for modifying the derivative spectrophotometer so it could also be used as a double-beam instrument for absorbance measurements of highly scattering samples. The integrating sphere then under discussion has been abandoned. Instead of the sphere, a different type of sample chamber was constructed to be particularly suitable for absorbance measurements of cell suspensions.

To reduce scattering errors the major requirement in the design of the sample holder was that it should gather a representative fraction of the transmitted light from the sample over a wide angle. Four other requirements were that it should (1) accommodate sample holders that could be used at liquid nitrogen temperature; (2) have a peak height of the plotted absorbance curve for a particular sample adjustable to any desired value and still have known scales in absorbance or transmission units; (3) by means of an entirely transistorized electronic measuring circuit eliminate maintenance problems of vacuum tube amplifiers; and (4) be simple enough for routine use by different people. The device, which meets these requirements reasonably well, has been in use since April 1967. A brief description is given here.

Optical system. The sample holders shown in Fig. 22 use Shibata's opal glass diffusion principle for both windows. The aluminum spacers also serve as heat-conducting elements and supports. Since the opal glass windows are held on with silicone grease and oiled modeling clay only, they do not break when the sample freezes. A holder for the usual 1-cm-square liquid absorption cells can be used in place of the opal glass sandwiches when the material is not to be frozen.

The sample chamber is shown in Fig. 23; just behind the sample holder is another opal glass window. Between this window and the photocell is a light-mixing tube with white walls. This tube helps to give uniform illumination on the photomultiplier tube for light falling on any part of the opal glass window at its entrance. The brass box in the sample chamber is thermally insulated by 2.5 to 4 cm of plastic foam. The lower part of this box can hold 100 cc of liquid nitrogen or other liquid for operation at a desired temperature. The entrance window is lucite 2.54 cm thick, preceded by a double-walled glass chamber to reduce fogging at low temperature. A turret can be rotated to bring any one of three multiplier
Fig. 22. The sample holder—an opal glass sandwich. The aluminum spacer and support is also a heat conductor.

tubes into position for different spectral regions. A filter-holder attachment can be used in front of the photomultiplier for special experiments.

The optical system, long in use for derivative spectrophotometry, has been kept. This is a tungsten lamp with a Bausch & Lomb monochromator having a $10 \times 10$ cm grating blazed for green. The slits isolate a beam of $1 \text{ m}_{\mu}$ half-width. Glass color filters are used to reduce the stray light which is inevitable from a single monochromator. The large monochromator is particularly valuable in that plenty of light is available for measurements of scattering samples with three sheets of opal glass in the beam.

A rotating half mirror alternates the beam from the sample to the reference cell about 10 times a second. Adjustable sectors on the mirror shaft pass light from separate bulbs to photocells that activate the appropriate electronic circuits while either the sample or the reference cell is illuminated.

**Electronics.** The electronic measuring system uses solid-state operational amplifiers throughout. The first stage is a preamplifier with automatic gain control that makes operation possible over a wide range of input currents from the photomultiplier. In Fig. 24 two block diagrams show the operating principles and controls for recording absorbance or transmittance. The operating zero-position control, not shown, is adjustable to facilitate recording of the difference spectra between two samples.

In many spectrophotometers the logarithm needed for absorbance plots is generated by a logarithmic slide-wire in the recorder. Change of scale can then be done only by chang-
ing slide-wires. In the instrument described the logarithm is produced by a transistor in the feedback circuit of an operational amplifier. The logarithmic signal appears as an electrical voltage that can be amplified or attenuated. This makes it possible to adjust the scale of the recording to compare curve shapes for different samples.

When the "curve size" control is at its extreme position, the 10-inch full-scale deflection of the recorder corresponds to the value of absorbance or transmission selected by the range switch. On any scale the actual plot can be reduced in size by the curve size control. When this is done calibration marks for either absorbance or transmission can be put on the record by setting a "calibrator" dial to the values desired.

**Absorption Spectra of Chlorophyll a in Algae**

_C. S. French_

Much of the present-day research on photosynthesis is concentrated on two closely related questions. One of these is to find means by which the light energy absorbed by photosynthetic pigments is transferred from one pigment to another, then trapped and converted to chemical energy as a change in the oxidation state of a reaction center. The other problem is to define the succeeding reactions of specific substances in the pathways of electron flow from the primary reaction center that makes products useful to plants.

There are two types of primary reaction centers believed to contribute energy to the electron flow system at
Fig. 24(A). Block diagram of the electronic measuring circuit for absorbance measurements.
Fig. 24(B). Block diagram of the electronic measuring circuit for transmission measurements. A single switch changes from the absorption to the transmission circuit.
two points in a series-linked system of redox compounds. These two reaction centers are activated by different pigment systems each of which contains predominantly, but not exclusively, one of the different "forms" of chlorophyll \( a \) and other pigments. Thus system 1 is largely powered by light absorbed by \( C_6\text{a} 680 \) and system 2 by light absorbed by chlorophyll \( b \), \( C_6\text{a} 670 \), and the "accessory pigments," carotenoids and phycobilins.

Considering the large amount of intensive work being done on the functioning of these photosynthetic systems it is remarkable that so little is known about the absorption spectra of the individual pigments initially catching the light used to drive the two systems of photosynthesis. Most of the available information on the partition of absorbed light between the two systems has come, not from absorption spectroscopy, but from measurements of action spectra. About all we know is that in green plants significantly more light is absorbed by system 1 than by system 2 at wavelengths beyond 685 m\( \mu \). However, system 2 still absorbs some light even at longer wavelengths because of the overlapping spectra of the pigments in the two systems. Furthermore, at 650 and 480 m\( \mu \), the bands of chlorophyll \( b \), system 2 absorption is larger than at other wavelengths. Some system 2 action spectra show a significant contribution from \( C_6\text{a} 670 \) and some others appear to involve \( C_6\text{a} 680 \). Beyond this qualitative information only a few attempts have been made to specify the exact proportion of any wavelength that goes to each functional system. Some few measurements of system 1 and system 2 action spectra have been made with precision comparable to that attainable with absorbance measurements. However, action spectra for the two systems in various species still have not been precisely correlated with well-measured absorbance spectra for the same cultures.

Ideally we would like to be able to separate each chlorophyll \( a \) form in a pure state without change of its spectral properties, measure its absorbance and fluorescence, and also, for a well-defined reaction, its action spectrum. Work on the difficult problem of separating the chlorophyll forms is progressing in several laboratories and some of it is described elsewhere in this report.

The present report will discuss some attempts to derive absorption spectra of the individual forms of chlorophyll \( a \) by analysis of new measurements of absorption spectra of algae, and mainly of those algae lacking chlorophyll \( b \). From past attempts, largely by derivative spectroscopy, it seems that each of the forms designated as \( C_6\text{a} 670 \), \( C_6\text{a} 680 \), \( C_6\text{a} 695 \), etc., may represent classes of pigment complexes rather than definite chemical compounds with identical spectra in different plants. This conclusion is, however, by no means certain because small amounts of unrecognized components could well shift the apparent position of the observed curves away from their true positions in the spectrum.

Some absorption spectra of intact algae. Spectra of some algae useful for characterizing the forms of chlorophyll \( a \) are given in Fig. 25; and Table 5 lists values of \( H \), the peak height ratio for comparison with chlorophyll \( a \) in solution. Figure 25(A) gives the spectra of Tribonema at two temperatures. These curves are probably flattened but the sharpening by low temperature clearly shows the doublet structure of the main red band, which is not evident at 22\( ^\circ \)C. The original record indicates a possible, but uncertain, doublet structure of the 625 band, perhaps with broad components at about 620 and 630 m\( \mu \). The bands at 496 and
TABLE 5. Ratios of the Orange Band Height to that of the Red Band for Chlorophyll a

<table>
<thead>
<tr>
<th>Chlorophyll a</th>
<th>22°C, H</th>
<th>-196°C, H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ether</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td>Acetone</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>Special Solvent*</td>
<td>...</td>
<td>0.30</td>
</tr>
</tbody>
</table>

Claes Chlorella

871 D ... 0.28
520 D 0.37 0.35
515 D 0.37 0.30
WT D 0.30 (0.36) ...

Tribonema 0.38 0.50
Botrydiopsis 0.36 0.47
Highkin barley 0.47 0.66
Ochromonas ... 0.43
Ochromonas refrozen ... 0.36

* Freed and Sancier, 1951.

463, and some of the absorption at shorter wavelengths, are presumably due to carotenoids, whereas the other labeled bands are attributed to forms of chlorophyll a.

Figure 25 (B) gives the spectra of Claes wild-type Chlorella at two temperatures. The low ratio of the peak absorbance at about 625 to about 670 m\(\mu\) (\(H = 0.31\), at 22°C) and the very small absorbance at 530 m\(\mu\) shows a surprising lack of flattening in this spectrum for a green alga. The 486- and 651-m\(\mu\) bands are attributed to chlorophyll b; those at 492, 467, 460, and some shorter absorption bands, to carotenoids. The 433 and 437 peaks might be due to different forms of chlorophyll a.

In Fig. 25 (C), we have spectra for the alga Botrydiopsis and for a leaf of Highkin's barley mutant, both lacking chlorophyll b. The curves appear strongly flattened but show the two major forms of chlorophyll a, and the barley has a band at about 710 m\(\mu\).

Claes Chlorella mutants 515, 520, and 871, when grown in the dark, contain very little chlorophyll and no chlorophyll b, thus providing excellent material for the study of the shapes of the chlorophyll a spectra. Furthermore, mutant 871 appears to be free of carotenoids absorbing in the visible spectrum. Figure 25 (D) shows large blue peaks, believed to be zeta-carotene, which obscure the blue chlorophyll a spectrum. Figure 25 (E) for mutant 520 shows longer wavelength carotenoid spectra.

In Fig. 25 (F) we have a close approximation to the spectrum of C\(_6\)72 with only a small percentage of C\(_6\)80 and probably very little interference from carotenoids. Its shape is discussed in detail below. An ethanol extract of chlorophyll from mutant 871 was chromatographed by Dr. Michel-Wolwertz who found two fractions—ordinary chlorophyll a and a component spectrally similar to, but chromatographically distinct from, pheophytin. This suggests that the spectrum for mutant 871, while it is the simplest we have yet seen, may be composed of other components besides C\(_6\)70. She found the other Claes mutants grown in the dark to contain only chlorophyll a.

Analysis of observed spectra. Our ultimate hope is to derive curve shapes for spectra of the individual chlorophyll forms that, when added together in correct proportions, would match any observed spectrum that was not distorted. Until that can be done it is helpful to compare experimental spectra by the relative proportions, peak positions, and half-widths of the minimum number of normal probability curves that, when added together, match the measured curves.

We have previously used derivatives of probability curves to analyze derivative spectra in order to approximate the relative content of different forms of chlorophyll a (Brown and French, 1959). The curves for the major components used for Chlorella were broader at 673 than at 683 m\(\mu\). Cederstrand et al.
Fig. 25. The absorption spectra of various algae; all except the wild-type Chlorella lack chlorophyll b. (A) Tribonema Sp. (Starr 639). The curves at two temperatures are directly comparable. The sharpening at low temperature shows the red chlorophyll a band to contain two forms of chlorophyll a not evident in the room temperature spectrum. (With D. C. Fork.) (B) Claes wild-
(1966) have matched absorbance spectra of Chlorella, plotted on a wave number scale, with probability curves peaking at 668 and 683 mµ and having equal half-widths of 21 mµ. For estimating the shape of the narrow bands here considered we have used wavelength rather than wave number scales. The discussion will be limited to spectra measured at -196°C that appear to be free of flattening.

Starting with two spectra having most of the chlorophyll as $C_670$ we see in Fig. 26 (A) that for mutant 871 a single probability curve does not fit the main peak. With the same probability curve at 672 mµ and 22 mµ half-width, an attempt was also made to match the spectrum of mutant 515 as shown in Fig. 26 (B). The misfit for both spectra is taken to mean that components other than a single chlorophyll a form are present.

Therefore, enough gaussian curves were taken to give a close fit for mutant 871 (disregarding the slight error in the sensitive trough region at 650 mµ). The curves used are given in Fig. 26(C) and Table 6. Then, with the same peak positions and half-widths as for 871, the heights were adjusted to come as near as possible to matching mutant 515 with unsatisfactory results that are not shown. However, the slight changes in band positions and widths recorded in Table 6 gave a good fit to 515, as shown in Fig. 26 (D). For mutant 515 the secondary component had to be slightly different, as shown in the figure and the table. The peak wavelength position, the half-widths, and the proportion of the total area for the gaussian curves of Fig. 26 are given in Table 6.

Mutant 520 appears to contain more $C_6680$ than do 871 and 515. Its best fit came from component curves nearly like those for 871 and 515 but slightly different, as shown in Table 6 and Fig. 26(D). Whether or not these small differences of 1 to 2 mµ in peak position and bandwidth required to match the spectra of different mutants are significant remains a question.

These curve analyses have pointed out the previously disregarded fact that a large fraction of the light incident on a photosynthetic cell is absorbed by the secondary chlorophyll a bands having broad maxima in the 625-mµ region. Table 6 shows that 40% of the total absorption from about 570 mµ to the red end of the spectrum should be attributed to these bands.

The secondary orange band is presumably different in peak position and width for the various forms of chlorophyll. It is so wide that wavelength peak differences probably amounting to about one fifth of its width are obscured. There certainly are, however, differences in the shape of the orange bands in different algae that may be worth further study after the variations in the red band are better understood.

A particularly interesting effect, susceptible to curve analysis, is re-
Fig. 26. Analyses of the red chlorophyll a absorption bands of several Claes Chlorella mutants grown in the dark and of Ochromonas danica, in collaboration with Dr. Brown. The characteristics of the gaussian component curves are listed in Table 1. The sum of these components is shown as a dotted line where it deviates from the measured curve being matched.

Reported in another section by Dr. Brown. This is in Ochromonas danica, an alga containing chlorophyll c rather than chlorophyll b. The three-peaked spectrum of the frozen sample changed to a two-peaked spectrum after thawing and refreezing. Attempts to match the three-peaked curve with a small number of gaussian curves were not fruitful. However, the spectrum of the refrozen material was fitted successfully as shown in Fig. 26(F). By changing only the relative heights, but not the peak positions or widths of these curves matching the refrozen sample, it was
not possible to match the three-peaked curve of the same sample before freezing. The nearest approach to a fit is illustrated in Fig. 26(G). This difference suggests that the spectra of the components themselves were affected by the treatment. Just from looking at the curves before and after refreezing we had the impression that the relative proportions of the bands but not their characteristics had been influenced. This does seem to be more nearly true for a repetition of this experiment with another culture shown in Fig. 34 of Dr. Brown's report.

Because mutant 871 seems to be free of carotenoids we fitted the blue part of its spectrum with gaussian curves with the results given in Fig. 27. Whether or not these represent only components of chlorophyll a remains to be decided when other carotenoid-free samples are found in algae or in preparations such as the S₁ fraction shown in Fig. 30 of Mr. Michel's report. Analysis of the blue and of the red spectra of carotenoid-less samples are needed to determine which of the blue bands correspond to the forms of chlorophyll that are now identifiable only by their red bands.

In conclusion we may say that the present best available approximation to the red part of the spectra of Cₐ

![Fig. 27. The blue spectrum of Claes Chlorella mutant 871 is approximately matched by the sum of the following gaussian curves described by peak position, half-width and percentage of the total area of their sum: 390 mμ, 27 mμ, 27%; 418 mμ, 29 mμ, 47%; 441 mμ, 18 mμ, 24%; 467 mμ (18 mμ), 2%.](image)
"670" is described by the sum of two Gaussian curves, with the following characteristics: (1) \( \lambda_{\text{max}} = 622 \text{ m} \mu \), \( W_{1/2} = 60 \text{ m} \mu \); (2) \( \lambda_{\text{max}} = 672 \), \( W_{1/2} = 22 \text{ m} \mu \), band height ratio \( (1) / (2) = 0.27 \) (because of overlap, the band-height ratio, \( (1) / (2) \), is not exactly the same as the total curve-height ratio, \( H \)).

References

**ELECTROPHORETIC STUDY OF THE CHLOROPHYLL-LIPROTEIN COMPLEXES OF EUGLENA**

**J. M. Michel**

Many investigations, some from this laboratory, have shown that the red part of the absorption spectrum of green organisms results from the juxtaposition of at least three discrete forms of chlorophyll \( a \), presumably lipoprotein complexes. They are identifiable only from differences in their absorption spectra. We know very little about their actual chemical properties. We do not know if the differences between these substances are due to different arrangements of chlorophyll bound to the same carrier, to different carriers for the same pigment molecule, or to some other set of conditions.

Most of the attempts to separate these forms have been by differential centrifugation using different ways of solubilizing the chloroplast material. Only a few attempts have used electrophoresis, although zone electrophoresis methods are now available with properties particularly adapted for the study of such components. Among these methods, acrylamide gel electrophoresis is of particular interest because it is possible to tailor the size of the pores inside the gel to the size of the particles being studied. (Ogawa et al., 1966; Thornber et al., 1967). We used acrylamide gel electrophoresis to study the protein-chlorophyll complexes of chloroplast fragments of *Euglena*.

**Methods**

*Euglena gracilis*, Indiana Culture Collection No. 752, grown for 21 days in low light intensity, was used. When so grown, *Euglena* contains an appreciable amount of a longer wavelength absorbing chlorophyll form, \( \text{Ch}_{695} \) (Brown and French, 1961). We prepared the chloroplast fragments using the needle-valve disintegrator and the anionic detergent Na-deoxycholate (DOC) as a solubilizing agent according to the method of Brown et al. (1965).

This method gives two fractions on centrifugation for 30 minutes at 30,000 \( g \); a sediment called here Sed 30,000 and a supernatant called Sup 30,000. Prior to electrophoresis, the fractions in tris-HCl buffer \( 0.01 \text{M} \), pH 8.9 were mixed with an equal volume of a solution of sucrose 40% in tris-glycine \( 0.022 \text{M} \), pH 8.9.

Disc electrophoresis was performed on the fractions Sed 30,000 and Sup 30,000, according to the method of Steward (1965). Before electrophoresis the sample was layered on top of the concentration gel; the buffer in the reservoir was tris-glycine \( 0.02 \text{M} \), pH 8.9.

**Results**

The electrophoretic patterns of the two fractions Sed 30,000 and Sup 30,000 are shown in Fig. 28. Sed 30,000 did not penetrate the gel. The stationary band is labeled \( S_0 \). The Sup 30,000 fraction, however, showed two clearly separated bands...
Sup 30,000  Sed 30,000

\[ S_1 \]
\[ S_2 \]

No Amido-black  No Amido-black

Fig. 28. Electrophoretic patterns of Sed 30,000 and Sup 30,000.

\( S_1 \) and \( S_2 \) moving to the anode and some material which did not move into the gel. This \( S_o \) band could be a contamination of Sup 30,000 by particles of Sed 30,000.

The \( S_1 \) band was blue-green and the \( S_2 \) band was green with a yellow front; under an ultraviolet lamp only the \( S_2 \) band showed a visible red fluorescence. Figure 28 shows also the pattern after coloration of the proteins by the specific dye Amido Black B10. This shows the absence of other mobile proteins in the centrifugal fractions used for electrophoresis.

The red part of the low-temperature absorption spectra of the bands \( S_1 \) and \( S_2 \) as well as the sample staying at the starting point for Sed 30,000, are shown in Fig. 29. The absorption spectra of the bands \( S_1 \) and \( S_2 \) at room temperature are given in Fig. 30 for the entire visible spectrum.

The absorption spectra of \( S_1 \) and \( S_2 \) are almost identical in the red, having a maximum at 670 m\( \mu \) and lacking the "long-wavelength" absorbing form. There is, however, a minor difference in that \( S_1 \) has relatively higher absorbance than \( S_2 \) near 660 and 690 m\( \mu \) and less at about 670 m\( \mu \). Sed 30,000 has the same absorption spectrum as the original extract; it contains most of the chlorophyll b.

Figure 30 shows clearly that the band \( S_2 \) contains most, if not all, of the carotenoids, whereas the band \( S_1 \) appears to be free of carotenoids. This result is similar to that of Ogawa et al. (1966) who found a differential separation of the carotenoids using sodium laurylsulfate as solubilizing agent for spinach chloroplasts. Brown et al. (1965) achieved a similar separation by differential centrifugation of DOC-treated Euglena chloroplasts.

The fluorescence spectra of the bands \( S_1 \) and \( S_2 \) are reported in Fig. 31; the maximum of emission is at 680 m\( \mu \). When compared with the original extract and Sed 30,000 the large emission band at 720 m\( \mu \) disappears in the fluorescence spectra of \( S_1 \) and \( S_2 \). Comparison of the ab-
sorption and fluorescence emission spectra of $S_1$ and $S_2$ gives direct evidence that the 670-m$\mu$ absorption maximum is correlated with the 680-m$\mu$ emission maximum.

From these data we can conclude that DOC solubilizes two components from the chloroplast lamellae of *Euglena*. The two components are different in their electrophoretic mobilities and in their pigments. Carotenoids are associated with the $S_2$ band, but the chlorophyll portion of the pigment system is essentially chlorophyll $a$ with nearly the same spectral properties in both cases. From the different electrophoretic mobilities it may be inferred that the protein portions of the complexes are different, whereas the spectra suggest that the chlorophyll $a$ molecules are bound to the protein in a similar or identical manner. However, we cannot yet exclude the possibility that the $S_2$ band could contain some lipids dissolved in detergent micelles.

Two more green bands have been separated by disc electrophoresis from material treated with sodium laurylsulfate. They are much less abundant than $S_1$ and $S_2$, which makes their study more difficult.

To see if the different forms of chlorophyll are chemically separable entities or if the complexes exist only in the highly ordered structure of the lamellar membrane, we are now involved in attempts to separate the $C_a672$, $C_a685$ and the $C_a695$ forms as such. Preliminary experiments show that, with appropriate experimental conditions, it is possible to separate two different electrophoretic bands from *Euglena* chloroplasts without using any detergent. However, so far these two bands having identical spectra each contain all three in vivo chlorophyll $a$ forms.

References


Ogawa, T., F. Obata, and K. Shibata,
THE CHLOROPHYLLS EXTRACTED FROM PLANTS BY ORGANIC SOLVENTS

Marie-Rose Michel-Wolwertz

Several studies reported in this Year Book and elsewhere have demonstrated the existence of different chlorophyll forms in vivo (for instance, Year Book 58, p. 278; Year Book 59, p. 330; Year Book 65, p. 483). It is generally considered that these "in vivo forms" differ in the arrangement and (or) state of aggregation of one single type of chlorophyll molecule with the lipoprotein carrier.

Meanwhile, Sironval et al. (1965) showed that two chemically different pigments, namely, chlorophyllide a and chlorophyll a, were responsible for the two forms C₆₈₄ and C₇₆₂ present in vivo during the initial period of greening of etiolated leaves.

Michel-Wolwertz and Sironval (1965) have isolated several chlorophyllous pigments from ethanol extracts of Chlorella by means of paper chromatography. These are in addition to the common chlorophylls a and b (called a₁ and b₁). They suggested that these "satellites" might be responsible for the different absorbing forms in vivo.

The purpose of the present study was to test this possibility.

1. We tried to find out whether the light regime received by a plant would influence the pigment composition of its extract.

2. We studied the effect of the presence of air or of nitrogen during extraction and chromatography upon the pigment composition of extracts of different species.

Method

Chlorella pyrenoidosa, Pringsheim (211/8b) and Euglena gracilis were used for extraction of pigments. For comparison we also used spinach leaves obtained from local stores.

Chlorella and Euglena were extracted with boiling 95% ethanol. Immediately after extraction, the pigments were transferred to petroleum ether (b.p. 45-60°C) by the addition of excess water. Spinach leaves were extracted by grinding with sand and pure acetone in a mortar. Pigments in acetone were directly transferred to diethyl ether by adding excess water. Extractions were made in dim white light. Pigments were separated by two successive paper chromatograms. In this study, we considered pigments of the chlorophyll a type almost exclusively, that is, pigments separated by rechromatography of the spot of chlorophyll a obtained from the first chromatogram.

For some experiments, we used a chamber to do both extraction and chromatography under nitrogen.

Results

Pigment composition of extracts from Chlorella pyrenoidosa cultivated under various light exposures. Table 7 compares the pigment composition of extracts from Chlorella pyrenoidosa cultivated under these conditions: (1) 11 days in continuous light; (2) 11 days in continuous light, then 2 days in complete darkness. (In this case the extraction was made in the dark); (3) 11 days in continuous light, then two days in complete darkness, after which the Chlorella received a 4-msec light flash before extraction. (The extraction was made in the dark immediately after the flash.)

The relative proportions of the different pigments are about the same in all the extracts. These results show that in Chlorella pyrenoidosa the pig-
TABLE 7. Pigment Composition of Extracts from Chlorella pyrenoidosa Grown Under Various Light Regimes

<table>
<thead>
<tr>
<th>Light Regime</th>
<th>Pigments, % of Total Chlorophyll a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$a_1$</td>
</tr>
<tr>
<td>Light 11 days</td>
<td>89.5</td>
</tr>
<tr>
<td>Light 11 days + dark 2 days</td>
<td>90.2</td>
</tr>
<tr>
<td>Light 11 days + dark 2 days</td>
<td>91.4</td>
</tr>
<tr>
<td>Light 11 days + dark 2 days + 1 flash of 4 ms</td>
<td>88.7</td>
</tr>
<tr>
<td></td>
<td>88.2</td>
</tr>
<tr>
<td></td>
<td>93.3</td>
</tr>
<tr>
<td></td>
<td>89.0</td>
</tr>
</tbody>
</table>

Pigment composition does not vary according to the light regime received by the algae.

**Effect of oxygen during extraction and chromatography on the pigment composition of extracts of different species.** Table 8 gives the relative proportions of the different pigments found in extracts of Chlorella, Euglena, and spinach, when all the procedures of extraction and chromatography were done in air. The results show that the proportions of the satellites are not the same for the three species. For example, the same solvent extracts of Euglena are rich in satellites compared to those of Chlorella. The fact that the proportions of the satellites vary from one organism to another had been interpreted as indicating either that these satellites were present in the living cells and the proportions varied in the different organisms studied or that the common chlorophylls $a$ and $b$ were differently altered from various plants during extraction and chromatography.

In order to decide between the two possibilities we extracted and chromatographed the extracts from Chlorella, Euglena, and spinach, both in the presence and in the absence of oxygen. The results (Table 9) show that extraction and chromatography in the absence of oxygen reduces or prevents formation of satellite chlorophylls. This is true also for the chlorophyll $b$ type satellites. In the absence of oxygen we found only $b_1$, but in the presence of oxygen, satellites $b_2$, $b_3$, etc., were also present. The absence of oxygen during extraction was especially effective in preventing the formation of satellites (Table 10).

**TABLE 8. Proportions of the Different Pigments in Extracts from Chlorella, Euglena, and Spinach Leaves**

<table>
<thead>
<tr>
<th>Material*</th>
<th>Pigments, % of Total Chlorophyll a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$a_1$</td>
</tr>
<tr>
<td>Chlorella pyrenoidosa</td>
<td>91.0</td>
</tr>
<tr>
<td></td>
<td>90.2</td>
</tr>
<tr>
<td>Euglena gracilis</td>
<td>69.6</td>
</tr>
<tr>
<td></td>
<td>71.0</td>
</tr>
<tr>
<td></td>
<td>70.0</td>
</tr>
<tr>
<td>Spinach leaves</td>
<td>93.6</td>
</tr>
</tbody>
</table>

* Chlorella and Euglena were extracted with boiling 95% ethanol; spinach leaves, with pure acetone (extraction and chromatography were done in air).
TABLE 9. Influence of the Presence or Absence of Air During Extraction and Chromatography on the Proportions of the Satellite Pigments

<table>
<thead>
<tr>
<th>Material*</th>
<th>Pigments, % of Total Chlorophyll a</th>
<th>Under Air</th>
<th>Under Nitrogen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(a_1)</td>
<td>(a_{2+3})</td>
</tr>
<tr>
<td>Chlorella pyrenoidosa</td>
<td></td>
<td>90.9</td>
<td>4.7</td>
</tr>
<tr>
<td>Euglena gracilis</td>
<td></td>
<td>71.2</td>
<td>16.2</td>
</tr>
<tr>
<td>Spinach leaves</td>
<td></td>
<td>93.6</td>
<td>3.9</td>
</tr>
</tbody>
</table>

* Chlorella and Euglena were extracted with boiling 95% ethanol; spinach leaves in pure acetone. For each extraction the same sample was used under air and under nitrogen.

However, satellites were also formed in the presence of oxygen during chromatography (Table 11). In this experiment the same volume of a chlorophyll solution was deposited on six paper chromatograms in the presence of air. The time required for depositing the solution was varied from 1 to 10 minutes. It is clear from the results that longer exposure to air destroys \(a_1\) and consequently creates more satellite chlorophyll, especially \(a_{2+3}\) and \(a_5\).

Conclusions

From these considerations, the conclusion is inescapable that all the satellites may be formed from the parent compounds by chemical alterations during extraction and also during chromatography. This does not exclude the possibility that enzymatic alteration may occur during extraction. Such alteration might account for the different proportions of satellites in extracts from different species. However, the extraction of spinach was made by grinding in acetone and this procedure was slower than extraction in boiling ethanol.

It appears very unlikely that these satellite chlorophylls are present in living cells, since they are almost completely eliminated if extraction and chromatography are done in the absence of oxygen. From this work it appears that they are not related to the different in vivo forms of chlorophyll \(a\).

References


TABLE 10. Influence of Oxygen During Extraction and Chromatography on the Proportions of the Satellite Pigments from Euglena gracilis

<table>
<thead>
<tr>
<th>Atmosphere</th>
<th>During Extraction</th>
<th>During 1st Chromatography</th>
<th>During 2nd Chromatography</th>
<th>Pigments, % of Total Chlorophyll a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(a_1)</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>nitrogen</td>
<td>nitrogen</td>
<td></td>
<td>96.2</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>nitrogen</td>
<td>air</td>
<td></td>
<td>90.5</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>air</td>
<td>air</td>
<td></td>
<td>71.5</td>
</tr>
<tr>
<td>Air</td>
<td>air</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
TABLE 11. Quantities of the Different Pigments Found on the Chromatogram as a Function of the Time in Air between Application of the Chlorophyll Solution to the Paper and the Start of Chromatography, optical density*

<table>
<thead>
<tr>
<th>Time, Min</th>
<th>$a_1$</th>
<th>$a_{2,3}$</th>
<th>$a_4$</th>
<th>$a_5$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.217</td>
<td>0.033</td>
<td>0.022</td>
<td>0.014</td>
</tr>
<tr>
<td>2</td>
<td>0.170</td>
<td>0.040</td>
<td>0.022</td>
<td>0.021</td>
</tr>
<tr>
<td>3</td>
<td>0.158</td>
<td>0.051</td>
<td>0.022</td>
<td>0.025</td>
</tr>
<tr>
<td>4</td>
<td>0.121</td>
<td>0.056</td>
<td>0.023</td>
<td>0.023</td>
</tr>
<tr>
<td>5</td>
<td>0.126</td>
<td>0.058</td>
<td>0.024</td>
<td>0.025</td>
</tr>
<tr>
<td>10</td>
<td>0.131</td>
<td>0.054</td>
<td>0.023</td>
<td>0.024</td>
</tr>
</tbody>
</table>

* The values given are the peak optical densities of the separated pigment bands dissolved in 4 ml of ether, 1-cm light path.

CHLOROPHYLL FLUORESCENCE IN ALGAE AND CHLOROPLASTS

J. S. Brown

Some fluorescence spectra of particular algae whose spectra show great contrasts with each other were measured at 20°C and at −190°C. Very small amounts of the algae were suspended in a wet paste of BaSO$_4$ to reduce the difference in light scattering between the room temperature and the frozen samples. The incident light was 436 mμ. The curves of Fig. 32 were all recorded at essentially the same size to facilitate comparison. However, the apparatus sensitivity adjustments were recorded to allow comparisons of the relative intensity of emission at the two temperatures. The ratios between the yields at −190°C and at 20°C are given in Table 12.

Any one of these curves is presumed to be the sum of a number of overlapping fluorescence spectra each of which is characteristic of or emitted by a particular form of chlorophyll. My objective was to determine the basic fluorescence spectrum for each of the pigment types present. The amount of fluorescent light radiated by any one pigment in comparison with that from other pigments depends directly on the relative number of incident quanta it absorbs or receives from other pigments, and inversely, on the rate at which the pigment transfers quanta to other pigments or photochemical traps in the chloroplast. The efficiency of energy transfer and trapping can vary with temperature. Therefore a direct proportionality between the amount of any chlorophyll form, as determined from absorption measurements, and the intensity of its fluorescence emission cannot be expected. The correlations sought are between the wave-

TABLE 12. Ratios of the Fluorescence Yield at −190°C to the Yield at 20°C for Selected Wavelengths and for the Total Fluorescence between 650 and 770 mμ

<table>
<thead>
<tr>
<th>Organism</th>
<th>680 mμ</th>
<th>691 mμ</th>
<th>720 mμ</th>
<th>740 mμ</th>
<th>Total Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phaeodactylum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High-light grown</td>
<td>0.23</td>
<td>0.53</td>
<td>4.2</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Low-light grown</td>
<td>0.40</td>
<td>0.71</td>
<td>12.</td>
<td>5.1</td>
<td>4.3</td>
</tr>
<tr>
<td>Euglena</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High-light grown</td>
<td>0.17</td>
<td>0.21</td>
<td>2.9</td>
<td>3.3</td>
<td>1.2</td>
</tr>
<tr>
<td>Low-light grown</td>
<td>0.05</td>
<td>0.05</td>
<td>1.6</td>
<td>2.1</td>
<td>1.5</td>
</tr>
<tr>
<td>Chlorella</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High-light grown</td>
<td>0.10</td>
<td>0.19</td>
<td>2.4</td>
<td>1.1</td>
<td>0.82</td>
</tr>
<tr>
<td>Low-light grown</td>
<td>0.23</td>
<td>0.64</td>
<td>2.2</td>
<td>1.7</td>
<td>0.93</td>
</tr>
<tr>
<td>Ochromonas</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spinach chloroplasts</td>
<td>0.50</td>
<td>0.55</td>
<td>2.9</td>
<td>4.5</td>
<td>1.3</td>
</tr>
</tbody>
</table>
length positions of absorption peaks and their corresponding fluorescence maxima.

Last summer Mr. Stephen J. Fulder attempted to derive the fluorescence spectra of the individual forms of chlorophyll by comparisons of a large number of fluorescence spectra at low temperature measured in different laboratories. His results

![Fluorescence spectra of several algae and spinach chloroplasts](image)

Fig. 32. Fluorescence emission spectra of several algae and spinach chloroplasts measured at 20°C and -190°C. Excitation at 436 μm.
showed wide variations in the shapes of the individual bands when they were deduced from different sets of data. The present work should provide a more coherent group of spectra for such curve analysis.

Some qualitative conclusions already have become evident. In the first place the composite nature of the major 685-μm peak (Year Book 58, p. 328) as the sum of components at longer and shorter wavelengths appears even more plausible. Secondly, since these measurements were made with a very dilute suspension of algae in a thick paste of BaSO₄, it is not likely that the difference between spectra at the two temperatures can be attributed entirely to reabsorption distortion by greater light scattering at low temperature. Therefore the actual lowering of the fluorescence at 680 and 691 μm shown in Table 12 is believed to show an increased efficiency in transfer of energy to longer wavelength forms at low temperature.

Long wavelength emission. The following evidence shows that there are several different long-wavelength emission bands in addition to F-680 and F-690. In Euglena gracilis, Phaeodactylum tricornutum, and Ochromonas danica, chlorophyll α-695 may accumulate to a proportion of the total chlorophyll easily detectable in absorption spectra, but it is also more labile than the chlorophyll forms absorbing at shorter wavelengths. When the amount of C₆695 is relatively large, the fluorescence band F-720 is also more evident, and when it is partially destroyed F-720 also decreases.

No one has yet observed an absorption band that would be a likely source of emission beyond 725 μm which is so evident in spinach chloroplasts and Euglena. Fluorescence between 720 and 740 μm may result from the second vibrational level of chlorophyll α to which Goedheer (1964) ascribes a long wavelength (780 μm) emission maximum observed from chlorophyll in solution. Brody (1962) observed this band at low temperature and attributed it to a chlorophyll dimer. However, Goedheer's experiments demonstrate that this band occurs in dilute as well as concentrated solutions and at both 20°C and -196°C. It is relatively greater in more concentrated solutions because of self-absorption of the main emission band at shorter wavelengths. Thus a significant proportion of long wavelength fluorescence in vivo may arise from the second vibrational level intrinsic in the chlorophyll α molecule. This hypothesis to account for a long wavelength emission band requires no corresponding long wavelength absorption band. On the other hand, if the emission is from a dimer or aggregate as Brody suggests, an absorption band for the dimer must exist. Possibly C₆695 is aggregated chlorophyll, but the nature of this absorbing form of chlorophyll has not yet been demonstrated. The wavelength position of the long wavelength fluorescence band in a particular sample depends on the proportion of the two types of fluorescence.

Short wavelength emission. Duysens (1952) first provided evidence that the chlorophyll α absorbing at longer wavelengths was largely non-fluorescent in red and blue-green algae. More recently Duysens and Sweers (1963) correlated the major portion of fluorescence with the activity of photosystem 2 in which the shorter wavelength absorbing chlorophyll α is considered to be functional. These findings led to the assumption that C₆670 is the source of F-685, the main fluorescence emission maximum at room temperature.

The variability in position of the emission maximum in different algae at room temperature shown in Fig. 32 and additional data from experi-
ments on *Ochromonas* discussed in another section of this report indicate that other forms of chlorophyll may contribute to the main emission band. A discrete fluorescence emission band between 690 and 700 m\(\mu\) at \(-190^\circ\text{C}\) has been observed in several laboratories. Goedheer (1964) has suggested that C\(_a\)680 is the chlorophyll form which fluoresces at about 690 m\(\mu\) (F-690) both at room temperature and \(-190^\circ\text{C}\). In *Ochromonas* the main emission maximum is at 690 m\(\mu\), suggesting that in this case C\(_a\)680 may be the major fluorescing chlorophyll form.

Thus we now have evidence that all three forms of chlorophyll \(a\) may fluoresce under physiological conditions but that the proportion of fluorescence contributed by each form varies considerably with the algal species and growth conditions. The same conclusion was reached several years ago concerning the relative absorption by each chlorophyll \(a\) form in different algae.

**Energy transfer.** Although no clearly consistent hypothesis concerning energy transfer emerges from these studies as yet, some postulations can be made. These corroborate and extend those of Goedheer (1966).

Upon cooling, the yield of the shorter wavelength fluorescence bands always decreases and that of the longer wavelength bands always increases. After the cells are damaged in some way such as heating to 50°C for 10 minutes, placing in 10% ethanol overnight, or freezing and thawing, the yield changes upon cooling are very different. For example, the *Euglena* grown in high light showed slightly more than a fivefold decrease in fluorescence yield at 680 m\(\mu\) when the cells were first cooled from 20°C to \(-190^\circ\text{C}\). But when this sample was thawed and brought again to \(-190^\circ\text{C}\), the yield at 680 m\(\mu\) was twice as great as from the cells at 20°C in the beginning. On the other hand, the increases in yield at 720 and 740, which were originally 2.9 and 3.3 (Table 12), were reduced to 1.2 and 1.5 after a second freezing. These yield changes are reflected in changes in shape of the emission spectra (as illustrated in Fig. 34 for *Ochromonas*).

The disruptive thawing and freezing process has either lowered the possibility of energy transfer between pigment molecules or has destroyed the longer wavelength absorbing pigment complex C\(_a\)695. Absorption spectra of *Euglena*, *Phaeodactylum*, and *Ochromonas*, measured at \(-190^\circ\text{C}\) and again after thawing and refreezing, do show a relative decrease in long wavelength absorption. Figure 33 shows an example for *Ochromonas*.

The structural or specially oriented nature of C\(_a\)695 is suggested. Only those organisms that are rather easily disrupted show a large change in shape of the emission spectrum at low temperature after a single thawing and refreezing. *Chlorella* or spinach chloroplasts suspended in isotonic sucrose buffer must be thawed and frozen several times before the yield changes that are induced by cooling are different from those observed after the first measurement at \(-190^\circ\text{C}\).

An explanation for these observations may be that at very low temperature the possibility of energy transfer between C\(_a\)670 and C\(_a\)695 increases, resulting in a decreased fluorescence yield at 680 m\(\mu\) and an increased yield at 720 m\(\mu\). Possibly some energy transfer between C\(_a\)670 and C\(_a\)695 occurs also at room temperature, and C\(_a\)695 acts as an energy collecting trap in the photochemical sense.

Last year we suggested that activity during photosynthesis of both C\(_a\)670 and C\(_a\)695 might be observed by
monitoring their respective fluorescence emission maxima. This should be possible with either Euglena or Phaeodactylum cultured in an appropriate manner. Now we can add that with Ochromonas fluorescence from C₆₈₈₀ may also occur as a major band at room temperature.

References

Absorption and Fluorescence Spectra of Ochromonas danica
J. S. Brown

The algal flagellate Ochromonas danica has a red absorption spectrum with three major components at 670, 682, and 692 m.μ. Furthermore, both its absorption and fluorescence spectra are very sensitive to mild disturbances such as freezing or gentle warming (Allen et al. 1960, and Year Books 57 and 58). The older measurements that produced these data were all done at room temperature and only derivative absorbance spectra were recorded.

We have recently extended these experiments with improved equipment and have measured both absorption and fluorescence at −190°C as well as at 20°C. The results, qualitatively similar to those reported earlier, are more precise and useful for comparison with absorption and fluorescence spectra of other algae discussed in other sections of this report.

Figure 33 shows absorption spectra of an Ochromonas suspension. The three adjacent bands evident in a derivative absorptive spectrum are difficult to distinguish in an absorbance spectrum at room temperature but are clearly visible in the low-temperature spectrum. The experimental derivative spectrum measured at 20°C was fitted previously with three gaussian derivative components with
peaks at 670, 682, and 692 m\(\mu\). Attempts to fit the low-temperature integral absorption spectrum with similar gaussian component bands have been unsuccessful.

Figure 32 in the preceding section of this report shows that at 20°C \textit{Ochromonas} has a single fluorescence emission band at about 690 m\(\mu\). At \(-190^\circ\text{C}\), a second broad emission maximum appears at about 720 m\(\mu\). The aliquot used for these measurements was from a culture in which essentially all of the cells were actively motile. Occasionally spectra had a shorter wavelength emission band between 670 and 680 m\(\mu\) in addition to bands at 690 and 720 m\(\mu\). The solid curve of Fig. 34 shows an example with a distinct shoulder on the short wavelength side of the 690-m\(\mu\) band. We found that the relative height of the 675-m\(\mu\) band was correlated with the number of rounded, nonmotile cells in the culture.

Thus a particular culture may show all three fluorescence maxima at low temperature, the relative height of the 675-m\(\mu\) band depending on the number of damaged cells. It is, of course, possible to damage all the cells by freezing and thawing or by homogenizing with the needle-valve press. After such treatment the emission is nearly all at about 675 m\(\mu\), as shown in Fig. 34 measured at \(-190^\circ\text{C}\), and \textit{Year Book} 58 (p. 329), measured at 20°C.

An absorption spectrum of cells that have been frozen, thawed, and cooled again to \(-190^\circ\text{C}\) is shown in Fig. 33. The shortest wavelength band near 670 m\(\mu\) increases, and the two longer wavelength bands decrease after refreezing. A comparison of the absorption spectra in Fig. 33 with the fluorescence spectra in Fig. 34 before and after thawing and refreezing indicates that the three absorption bands are the sources of emission of the three fluorescence bands. In addition, changes in absorbance throughout the visible spectrum are evident. When more comparative spectra are available, we hope, by correlating some of these changes with those in the red region, to determine other absorption maxima for the forms of chlorophyll \(a\).

Chlorophyll \(a\)-680 may be the primary fluorescent form in \textit{Ochromonas}. Since \(\text{C}_6\text{a}\)680 is thought to be the major pigment in photosystem 1, the correspondence between fluorescence and photosynthetic activity may be different from that in other algae where \(\text{C}_6\text{a}\)670, which functions in photosystem 2, is the main fluorescing form.

\textbf{Reference}


\textbf{THE ACTION SPECTRUM FOR BLUE-LIGHT-STIMULATED OXYGEN UPTAKE IN \textit{Chlorella}}

James M. Pickett

Emerson and Lewis (\textit{Year Book} 40, p. 158; and 1943) found the rate
of oxygen uptake by *Chlorella pyrenoidosa* to increase after an exposure to low-intensity blue light. Stimulation of oxygen uptake by 480-\(\mu\)m light was increased by keeping cells in the dark for 90 minutes. Following an exposure to 480 \(\mu\)m, the rate of oxygen uptake remained greater than the former dark respiration rate for approximately 1 hour.

Ried (Year Book 64, p. 399) reported that *Chlorella pyrenoidosa* kept in the dark, then exposed to flashes of blue light (\(\lambda < 540 \mu\)m), gave increased rates of oxygen uptake. The maximum increase in rate of uptake occurred 6 to 10 minutes after the flash. This increase was not inhibited by \(5 \times 10^{-8} M\) DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea]. Under optimal conditions the extra amount of oxygen consumed after a short-wavelength flash was more than 500 times greater than the amount evolved photosynthetically during the flash.

Kowallik and Gaffron (1966) found no blue-light stimulation of oxygen uptake in the nonphotosynthetic alga *Prototheca*, which has little absorption in the visible. However, after dark adaptation for more than 1 hour, the dark yellow mutant of *Chlorella vulgaris* (211-11h, M.20), which also lacks chlorophyll, did show the blue-light stimulation of oxygen uptake. Kowallik and Gaffron found that the rate of dark respiration decreased about 60% in algae kept in a glucose-free medium for 6 hours. However, in blue light, the initial rate of oxygen uptake was stimulated as much as 30% for this time.

Exploratory experiments to develop the procedure of measurement. A typical response to a 5-second flash of blue light is shown in Fig. 35. The maximum rate of oxygen uptake, reached 10 to 15 minutes after the flash, was measured by the maximum difference between the extrapolated dark current before the flash and the measured current after the flash, as shown in Fig. 35. The rate of oxygen
uptake remained high for at least 30 minutes. The maximum rate of oxygen uptake stimulated by a 5-second flash of 469 mµ was about 1% of the light-saturated rate of oxygen evolution.

The stimulation of oxygen uptake by blue light developed slowly after the cells were placed in darkness, as seen in Fig. 36. Almost all data used for the action spectrum were measured with cells which had been dark for 15 to 48 hours. Figure 36 also shows that it was necessary to correct the measured rates of uptake for the change in response which occurred during each 6- to 8-hour experiment. Preliminary experiments indicated that 469 mµ was near the peak of the action spectrum. Figure 37 shows that the response to 469 mµ was nearly linear up to 0.75 nein/cm²/sec. These data provided the basis for selection of approximately 0.5 nein/
Fig. 37. Light saturation curve for uptake after 5-sec flashes of 469 mμ for cells kept dark for 48 hr. Corrected for change in response with time.

The resulting action spectrum is shown in Fig. 38(A) and is in general agreement with Ried's recent results (personal communication). The curve was drawn to fit the mean effectiveness at each wavelength. The bar at 469 mμ represents the mean ±3 standard deviation units (n = 39). All but two measurements at 469 mμ were within two standard deviation units. The scatter at other wavelengths is generally greater than at 469 mμ, which probably indicates that some of the scatter was introduced by the normalization process. If so, the shape of the action spectrum for a single suspension must change somewhat with time. The measurements with the mercury lines are in good agreement with the measurements made with bands (half-band width approximately 10 mμ) isolated from tungsten emission. The effectiveness beyond 550 mμ was always less than 5% of the effectiveness at 460 mμ. The action spectrum was not changed by using suspensions of 25 μl cells/ml instead of 50 μl cells/ml.

The spectrum in Fig. 38(A) sug-
suggests that the pigment responsible for the blue-light-stimulated increase in rate of oxygen uptake is a flavin. The near-ultraviolet peak at 375 mμ distinguishes the action spectrum from that of a carotenoid. If a cis-carotenoid were responsible for the effect, this peak should occur below 330 mμ.

Shading by chlorophylls and carotenoids would shift the observed peak of the action spectrum toward the red. The absorption spectrum of whole cells was therefore measured in the Beckman DK-2 spectrophotometer with opal glass plates. The corrected action spectrum (Fig. 38B) was estimated by multiplying the observed effectiveness (Fig. 38A) by the reciprocal of the transmission of a 10 μ-thick layer of cells (absorbance at 440 mμ = 0.575). This correction for shading, by a layer half the estimated thickness used, shifts the visible absorption peak from 460 to 440 mμ and introduces a shoulder at 470 mμ. The location of the near-UV peak remains unchanged at about 375 mμ. However, the ratio of the visible to near-UV peak is increased from 1.15 to 1.42. The corresponding value for riboflavin (Fig. 38B) is 1.17. Correction for shading by a thinner layer would have given smaller shifts. The fact that the corrected spectrum lies within the scattering of the measurements explains why dilution of the cell suspension from 50 μl cells/ml (20μ-thick cell layer) to 25μl /ml did not change the action spectrum.
significantly. It should be noted that the absorption spectrum of flavoproteins is generally more complicated than that of riboflavin itself. The peak height ratio in different flavoproteins is variable, and shoulders may occur on the short- or long-wavelength sides of the visible peak.

Because of the scatter in the data, one can only conclude that the pigment responsible for the blue-light-stimulated increase in rate of oxygen uptake is probably a flavin.

Effect of poisons. After adding $10^{-5} \text{M DCMU}$ in ethanol (final ethanol concentration, 1%) the steady-state rate of photosynthetic oxygen evolution from \(0.62 \text{ ein/cm}^2/\text{sec}\) at 469 m\(\mu\) was inhibited by 94%. The rate of oxygen uptake caused by a 5-second flash of \(0.62 \text{ ein/cm}^2/\text{sec}\) was not inhibited under the same conditions (Fig. 39).

By contrast with DCMU, cyanide poisons the light-stimulated uptake of oxygen much more than photosynthesis. With \(2 \times 10^{-4} \text{M KCN}\) the inhibition of light-stimulated oxygen uptake was 97%, but steady-state oxygen evolution only 28%. A further indication that the prolonged increase in oxygen uptake by blue light is related to respiration is presented in Fig. 40. Addition of 1% glucose to starved cells stimulated the blue-light effect 63%. In the presence of glucose the rate of dark respiration was increased by an amount 10 times greater than the maximum rate of light-stimulated oxygen uptake. The ratio of the rate of respiration before addition of glucose to that after addition of glucose is unknown. Only 21% of the increase in dark respiration was rapidly inhibited by cyanide. However, cyanide sensitivity of light-stimulated oxygen uptake and the sensitive part of respiration were identical (Fig. 40).

Discussion. Our results probably concern the same stimulation of oxygen uptake by blue light measured by Emerson and Lewis (1943), Ried (Year Book 64, p. 399), and also Kowallik and Gaffron (1966).

Our data indicate that the prolonged increase in the rate of oxygen uptake observed after exposure to

![Fig. 39](image_url) Effect of DCMU on oxygen uptake. Oxygen uptake after a 5-sec flash of \(0.62 \times 10^{-9} \text{ ein/cm}^2/\text{sec}\) of 469 m\(\mu\) light is not inhibited by \(1 \times 10^{-4} \text{M DCMU}\), which inhibited oxygen evolution 94%. Reservoir dark 72 hr.
small doses of blue light is activated by a flavin. The action spectrum (Fig. 38A) is almost identical to that of Kowallik (1967a) for increased oxygen consumption by a chlorophyll-less mutant of *Chlorella* exposed to continuous blue light. The effect does not appear to be coupled to system 2 of photosynthesis because the uptake is not inhibited by DCMU. Kowallik (1967b) found that light-stimulated oxygen uptake in *Chlorella* had a respiratory quotient of one. These results are consistent with our data for cyanide sensitivity.

Although the data suggest that oxygen consumption stimulated by blue light is probably mediated by mitochondrial respiration, there is no evidence that the responsible flavin is directly involved in respiration.

**Summary.** The action spectrum for the stimulation of oxygen uptake by blue light was determined for *Chlorella pyrenoidosa*. The action spectrum is limited to wavelengths shorter than 550 mµ and has peaks at 460 and 375 mµ. The peaks are separated by a definite minimum at 400 mµ. The action spectrum indicates that the responsible pigment is a flavin.

The effect is not closely coupled to system 2 of photosynthesis because the uptake was not inhibited by 10⁻⁵ M DCMU. Light-stimulated oxygen uptake may be closely related to cyanide-sensitive dark respiration, since both exhibit the same cyanide sensitivity.

**References**


**INTERMEDIATES IN PHYTOCHROME TRANSFORMATION IN VIVO AND IN VITRO**

*Winslow R. Briggs and David C. Fork*

The plant pigment phytochrome, a chromophoric protein, mediates a wide range of physiological processes in green plants from the algae through the higher angiosperms. It can exist in either of two spectrally distinct forms: a red-absorbing form Pr with maximum absorption between 650 and 670 mµ, and a far-red-
absorbing form Pfr with maximum absorption between 715 and 735 mJ. The two forms are readily interconvertible by red and far-red light. Both have absorption peaks in the long ultraviolet or blue, attributable to the chromophoric group, a linear tetapyrrole. These latter peaks show spectral shifts comparable to those in the red and far red upon appropriate illumination.

Previous work (Year Book 64, pp. 406-412) has shown that a mixture of high-intensity red and far-red light causes rapid cycling of the pigment from one spectral form to the other, producing measurable steady-state levels of spectrally detectable intermediates in partially purified phytochrome from dark-grown seedlings of *Avena sativa* L. Actinic light, provided by a Sun-Gun lamp, was passed through a 5-cm water filter, and a Corning 2030 cutoff filter to remove wavelengths below 620 mJ. Absorbancy changes were measured at 543 mJ by means of previously described instrumentation (Year Book 63, pp. 435-441). The intermediates were detected by their increase in absorbancy at this wavelength when the actinic beam was turned on, and their subsequent absorbancy decrease when it was turned off. Appropriate filters between the sample and the phototube prevented actinic light from reaching the phototube, but allowed passage of the low-intensity 543-mJ measuring beam. Except for Q10 experiments, sample temperature was maintained at approximately 5°C.

A kinetic analysis of absorbancy decay after illumination suggested that two intermediates with different rate constants were decaying in parallel to Pfr. Time course studies for the formation of the intermediates in the light showed that the more rapidly decaying one increased to its maximum concentration within 2 seconds and then decreased to a steady-state level approximately one half as high by the end of 12 seconds of illumination. The more slowly decaying intermediate appeared less rapidly, reaching its maximum level at about 12 seconds and then remaining there. The decay constant τ for one intermediate was about 0.2 and for the other about 1.1 seconds at 5°C. The Q10 for dark decay (between 5°C and 25°C) of the intermediates was between 1.9 and 2.3, although the overall Q10 for transformation of the pigment in either direction was extremely close to 1.0. Thus, under the conditions of the experiment, decay of intermediates could not have been limiting the rate of light-induced pigment transformation. The two intermediates studied appeared to correspond to the two slowest-decaying intermediates on the Pr or Pfr pathway, as described by Linschitz, Kasche, Butler, and Siegelman (1966) from flash photolysis experiments.

**Phytochrome intermediates in vivo.** It was of some interest to determine whether the intermediates studied with partially purified phytochrome could also be detected for native phytochrome in vivo, and if they could, whether or not their kinetics were similar to those in solution. For this purpose we used approximately 1.5 grams of coleoptile tips between 2 and 4 mm in length, which were excised from oat seedlings grown for 5 days in complete darkness. Care was taken to eliminate any fragments of the primary leaves, since these contain substantial amounts of protochlorophyll, which would interfere with the desired spectral measurement. The coleoptile tips were packed into a cuvette approximately 2 cm in diameter, forming a layer of tissue about 2 mm thick. The sample was spread carefully in the cuvette to eliminate any small light leaks. The cuvette was then placed over the photomultiplier
tube and kept surrounded with ice during the measurements. Although it was not possible to monitor tissue temperature directly, experience with liquid samples showed that the average temperature was probably between 5°C and 8°C.

Figure 41 shows records of absorbancy changes during and after three different durations of actinic illumination. It is obvious that with the shortest period of illumination the decay rate is substantially higher than that found after the two longer periods. A plot of decay half-time against concentration of intermediate at the end of illumination shows a steep increase in decay half-time with increasing concentration of intermediate. These observations are in close agreement with those previously obtained with partially purified phytochrome. In the experiment shown in Fig. 41, light intensity was kept constant, and concentration of intermediates was varied by varying exposure time. When the reciprocal experiment was done (exposure time kept constant and concentration of intermediates varied by varying light intensity) decay half-times remained constant and were independent of intermediate concentration. These results are also in agreement with those previously obtained with the in-vitro system. Since the signals obtained with the tissue sample were smaller and substantially noisier than those obtained with phytochrome solutions, it was not possible to do any further kinetic analysis on the decay patterns found. However, since the kinetic data that were available closely matched those previously obtained with solutions, it seems reasonable to conclude that the same two intermediates were being observed in vivo as had previously been observed in vitro. Absorption spectra of this and similar preparations of oat coleoptile

![Figure 41](image)

**Fig. 41.** Absorbancy changes at 543 mµ induced in intact oat coleoptile tips by high-intensity actinic light (620 to 800 mµ, 4 × 10⁶ erg cm⁻² sec⁻¹).
tips showed almost no protochlorophyll (before initial illumination) or chlorophyll (after illumination) but did have very high absorbancy at 667 m\(\mu\) after far-red irradiation or at 735 m\(\mu\), after red irradiation. Thus the predominant pigment absorbing at the longer wavelengths was clearly phytochrome.

One important difference between the behavior of the intermediates in vivo and that in vitro should be noted: The steady-state level of intermediates in vivo, per unit of measurable phytochrome in the sample, is approximately five times as high as that in vitro, although the measured light intensity at the sample surface in the current experiments was less than one half that used previously with phytochrome solutions. One could account for this difference in steady-state levels in two ways: either slower decay or more rapid formation in vivo. A comparison of intermediate behavior in the tissue and in solution (cf. Fig. 41 and Year Book 64, Fig. 31) reveals that both explanations obtain. Decay in vivo is somewhat slower, and rise time is significantly shorter. Since rise time is independent of length of exposure time for illuminations longer than 1 second, the comparison for this parameter is easily made: steady state is reached in vivo in approximately 0.3 second, while requiring well over 1 second in vitro. Hence for phytochrome in the intact tissue, formation of intermediates is facilitated and decay is hindered; however, speculation on possible mechanisms is premature. The differences in rise time are actually more dramatic than appears on first inspection of the figures, since the total absorbancy of the tissue sample is far higher than that of the phytochrome solution. Thus in the current experiments the average light intensity integrated across the sample is far lower than that in previous experiments with phytochrome solutions.

A difference spectrum for intermediates in vitro. Partially purified phytochrome was obtained from dark-grown oat seedlings as described earlier (Year Book 64, pp. 406–412) for investigation of the spectral properties of the intermediates between 365 and 580 m\(\mu\). The source of actinic light was the same as above, although the intensity was somewhat lower (2.9 \(\times\) \(10^5\) ergs cm\(^{-2}\) sec\(^{-1}\)). Preliminary experiments showed that high concentrations of sucrose or glycerol substantially slowed intermediate decay, allowing formation of considerably higher steady-state levels than obtainable in buffer alone. Figure 42, top two tracings, shows absorbance changes at 560 m\(\mu\) for identical amounts and concentrations of phytochrome diluted 50% either with buffer or with glycerol (7°C). At this wavelength glycerol almost doubles the height of the signal. Thus the difference spectrum was obtained both with glycerol, to obtain maximum signal size, and without, to determine whether or not glycerol spectrally altered the intermediates. The bottom two tracings of Fig. 42 show the absorbancy changes found at 418 and 385 m\(\mu\) in the presence of glycerol. A sharp absorbance decrease was found at 418 m\(\mu\) and increases at 385 and 560 m\(\mu\). The complete difference spectra with and without 50% glycerol are shown in Fig. 43. Each point represents the average height of a minimum of six signals, obtained in every case with a 3-second light period followed by 6 seconds of darkness (the regime illustrated in Fig. 42). Though the signals with glycerol are significantly larger, the two curves are otherwise essentially the same, with minima near 418 m\(\mu\), max-
ima near 380 mµ, and isobestic points near 398 and 485 mµ.

Several points should be made about these difference spectra. First, they represent the difference in absorbancy between the intermediates and Pfr, to which both decay. Second, it is not possible to obtain values above 580 mµ, since the technique restricts one to measurements at wavelengths well below the range of the actinic beam. Third, the spectra are composite, representing both the fast and slow intermediates previously described. Though it should be possible to separate these two components by detailed kinetic analysis of the records, such analysis has not yet been done. Fourth, these difference spectra are not identical with the difference spectrum between Pr and Pfr. The latter spectrum has a minimum at about 410 mµ, a maximum at about 385 mµ, and isobestic points at 398 and 495 mµ. Finally, 3 seconds of illumination were not sufficient to cause the maximum possible absorbancy changes in the glycerol preparation. Experiments that allowed the change to go to completion gave much larger changes but the spectra were the same.

Reference

Fig. 43. Difference in absorbancy between intermediates and Pfr from 365 to 580 μ. The absorbancy changes were measured from records similar to those shown in Fig. 42, by means of the same actinic light.

EXPERIMENTAL TAXONOMY INVESTIGATIONS

GROWTH RESPONSES OF Mimulus RACES AND F₁ HYBRIDS AT THE STANFORD, MATHER, AND TIMBERLINE TRANSPLANT STATIONS

Malcolm A. Nobs and William M. Hiesey

A segment of the long-range investigations on Mimulus is concerned with the comparative performance of parental and F₁ hybrids of a wide array of crossing combinations at the three altitudinal transplant stations. These studies, begun in 1961 and completed in 1966, will be briefly summarized.

The questions. Earlier inquiries on the genetic structure of ecological races of Potentilla (Carnegie Publication 615) and Achillea (Year Book 51, pp. 122–124) dealt mainly with crosses between extreme altitudinal and latitudinal races. Studies on crosses between ecological races only slightly or moderately differentiated from each other in comparison with crosses between ecological extremes have been mostly lacking. Because of its biosystematic structure, the Mimulus cardinalis–M. lewisii complex is particularly well suited to investigations aimed at filling this gap in our knowledge (Year Book 64, pp. 427–428). Questions which this work was designed to clarify include the following:

1. How do first-generation hybrids between closely related yet distinguishable ecologic races of the same species but from distinct habitats compare with hybrids between highly diverse races in their capacity to survive and grow at the altitudinal transplant stations at Stanford, Mather, and Timberline?
of the F₁ population of *M. cardinalis Los Trancos × M. cardinalis Yosemite*, which is shown in the lowermost bar diagram of the same figure for comparison.

At Mather the heterosis of the two combinations is drastically reversed as compared with Stanford: *M. cardinalis Los Trancos × M. lewisii Yosemite* is strikingly more vigorous than *M. cardinalis Los Trancos × M. cardinalis Yosemite*. At Timberline the relative performance of the two F₁ combinations is approximately the same as at Mather although the actual total dry weight yield at Timberline in both instances is reduced by a factor of nearly 10 because of the much shorter growing season.

2. *M. cardinalis Los Trancos × M. lewisii Timberline*. When the Los Trancos clone of *M. cardinalis* (6546-5) is crossed with the high-Sierran *M. lewisii* originally from Timberline at 3300 m elevation (7405-4), the resulting F₁ progeny show about the same degree of vigor as the progeny of the cross *M. cardinalis Los Trancos × M. lewisii Yosemite*. The parallelism in the performance of these two F₁ populations when grown at the Stanford, Mather, and Timberline transplant stations is striking (Fig. 45). This result fails to support an earlier hypothesis that the F₁ between *M. cardinalis* and *M. lewisii* are of greater magnitude and evolutionary significance than the genetic differences between ecological races within either species.

2. The genetic differences between ecological races of either species are, nevertheless, of sufficient magnitude to be of ecological significance in natural selection.

3. *M. cardinalis Yosemite × M. lewisii Yosemite*. The combination between *M. cardinalis* originally from Yosemite National Park at 880 m elevation (clone 6694-105) and *M. lewisii* (clone 7121-5) from the same area is of particular interest in studying the effect of combining the genomes of these two distinct but closely related taxa originally growing in essentially the same natural habitats in comparison with crosses between ecological races native to different altitudes and latitudes.

The most striking feature of the F₁ population from this cross is its marked similarity to the responses of the F₁ hybrids between the two *cardinalis × lewisii* combinations previously discussed at the three transplant stations (Fig. 45).

**Conclusions.** From the available data, the following conclusions regarding the genetic structure of natural populations of *Mimulus cardinalis* and *M. lewisii* appear to be valid:

1. The genetic differences that have evolved between *M. cardinalis* and *M. lewisii* are of greater magnitude and evolutionary significance than the genetic differences between ecological races within either species.

2. The genetic differences between ecological races of either species are, nevertheless, of sufficient magnitude to be of ecological significance in natural selection.

3. Hybrid vigor, or heterosis, is the general rule in crosses within the *Mimulus cardinalis–M. lewisii* complex in both intraspecific and interspecific crosses. The expression of heterosis in a given F₁ population may be profoundly influenced by climate as, for example, in intra-*cardinalis* hybrids grown at Stanford as compared with Mather, or in intra-*lewisii* hybrids grown at Timberline as compared with Mather. In other instances heterosis may be almost equally expressed over the entire range of climates at Stanford, Mather, and Timberline, as in combinations between *M. cardinalis* and *M. lewisii*. In such combinations heterosis is espe-
cially evident at Mather where most of the parental races of both *M. cardinalis* and *M. lewisii* fail to be successful.

4. There is no evidence to support the hypothesis that the survival of F₁ hybrids at contrasting altitudes is related in a simple way to the altitudinal origin of the particular ecological races that enter into the crosses.

**PHOTOSYNTHETIC RATES OF Mimulus RACES AND HYBRID DERIVATIVES**

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

The higher rates of light-saturated photosynthesis of coastal *Mimulus cardinalis* as compared with subalpine *M. lewisii* described last year (*Year Book 65*, pp. 461–468) have been confirmed by means of different preconditioning treatments.

As previously emphasized, such differences in photosynthetic capacity of contrasting races of *Mimulus* and hybrids are revealed only when the experimental plants are previously conditioned by growing them under a high light intensity as, for example, at approximately 100,000 ergs/cm²/sec, or about one third the intensity of full sunlight. Leaves of the same individual plants grown under a weak light of 25,000 ergs/cm²/sec intensity have a considerably lower light-saturated rate, and the differences in light-saturated photosynthetic rates of ecological races and hybrids tend to disappear. The ratio between the light-saturated photosynthetic rate of a given clone grown under high versus low light intensity can therefore be used as one measure of its performance.

Table 13 shows light-saturated photosynthetic rates of *M. cardinalis*, subalpine *M. lewisii*, their reciprocal F₁ hybrids, and two F₂ individuals that differ widely from each other in their morphology and in their growth responses at the Stanford, Mather, and Timberline transplant stations. All of these clones were previously grown under the same conditions, under high light intensity in controlled cabinets, before measurement. The F₂ clone 7135-35 resembles *M. cardinalis* both morphologically and in its survival, and in interstation transplant responses. It is a vigorous

| TABLE 13. Photosynthetic Rate and Chlorophyll Content of Mimulus Parents and Hybrids |
|---|---|---|---|
| **Gross Light-Saturated Photosynthetic Rate** | **Ratio, P Rate of Leaves Grown under High and Low Light Intensity** | **Chlorophyll Content of Leaves** |
| mg CO₂/dm²/hr* | | mg/dm² surface | mg/g fresh weight |
| **Mimulus cardinalis**, Los Trancos, 6546-5 | 38.9 | 1.8 | 5.07 | 1.60 |
| **Mimulus lewisii**, Timberline, 7405-4 | 26.3 | 1.4 | 3.40 | 0.90 |
| **Mimulus cardinalis** × *lewisii*, F₁ Hybrid, 6546-3 | 29.0 | 1.5 | 4.35 | 1.41 |
| **Mimulus lewisii** × *cardinalis*, F₁ Hybrid, 6547-1 | 30.1 | 1.5 | 4.00 | 1.42 |
| **Mimulus cardinalis** × *lewisii*, F₂, cardinalis-like, 7135-35 | 33.1 | 1.9 | 4.94 | 1.62 |
| **Mimulus cardinalis** × *lewisii*, F₂, lewisis-like, 7111-16 | 31.3 | 1.8 | 3.93 | 1.05 |

*Plants previously grown under high light intensity. All values are means of several independent measurements.*
survivor at Stanford but dies at the Mather and Timberline stations. In contrast, the F₂ clone 7111-16 strongly resembles the *M. lewisii* parent. The resemblance is so close that it would be classified as a member of this species, and is a nonsurvivor at Stanford and Mather.

The two F₂ individuals yield segregating F₃ progenies that differ widely from each other in overall genetic composition. The growth responses and survival of samples of these F₃ progenies are currently being tested at the altitudinal transplant stations.

The ratio between the light-saturated photosynthetic rates of propagules of these clones grown under high light intensity to those grown under low light intensity is given in the second column of figures from the left in Table 13. These ratios differ, especially in that *M. cardinalis* has a higher ratio than *M. lewisii*. The ratios of the F₁ hybrids are intermediate between the parents, but both of the contrasting F₂ individuals have ratios as high as the *M. cardinalis* parent.

The bulk chlorophyll content of the leaves also differs greatly between the two parent clones and can be regarded as another segregating characteristic in the hybridization studies independent of the light-saturated photosynthetic rate. The chlorophyll content affects the capacity of the leaves to absorb light and therefore influences the rate at low intensities of incident light, but not at saturating light intensities (*Year Book 65*, pp. 461-468). The chlorophyll content of the leaves is shown in the two columns at the right of Table 13 and is expressed on both a leaf-area and a fresh-weight basis. The differences in relative values between these two methods of computing chlorophyll content are due primarily to hereditary differences in leaf thickness between the clones, which result in different leaf-surface to leaf-volume ratios.

The major points of interest shown by the data in Table 13 may be summarized:

1. Coastal *M. cardinalis* has a substantially higher light-saturated photosynthetic rate than does subalpine *M. lewisii*. Likewise, the ratio between light-saturated rates of photosynthesis of propagules grown at high light intensities to those grown at low light intensities is much higher in the coastal clone.

2. The two reciprocal F₁ hybrids have a light-saturated photosynthetic rate that is intermediate between the parents, as do also the two very unlike F₂ individuals. The ratio between high and low light-grown propagules differs markedly between the F₁ and the two F₂ individuals, being as high as in the *M. cardinalis* parent in both F₁ plants.

3. Chlorophyll content is markedly higher in the coastal *M. cardinalis* parent than in *M. lewisii*, and intermediate in both reciprocal F₁ individuals. In the *cardinalis*-like F₂ chlorophyll content is essentially the same as in the *M. cardinalis* parent, and in the *lewisii*-like F₂ it is nearly as low as in the *M. lewisii* parent.

These data suggest that differences in photosynthetic characteristics in *Mimulus* are inherited in much the same manner as morphological markers (*Year Book 63*, pp. 432-435), and that one may expect the principle of genetic coherence (*Year Book 62*, pp. 387-389) to apply to the inheritance of factors controlling various steps of the photosynthetic processes that determine observed measured rates under specified controlled conditions.

Studies such as those described above and biochemical investigations comparable with those reported below for *Solidago* are being extended to include *M. nelsonii* from Mexico, F₁ hybrids between *nelsonii* and subal-
pine *M. lewisii*, and the tetraploid amphiploid derived from these species as described in last year's report. Data from these studies are still too incomplete to be reported.

**CARBOXYDISMUTASE ACTIVITY IN SUN AND SHADE ECOTYPES OF Solidago**
Olle Björkman and Monika Björkman

Preliminary studies, reported last year, indicated that ecotypes of *Solidago virgaurea* originally from open as contrasted with shaded habitats may differ in their capacity to produce the photosynthetic enzyme carboxydismutase (ribulose diphosphate carboxylase). Of the two *Solidago* clones investigated, the clone from an open habitat showed a higher activity of the enzyme than the one from a shaded habitat, in extracts prepared from the leaves when grown under a high light intensity. Unrelated species limited in natural distribution to open locations, such as *Plantago lanceolata*, showed a higher activity of carboxydismutase than species limited strictly to shaded habitats, such as *Lamium galeobdolon*.

This year we have studied this problem in greater detail. An improved assay procedure for enzyme determinations has been developed, additional *Solidago* clones have been studied, and photosynthesis measurements and enzyme determinations have been made on the same leaves.

**Plant materials.** The recent measurements were made with cloned individuals of two populations of two races of *Solidago virgaurea*, "Beskades," native to an exposed alpine-arctic heath in northern Norway, and "Hallands Väderö," native to a dense oak forest in southern Sweden. Several clones of each race were grown in controlled cabinets at two different light intensities, 25,000 and 110,000 erg cm\(^{-2}\) sec\(^{-1}\) (400–700 m\(\mu\)). Other conditions were identical and as described earlier (*Year Book 64*, p. 420). In all measurements of photosynthesis, single rosette leaves, attached to the intact plants, were used. Recently matured leaves (about 80% of full expansion) were used both for photosynthesis measurements and enzyme determinations, except where specified.

**Photosynthesis measurements.** Light-saturated rates of photosynthetic CO\(_2\) uptake were measured with the apparatus described in *Year Book 63* (pp. 430–431) modified to meet the requirements of the present measurements. Light intensities, providing up to \(1.5 \times 10^{-7}\) absorbed einsteins cm\(^{-2}\) sec\(^{-1}\) at 665 m\(\mu\), half-bandwidth 35 m\(\mu\), were obtained from a quartz-iodine lamp (DWY), appropriate lenses, and water and interference filters. To minimize undesirable gradients in CO\(_2\) concentration in the leaf chamber its volume was kept small (15 ml), and high flow rates (0.5 to 1.0 l/min) were used. The average CO\(_2\) concentration of the air in the leaf chamber was 0.030%.

**Enzyme determinations.** Crude enzyme extracts were prepared from each separate leaf by homogenizing the pre-weighed leaf sample (approximately 200 mg fresh tissue) in 10 ml of a mixture of 0.04 M tris-HCl, 0.01 M MgCl\(_2\), 0.25 mM EDTA, and reduced glutathione (GSH). The final pH was 7.8 at 23°C. Addition of GSH to the mixture used for homogenization was essential for high and reproducible enzyme activities. Although in most cases 1.0 mM GSH was sufficient for maximum activity, higher concentrations were required with some samples. A concentration of 5.0 mM GSH proved to be sufficient for all clones and was used throughout the investigation. The supernatant, obtained after spinning the homogenate at 30,000 g for 20 minutes, was used for the enzyme assays without
further purification. All preparative procedures were carried out at 0° to 2°C.

The enzyme assays were consistently made 40 to 60 minutes after the start of the homogenization. As a standard procedure, the reaction was started by the addition of 0.1 ml of the enzyme extract to 0.4 ml of a freshly prepared mixture of 2.5 μmole NaHCO₃O₃ (0.5 μcurie/μmole), 0.14 μmole ribulose-1,5-diphosphate, 1.25 μmole GSH, 0.1 μmole EDTA, 10 μmole tris-HCl, and 2.5 μmole MgCl₂. The pH was 7.8 at 23°C. The sodium salt of ribulose-1,5-diphosphate was obtained by conversion of the barium salt. This was either purchased from Sigma or was kindly supplied by Dr. Daniel McMahon, University of Chicago. The latter preparation yielded somewhat higher activities with given enzyme preparations in comparison with the former and was used as a standard throughout the investigation.

After two minutes' incubation at 23°C the reaction was stopped by the addition of 0.1 ml 6 M acetic acid. A 0.25-ml aliquot was pipetted into a liquid scintillation counting vial and dried at 90°C for 2 hours. Then 0.25 ml of water was added to each vial, followed by 10 ml of Bray's solution. The radioactivity measurements were made with a Packard Tri-Carb Liquid Scintillation Spectrometer.

The rate of C¹⁴ incorporation was entirely linear with enzyme concentration in the range used, but declined somewhat with increasing incubation time. No change in the activity of the enzyme preparation was observed during a period of 1 hour if the enzyme was kept at 0° to 2°C. At least four replicate assays were run for each preparation, the typical variation among replicates being about 5%. The rate of C¹⁴ incorporation in the absence of ribulose diphosphate in all cases was less than 1% of the rate in the presence of this substrate.

Results. The carboxydismutase activities in preparations from selected clones of the Beskades population from an open habitat, and of Hallands Väderö from a densely shaded habitat are compared in Table 14. The three clones of each population were selected to include the extremes of variation with regard to leaf anatomy as well as to their photosynthetic characteristics as found in previous extensive work.

It is evident that the Beskades plants grown at a high light intensity show the highest enzyme activities regardless of the basis used for expressing them. The enzyme activities of the Hallands Väderö clones are relatively low when preconditioned to either high or low light intensities. Two of the Beskades clones (033 and 039) seem to require a high light in-

<table>
<thead>
<tr>
<th>Light intensity for growth</th>
<th>Clone</th>
<th>μmole CO₂/min/g Tissue</th>
<th>μmole CO₂/min/mg Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>high</td>
<td>low</td>
</tr>
<tr>
<td>Beskades [sun race]</td>
<td>033</td>
<td>5.0</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>039</td>
<td>5.2</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>076</td>
<td>7.0</td>
<td>6.4</td>
</tr>
<tr>
<td>Hallands Väderö [shade race]</td>
<td>124</td>
<td>2.8</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>132</td>
<td>2.2</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>014</td>
<td>4.6</td>
<td>3.2</td>
</tr>
</tbody>
</table>

* Each value represents the mean of independent experiments with two or more different plants of the same clone.
tensity to produce maximum enzyme activity, whereas in the remaining clone (076) the activity is high whether the clone has been previously grown in strong light or weak light.

The specific activity of the enzyme, that is, the activity on the basis of soluble protein, shows considerably less variation among individual clones and the differences between the two races become more distinct than when the activity is expressed on the basis of fresh weight. When grown at high light intensity the Beskades clones have nearly twice the specific activity of the Hallands Väderö clones. This is not associated with a lower level of soluble protein in the Beskades clones; the total as well as the soluble fraction of the leaf protein is generally higher rather than lower in the Beskades as compared with the Hallands Väderö plants.

It is interesting to note that even though the assays were carried out at bicarbonate concentrations several times below saturation, and also at a suboptimal temperature and ribulose-diphosphate concentration, the specific activities of the Beskades plants are among the highest reported in the literature for any plant species. It should be pointed out, however, that if compared under physiological CO₂ concentrations the light-saturated rate of CO₂ fixation by the intact leaf is much higher than the rate of CO₂ fixation catalyzed by the enzyme extract of the same leaf.

We investigated the possibility that the apparently lower specific activities of Hallands Väderö in comparison with Beskades leaves might be caused by the presence of an inhibitor in Hallands Väderö not present in Beskades, or conversely, to an activator present in Beskades but not in Hallands Väderö. For this purpose homogenates of leaves from the two populations were mixed, and the resultant activity compared with the activities of the separate extracts. The results presented in Table 15 show that the activity of the combined homogenates is very close to the calculated mean of the activities of the separate extracts. This is evidence for the absence of any such inhibitor or activator. Also, the presence of a competitive inhibitor would be expected to shift the apparent value of the $K_m$. No differences of $K_m$ have been found among extracts from the different clones.

As has been reported earlier (Björkman and Holmgren, 1963), pigment bleaching may result when shade habitat plants of Solidago are grown under light of high intensities. This effect increases with leaf age and, at advanced stages of bleaching, deterioration of the chloroplasts is often apparent. Precautions were therefore taken to avoid complications that might result from such secondary effects. The light intensity was kept at a somewhat lower level than in previous investigations, and only recently matured leaves showing no visible evidence of chlorophyll bleaching or chloroplast disturbances were used for enzyme determinations.

To test to what extent a low carboxydismutase activity may be the result of light damage of the chloroplasts, enzyme activity was determined in extracts of leaves in which

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Activity, cpm/vial</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Hallands Väderö 132</td>
<td>3839 ± 56*</td>
</tr>
<tr>
<td>(2) Beskades 076</td>
<td>7676 ± 140</td>
</tr>
<tr>
<td>(3) Mean of (1) and (2)</td>
<td>5758</td>
</tr>
<tr>
<td>(4) Hallands Väderö 132 + Beskades 076 1:1</td>
<td>5865 ± 175</td>
</tr>
</tbody>
</table>

* Mean and standard deviation, four independent determinations.
increasing degrees of bleaching had been induced. As shown in Table 16, the specific enzyme activity remains the same even to a stage where the chlorophyll content is less than one third of the normal, and severe structural disturbances of the chloroplasts have become apparent.

It thus seems highly probable that the lower enzyme activity found in the Hallands Väderö as compared with the Beskades plants when grown at high light intensities is caused by a genetically determined lower capacity of Hallands Väderö to produce the enzyme. It is tempting to speculate that these differences have arisen as a result of an adaptation of the photosynthetic machinery to the light environment of the plants in their native habitats. Under the low light intensities prevailing on the floor of a dense forest, efficient utilization of light for photosynthesis would require a high capacity of those steps concerned with the absorption and utilization of the light in primary photoacts, whereas the capacity of enzymic steps could be relatively low. With abundant light, on the other hand, the capacity of nonphotochemical steps must be high for efficient photosynthesis.

However, the extent, if any, to which the comparatively low level of carboxydismutase of the Hallands Väderö leaves actually does limit the light-saturated rate of photosynthesis under conditions of normal—and thus limiting—carbon dioxide concentrations is a question yet unanswered. In an attempt to gain some information on this problem we determined the relationship between enzyme activity and photosynthesis in Solidago clones. Figure 46 shows the results obtained when the rate of light-saturated photosynthesis of leaves from a number of different Solidago clones of both races is plotted against the specific activity of the enzyme in extracts prepared from the same individual leaves. The two variables show a very high correlation. The results of a detailed study of two clones of each population when grown under high and low light intensities (Table 17) further support the conclusion that there is a strong relationship between the light-saturated rate of photosynthesis and the specific activity of the enzyme. Both show a parallel variation regardless of whether the differences are due to genetically distinct clones or to preconditioning by light.

If the assumption is valid that the enzyme concentrations in vivo are proportional to the enzyme activities measured in vitro, these findings strongly suggest that the failure of the shade clones to adjust to the efficient use of strong light for photosynthesis may be caused, at least in part, by a limited capacity to produce the carboxylation enzyme.

Whether Solidago clones from shaded and exposed habitats differ in their capacity to produce photosynthetic enzymes other than carboxydismutase is not known. In considering the question of what internal

<table>
<thead>
<tr>
<th>Leaf No.</th>
<th>Total Chlorophyll, mg/mg protein</th>
<th>Carboxydismutase Activity, μmole CO₂/min/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.17</td>
<td>0.14</td>
</tr>
<tr>
<td>2</td>
<td>0.12</td>
<td>0.17</td>
</tr>
<tr>
<td>3</td>
<td>0.05</td>
<td>0.17</td>
</tr>
</tbody>
</table>
factors may limit the rate of light-saturated photosynthesis, it should also be kept in mind that with normal \( \text{CO}_2 \) concentrations these factors include not only the capacity of enzymic steps but also the barriers to the diffusion of \( \text{CO}_2 \). Of great interest in this connection are the findings by Dr. Holmgren in Uppsala, Sweden, that shaded habitat clones of \textit{Solidago} when grown in strong light show a greater stomatal resistance to diffusion than do exposed habitat clones. Perhaps a simple explanation for these differences can be found in the physical structure of the leaves. However, the stomatal aperture is under metabolic control and may very well be strongly influenced by the rate at which biochemical steps of photosynthesis operates. The possibility cannot be ruled out, therefore, that the higher stomatal diffusion resistance found in the shaded habitat clones is associated with a lower capacity of one or several enzymic steps in photosynthesis.

**FURTHER STUDIES OF THE EFFECT OF OXYGEN CONCENTRATION ON PHOTOSYNTHETIC CO\(_2\) UPTAKE IN HIGHER PLANTS**

\textit{Olle Björkman}

Last year we reported that under atmospheric \( \text{CO}_2 \) concentrations the rate of photosynthetic \( \text{CO}_2 \) uptake was about 30\% inhibited by the oxygen in the air in a number of species of higher plants. The degree of inhibition was remarkably constant over a wide range of light intensities and among various species differing widely in their maximum photosynthetic rate. However, two species of green algae tested showed no inhibiting effect of 21\% oxygen when photosynthesis was measured under conditions similar to those used for the higher plants. Although no conclusive evidence was obtained with regard to the mechanism of the inhibitory

### Table 17. Light-Saturated Rate of Photosynthesis and Carboxydismutase Activity in Shade and Sun Clones of \textit{Solidago}

<table>
<thead>
<tr>
<th>Origin of Clone</th>
<th>Photosynthesis, ( \mu \text{mole CO}_2/\text{min/mg protein}^* )</th>
<th>Carboxydismutase Activity, ( \mu \text{mole CO}_2/\text{min/mg protein}^* )</th>
<th>Photosynthesis, ( \mu \text{mole CO}_2/\text{min/mg protein}^* )</th>
<th>Carboxydismutase Activity, ( \mu \text{mole CO}_2/\text{min/mg protein}^* )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beskades</td>
<td>033</td>
<td>0.20</td>
<td>0.26</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>076</td>
<td>0.24</td>
<td>0.26</td>
<td>0.20</td>
</tr>
<tr>
<td>Hallands</td>
<td>132</td>
<td>0.12</td>
<td>0.17</td>
<td>0.10</td>
</tr>
<tr>
<td>Väderö</td>
<td>124</td>
<td>0.11</td>
<td>0.14</td>
<td>0.08</td>
</tr>
</tbody>
</table>

* All values represent means obtained from several ramets of each clone. Photosynthesis and enzyme determinations were carried out on comparable but not necessarily the same leaves.
effect, some of our results suggested that the inhibition might be caused by a leakage of electrons from highly reduced photosynthetic intermediates back to molecular oxygen, possibly at a site between the two photosystems. That an enhancement of the rate of the normal respiratory process by an increased oxygen concentration would be responsible for the apparent inhibition of CO₂ uptake was not considered as a likely explanation, since it is well established that respiration is saturated at a low partial pressure of O₂.

As part of their extensive work directed toward uncovering the relationship between photosynthesis and respiration in higher plants, Krotkov and co-workers investigated the dependence of the CO₂ compensation point on oxygen concentration. They found that this point (i.e., the CO₂ concentration at which there is no net uptake or release of CO₂) was close to zero at very low oxygen concentrations and that it increased linearly with concentration up to 100% O₂ (Tregunna, Krotkov, and Nelson, 1966; Forrester, Krotkov, and Nelson, 1966a, b). One exception to this rule was corn (Zea mays) in which the CO₂ compensation point is zero even at an O₂ concentration of normal air. From these and other data they concluded that the main effect of increasing oxygen on CO₂ exchange is a stimulation of the rate of CO₂ evolution in light. They also concluded that the process underlying this evolution of CO₂ in light operates through a pathway different from that of the normal respiratory process. Fock and Egle (1966), working with bean plants and the liverwort Conocephalum, arrived at similar conclusions.

Several years ago Zelitch showed that glycolic acid accumulates in the leaves of higher plants in the presence of hydroxysulfonates, which are effective inhibitors of the enzyme glycolic acid oxidase. Recently he reported (Zelitch, 1966) that the addition of this inhibitor to leaf discs of tobacco stimulated the rate of net CO₂ uptake in air, at least temporarily. Moreover, if the leaves were fed with C¹⁴-labeled glycolate, the rate of CO₂ evolution in light and CO₂-free air increased, as did the radioactivity of the CO₂ evolved. Thus, there are several lines of evidence that strongly indicate a close similarity between the effects on CO₂ exchange obtained when the oxidation of glycolate is inhibited, and those obtained when oxygen concentration is reduced. Also, it has been shown by Hess and Tolbert (1967) that the green alga Chlorella, in which the CO₂ exchange was found not to be affected by 21% oxygen, lacks glycolate oxidase. Some of our studies this year have therefore been concerned with the question of whether a stimulation of glycolate oxidation is, in fact, the primary cause of the inhibitory effect of oxygen on photosynthetic CO₂ uptake in higher plants.

Quantitative measurements of CO₂ exchange and glycolic acid content under air and low-oxygen concentration. If the increase in the rate of CO₂ uptake, which takes place when oxygen concentration is reduced, is caused by inhibition of the rate at which glycolate is oxidized to glyoxylate, glycolate should accumulate in the same way as it does when the enzyme mediating this step is inhibited. If the rate of glycolate formation is not strongly affected by oxygen concentration, the rate of glycolate accumulation would be expected to be proportional to the difference between the rates of CO₂ uptake in low oxygen and in air.

To test whether such an accumulation actually takes place, the following experiment was carried out: The rate of steady-state CO₂ uptake in
air at 22°C was determined on a detached leaf of *Solidago multiradiata* whose petiole was placed in a small volume of water, and the effect of changing the oxygen concentration between 21% and 1.5% was measured repeatedly. The leaf was then kept at 1.5% O₂ and the rate of CO₂ uptake recorded continuously for 1 hour. After this time the leaf was killed by rapid immersion (<2 sec) in boiling 1% NaHSO₃ solution. The water in which the petiole was inserted during the experiment was added to the solution, which was then kept at 95°C for 5 minutes. The leaf was homogenized, and the supernatant obtained after centrifugation was assayed for glycolate, by the technique described by Zelitch (1958). The controls were matched leaves kept in air during the experiments. The results in Table 18 show that no increase in glycolate content took place when the leaf was kept under low O₂ concentration, even though the rate of CO₂ uptake was 41% higher than in air.

Experiments in which *Mimulus* plants were grown under 4% and 21% oxygen concentration over a 10-day period (this report, p. 232) likewise yielded negative results. No significant difference in glycolate content of the leaves was found between plants grown under 4% and 21% O₂.

**Effect of temperature on the inhibition of CO₂ uptake by oxygen.** With saturating light and normal CO₂ pressure most higher plants have a flat temperature optimum, usually between 15° and 30°C. An attractive hypothesis to explain why the rate of CO₂ uptake declines at a relatively low temperature is that the rate of CO₂ evolution in the light increases more strongly with increasing temperature than does photosynthesis. Zelitch's findings that the stimulation of CO₂ uptake by the addition of a glycolate oxidase inhibitor increases strongly with temperature appears to be in good agreement with this explanation. In Zelitch's experiment with tobacco leaf discs, CO₂ uptake was stimulated several fold by the inhibitor at 35°C but was not affected at 25°C.

If such a strongly temperature-dependent photorespiratory process underlies the observed inhibitory effect of oxygen on CO₂ uptake, this effect should be small or nonexistent at a low temperature (in tobacco leaves at 25°C) and should increase strongly with temperature. The data shown for *Solidago* and *Mimulus* in Fig. 47 indicate that this is not the case as far as the steady-state rate of CO₂ uptake is concerned. The degree of inhibition caused by 21% O₂ is almost independent of temperature.

The results thus obtained both in the experiments on glycolate accumu-

### TABLE 18. Glycolate Content in Detached Leaves of *Solidago multiradiata*, T. L. 7621-2, in Relation to CO₂ Uptake in Low O₂ and in Air

<table>
<thead>
<tr>
<th></th>
<th>µmole/leaf</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) CO₂ fixed during 1 hour in 1.5% O₂</td>
<td>48</td>
</tr>
<tr>
<td>(2) CO₂ fixed during 1 hour in 21% O₂</td>
<td>34</td>
</tr>
<tr>
<td>[3] Increase in CO₂ fixation due to low O₂ concentration (1) minus (2)</td>
<td>14</td>
</tr>
<tr>
<td>(4) Glycolate content expected if 1 mole CO₂ is formed for each mole of glycolate oxidized</td>
<td>&gt; 14</td>
</tr>
<tr>
<td>(5) Glycolate content found in 1.5% O₂</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>(6) Glycolate content found in 21% O₂ (controls)</td>
<td>&lt; 1</td>
</tr>
</tbody>
</table>
Fig. 47. Light-saturated rate of CO₂ exchange as a function of temperature in low O₂ concentration and in air. Light: 665 mp, half bandwidth 35 mp, 2.3 × 10⁵ erg cm⁻² sec⁻¹, CO₂ concentration: 0.03%. Temperature changes were carried out in air. (A) Solidago virgaurea, Beskades 039. (B) Mimulus cardinalis, 7210.1.
lation in low O₂ concentration and in those on temperature dependence of CO₂ uptake fail to support the supposition that the primary cause of the observed inhibition of CO₂ uptake by oxygen in normal air is stimulation of a "photorespiratory process" mediated by the enzyme glycolate oxidase.

It is noteworthy, however, that the time course of the change in CO₂ uptake that results when CO₂ concentration is altered is markedly affected by temperature. The curves shown in Fig. 48 typify the time course obtained with Mimulus and Solidago leaves, but it should be pointed out that the magnitude of the transient changes varies considerably among different leaves, and also varies with the detailed experimental conditions.

**Effect of wavelength on the inhibitory effect of CO₂ uptake by oxygen.** The steady-state rate of CO₂ uptake in normal air seems to be a remarkably constant fraction of the rate in low O₂. The degree of inhibition caused by 21% O₂ was found to be about the same among diverse species of higher plants even though their light-saturated rate of CO₂ uptake varied greatly. Furthermore, the degree of the inhibition is constant over a wide range of light intensities and, as already mentioned, also of temperatures. The results shown in Tables 19 and 20 indicate that the inhibition is also about the same at different wavelengths of the light over the range investigated. Thus there is no indication that the observed inhibition of CO₂ uptake, at least under rate-limiting light intensities, is due to a photorespiratory process sensitized by pigments other than those operating in photosynthesis. Whether or not the spectral distribution of the light influences the degree of inhibition at or beyond saturating light intensities is not yet known.

**Absence of an inhibition of CO₂ uptake by 21% oxygen in certain higher plant species.** The thermophilic grasses corn and sugar cane, which exhibit unusually high light-saturated rates of photosynthesis, are capable of reducing the CO₂ content in a closed system to zero. The results of Forrester et al. (1966b) also indicated that the rate of CO₂ uptake in corn was little affected when O₂ concentration was changed from 1% to 21% O₂. The absence in corn of an inhibitory effect of O₂ in this range was confirmed in this laboratory. No significant inhibition was found at temperatures ranging from 10° to 40°C, light intensities from 2 × 10³ to 1.2 × 10⁶ erg cm⁻² sec⁻¹, and wavelengths from 430 to 700 mµ. Similarly, the dicotyledonous species Amaranthus edulis, which, like corn and sugar cane, is capable of high rates of CO₂ fixation and has a CO₂ compensation point close to zero in air (El-Sharkawy et al., 1967), was found to lack an apparent inhibition of CO₂ uptake by 21% O₂.

Figure 49 shows the different response pattern in the rate of CO₂ exchange to changes between light and dark in the three species Mimulus cardinalis, corn (Zea mays), and Amaranthus edulis. Mimulus was chosen to represent species whose CO₂ uptake is inhibited by 21% O₂. Particularly noteworthy are the differences among the species with regard to the presence of a marked transient high rate of CO₂ evolution during the first minute after the light is turned off. The presence of such a post-illumination burst of CO₂ has been taken as evidence for a stimulation of the rate of CO₂ production in the light (e.g., Tregunna et al., 1964). The fact that the extent of the burst decreases with decreasing O₂ concentration (Fock and Egle, 1966, and Tregunna et al., 1966) and that it is absent in corn, even in the presence of air, at first sight seems to be con-
sistent with the hypothesis that there is a close connection between the \( \text{CO}_2 \) burst and the inhibitory effect of \( \text{O}_2 \) on net \( \text{CO}_2 \) uptake. It is known, however, that glycolysis is inhibited in the light. Darkening relieves this inhibition and may lead to an overshoot of oxidation, which may, in part, explain the observed \( \text{CO}_2 \) burst. Moreover, the present finding that *Amaranthus*

![Graph showing time course of CO2 exchange](image)

Fig. 48. Time course of the rate of \( \text{CO}_2 \) exchange in response to changes in oxygen concentration at different temperatures. Same leaf and conditions as in Fig. 47(A).
TABLE 19. Photosynthetic CO₂ Uptake under 0.2% O₂ and under Air at Different Wavelengths in Solidago virgaurea, Hallands Väderö 124

<table>
<thead>
<tr>
<th>Wave-length, mµ</th>
<th>Half band-width, mµ</th>
<th>Rate of CO₂ Uptake, * nano-mol cm⁻² sec⁻¹</th>
<th>In low O₂</th>
<th>In Air</th>
<th>Inhibition, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>440</td>
<td>37</td>
<td>0.39</td>
<td>0.24</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>493</td>
<td>15</td>
<td>0.38</td>
<td>0.26</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>535</td>
<td>12</td>
<td>0.36</td>
<td>0.25</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>554</td>
<td>12</td>
<td>0.37</td>
<td>0.25</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>575</td>
<td>13</td>
<td>0.36</td>
<td>0.24</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>605</td>
<td>13</td>
<td>0.36</td>
<td>0.24</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>633</td>
<td>14</td>
<td>0.37</td>
<td>0.24</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>665</td>
<td>13</td>
<td>0.38</td>
<td>0.25</td>
<td>33</td>
<td></td>
</tr>
</tbody>
</table>

* Measurements were made at 22°C and 0.03% CO₂. Light intensity at 665 mµ was 6 × 10^3 erg cm⁻² sec⁻¹. At other wavelengths the intensity was adjusted to give the same rates of CO₂ uptake as at 665 mµ. The rates were linearly related to light intensity.

edulis leaves exhibit a pronounced burst of CO₂ immediately following illumination, despite the fact that 21% O₂ does not inhibit the net CO₂ uptake in these same leaves, shows that such a burst does not necessarily indicate an inhibitory effect of O₂.

Conclusions. Experiments designed to test the hypothesis that the inhibition of photosynthetic CO₂ uptake by O₂ in normal air can be explained simply by an enhancement of the rate of glycolate oxidation mediated by the enzyme glycolate oxidase have yielded negative results. An increased rate of CO₂ fixation in low O₂ concentration was not correlated with accumulation of glycolate. Nor does the inhibitory effect of O₂ on CO₂ uptake show a temperature dependence consistent with the finding that inhibition by glycolate oxidase inhibitors of the rate of CO₂ production in light is strongly increased with temperature.

Measurements of the inhibition of net CO₂ uptake caused by 21% O₂ at different wavelengths of light give no indication that a “photorespiratory process” whose action spectrum differs markedly from that of photosynthesis causes the inhibition.

Although no new evidence supporting such a conclusion has been obtained, it seems reasonable that the inhibition is caused primarily by a back-reaction between a highly re-

TABLE 20. Photosynthetic CO₂ Uptake under 0.2% O₂ and under Air at Different Wavelengths in Mimulus cardinalis 7120-7

<table>
<thead>
<tr>
<th>Wave-length, mµ</th>
<th>Half band-width, mµ</th>
<th>Rate of CO₂ Uptake, * nano-mol cm⁻² sec⁻¹</th>
<th>In low O₂</th>
<th>In Air</th>
<th>Inhibition, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>440</td>
<td>37</td>
<td>1.39</td>
<td>0.93</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>540</td>
<td>48</td>
<td>1.33</td>
<td>0.89</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>590</td>
<td>30</td>
<td>1.38</td>
<td>0.94</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>650</td>
<td>49</td>
<td>1.38</td>
<td>0.93</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>700</td>
<td>55</td>
<td>1.40</td>
<td>0.91</td>
<td>35</td>
<td></td>
</tr>
</tbody>
</table>

* Measurements were made at 23°C and 0.03% CO₂. Light intensity at 650 mµ was 4 × 10^4 erg cm⁻² sec⁻¹. At other wavelengths the intensity was adjusted so that the rates of CO₂ uptake were approximately the same as those at 650 mµ. The rates were partially light saturated.
Fig. 49. Time course of the rate of CO₂ exchange in response to light and dark in *Mimulus cardinalis*, 7211-4; *Zea mays*, Ferry Morse hybrid 901; and *Amaranthus edulis*. Seeds of *Amaranthus*, native to Argentina, were kindly supplied by Dr. R. S. Loomis and Dr. W. A. Williams of the University of California at Davis. Measurements were made at 24°C and 0.03% CO₂. White light of an intensity of $3 \times 10^5$ and $5 \times 10^5$ erg cm⁻² sec⁻¹ (400–700 mμ) was used with *Mimulus* and the two other species, respectively.
duced photosynthetic intermediate and molecular oxygen that increases with O₂ concentration. Hydrogen peroxide resulting from such a back-reaction could have several secondary effects. For example, it could increase the rate of oxidation of glyoxylate; it also could stimulate the production of glycolate from intermediates of the Calvin cycle as proposed by Coombs and Whittingham (1966).

Comparative studies of photosynthesis among species differing in their response to oxygen concentration appear to be a promising approach to gaining new insight into the mechanism of inhibition by O₂. Of particular interest in this connection are the results of Hatch and Slack (1967), which indicate that certain tropical grasses, including corn and sugar cane, which lack an apparent inhibition of CO₂ uptake by 21% O₂, utilize a different pathway for photosynthetic CO₂ fixation than most other plants.

References

EFFECT OF OXYGEN CONCENTRATION ON DRY MATTER PRODUCTION IN HIGHER PLANTS
Olle Björkman, William M. Hiesey, Malcolm Nobs, Frank Nicholson, and Richard W. Hart

The fact that the rate of photosynthetic CO₂ uptake in most higher plants is strongly enhanced by low O₂ content in the atmosphere propounds the question of whether or not the rate of dry matter production is also enhanced. An increase would be expected if (1) the enhancement of CO₂ uptake is due to a net increase in the yield, and not merely an effect caused by a greater portion of the reducing power and phosphate bond energy formed in photosynthesis being used for the reduction of CO₂ at the expense of other vital endergonic processes, and (2) if low O₂ concentration does not adversely affect secondary growth processes.

Although a great deal is known about the effects of O₂ partial pressure in the root medium, few investigations have been concerned with the dependence of growth on the O₂ concentration of the atmosphere. In recent years Siegel et al. (1963) have shown that a number of plant processes such as germination, root, and coleoptile elongation are essentially unaffected or sometimes even enhanced, by subatmospheric O₂ levels, whereas senescence is suppressed. In some cases early seedling growth was found to be somewhat greater at 10% as compared with 21% O₂, and young seedlings were able to grow at concentrations as low as 5%. Before any conclusions can be drawn as to whether the enhancement of CO₂ uptake by low O₂ is matched by an enhancement of dry matter production, however, further experimentation under precise control not only of O₂ concentration but also of other external variables, particularly the concentration of CO₂, is needed.
To make such studies feasible, new growth chambers were developed during the year. A brief description of their design and the results of exploratory growth experiments under different O₂ concentrations are given below.

**Growth cabinets with controlled O₂ and CO₂ concentration.** The basic construction of the cabinets is the same as described previously (Hiesey and Milner, 1962), except that the air circulation system is closed to the external atmosphere. In addition, the cabinets are connected with large collapsible plastic bags (approximately 500 l) to equalize the internal and external pressures. This prevents leakage of external air into the cabinets, which would otherwise take place when the external pressure increases or when the internal pressure decreases due to decreases in temperature.

A schematic diagram of the control system is given in Fig. 50. Thermistor-operated controllers provide precise temperature control. Gas from the cabinets is continuously pumped through a paramagnetic O₂-analyzer (Beckman, Model F3) and an infrared CO₂-analyzer (Beckman, Model 15 A or LIRA, Model 300). Both the O₂-analyzer and the CO₂-analyzer are equipped with electronic controllers, specifically designed for this purpose.

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**Fig. 50.** Block diagram of gas control system. The valve positions are shown for the control cycle of cabinet A.
by Mark Lawrence. Each consists of a solid-state operational amplifier used in a voltage comparator circuit which compares the output of the gas analyzer with an internal reference. When the gas concentration deviates from the preset level, a transistor switch activates a solenoid in the controlling gas lines to correct this condition.

To avoid undesirable differences in CO$_2$ or O$_2$ concentration between the two cabinets, the same analyzers are used for both cabinets. An electronic timer switches the gas sampling and controlling circuits between the two cabinets at preset intervals (usually 1 min). When a cabinet is being operated at or above atmospheric O$_2$ concentrations, CO$_2$-free air is slowly fed into the cabinet to prevent the CO$_2$ concentration from increasing beyond the preset value; at subatmospheric O$_2$ concentrations, the CO$_2$-free air is replaced with CO$_2$-free N$_2$. The absolute accuracy of CO$_2$ control is about ±5 ppm in the range 200 to 400 ppm. The maximum difference between cabinets (if both are set to the same value) is only about ±1 ppm. The accuracy of the O$_2$ control is about ±0.5% in the range 0% to 25% O$_2$.

Light is provided by 96-inch Sylvania VHO fluorescent tubes (2.4 m long), supplemented with incandescent lamps. Since a single light bank is used for the illumination of both cabinets, differences in light intensity and quality between the two cabinets can be kept very small.

The root medium can be aerated with gas whose composition is independent of that of the cabinet atmosphere. Gas-tight seals between root and shoot are obtained with foam neoprene gaskets (4 mm thick). Prior to an experiment, cuttings or seedlings are inserted through a tiny hole in each gasket at an early stage of development. As the stems expand, tight seals are obtained by the neoprene gaskets without undesirable effects on the plants.

Transpiration water is condensed on the cooling coils of the refrigeration system and drained through a trap. The cabinets work well, and several successful runs have been completed with Phaseolus, Mimulus, and Zea.

**Effect of O$_2$ concentration on growth rate.** In all experiments described below, the plants were grown under continuous light and 0.03% CO$_2$. Except where specified, the roots were immersed in a large volume of nutrient solution which was aerated with normal air (21% O$_2$, 0.03% CO$_2$). Within each experiment all factors but O$_2$ concentration were kept constant.

The short-term response of growth to different O$_2$ concentration was studied on bean seedlings (*Phaseolus vulgaris*, var. Mexican Red). Carefully matched 5-day-old seedlings were grown under 2.5% and 21% O$_2$ at 29°C and a light intensity of 5 × 10$^4$ erg cm$^{-2}$ sec$^{-1}$. Plate 1 illustrates the response obtained in a 6-day period. Clearly the growth was strongly enhanced at the low O$_2$ concentration. As shown in Table 21, the net increase in the total dry weight of the seedlings was about twice as high with 2.5% as compared with 21% O$_2$. All organs showed an enhanced growth in low O$_2$; there were no major differences in their relative proportions. Root growth was strongly increased, even though the roots themselves were aerated with normal air.

The effect of low O$_2$ concentration on the growth of bean seedlings over an extended period was studied in another experiment. In this case 12 pairs of 6-day-old seedlings (*Phaseolus vulgaris*, var. Ferry Morse Bush 869), sampled at random, were grown in perlite with nutrient solu-
TABLE 21. Effect of \( \text{O}_2 \) Concentration on Dry Weights of the Bean Seedlings, 6 days' growth (Plate 1)

<table>
<thead>
<tr>
<th></th>
<th>( 21% \text{O}_2 ) grams</th>
<th>( 2.5% \text{O}_2 ) grams</th>
<th>Ratio, 2.5% \text{O}_2/21% \text{O}_2</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Leaves, final weight</td>
<td>0.288</td>
<td>0.520</td>
<td>1.80</td>
</tr>
<tr>
<td>(2) Roots, final weight</td>
<td>0.118</td>
<td>0.237</td>
<td>2.00</td>
</tr>
<tr>
<td>(3) Stem, final weight</td>
<td>0.130</td>
<td>0.164</td>
<td>1.26</td>
</tr>
<tr>
<td>(4) Entire plant, final weight</td>
<td>0.536</td>
<td>0.921</td>
<td>1.72</td>
</tr>
<tr>
<td>(5) Entire plant, original weight</td>
<td>0.203</td>
<td>0.221</td>
<td></td>
</tr>
<tr>
<td>(6) Growth in 6 days, (4) minus (5)</td>
<td>0.333</td>
<td>0.700</td>
<td>2.10</td>
</tr>
</tbody>
</table>

TABLE 22. Effect of \( \text{O}_2 \) Concentration on Dry Weights of Mimulus

<table>
<thead>
<tr>
<th></th>
<th>( 21% \text{O}_2 ) grams</th>
<th>( 5% \text{O}_2 ) grams</th>
<th>( 5% \text{O}_2 )/21% \text{O}_2</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Entire plant, final weight*</td>
<td>0.675* ± 0.029</td>
<td>1.186* ± 0.043</td>
<td>1.76</td>
</tr>
<tr>
<td>(2) Entire plant, original weight</td>
<td>0.110</td>
<td>0.110</td>
<td></td>
</tr>
<tr>
<td>(3) Growth in 10 days, (1) minus (2)</td>
<td>0.565</td>
<td>1.076</td>
<td>1.90</td>
</tr>
</tbody>
</table>

* Means are significantly different at \( P = 0.01 \).

ation fed automatically. The roots were kept at the same \( \text{O}_2 \) concentration as the shoots. Light intensity was \( 8 \times 10^4 \text{ erg cm}^{-2} \text{ sec}^{-1} \), and temperature was \( 27°C \). Plate 2 illustrates the typical appearance of the plants after 17 days under 5\% and 21\% \( \text{O}_2 \). No adverse effects of growing the plants under low \( \text{O}_2 \) concentration were found. The plants grew very rapidly under these conditions, and produced an abundance of flowers. The net increase in shoot dry weight was about twice as great under 5\% \( \text{O}_2 \) as under air. No quantitative measurements of root weights were made, but it was obvious that root growth also was generally greater in the plants grown in low \( \text{O}_2 \). With beans chlorosis became increasingly apparent in developing shoots after about 10 days in normal air. Presumably the effect is caused by growing the bean plants under continuous light. Interestingly, no signs of chlorosis could be detected in the plants grown under 5\% \( \text{O}_2 \).

Another noteworthy effect of growing the bean plants under low \( \text{O}_2 \) is that the rhythmic movement of primary leaves was arrested after about two days. In air this movement continued for at least five more days.

\textit{Mimulus cardinalis}, Jacksonville 7211-4, provides an experimental material much better suited for the present studies than \textit{Phaseolus}. Uniform cuttings of a single clone can easily be obtained, and the problem of genetically variable seedling material can be avoided. Moreover, continuous light has no adverse effects on \textit{Mimulus}; on the contrary, it results in a very rapid growth.

The results obtained with \textit{Mimulus} thus far indicate that the yield in dry matter production is strongly enhanced by low \( \text{O}_2 \). As shown in Table 23 the net gain in dry weight was much higher in 5\% and 2.5\% \( \text{O}_2 \) than
in air, under the conditions tested (7.2 \times 10^4 \text{ erg cm}^{-2} \text{ sec}^{-1}, 24^\circ \text{C}). It appears that 2.5\% \text{ O}_2 might be too low for maximum enhancement of dry matter production.

Growing \textit{Mimulus} under 2.5\% and 5\% \text{ O}_2 resulted in a greater root-to-shoot ratio, and a lower water content of the shoots, than in air (9.8\% dry matter in low \text{ O}_2 versus 8.4\% in air). The plants grown under low \text{ O}_2 had thicker leaves and a more compact growth habit than those grown in air.

Photosynthesis measurements on \textit{Mimulus} and \textit{Phaseolus} leaves showed that the degree of inhibition of net \text{ CO}_2 uptake by 21\% \text{ O}_2 was unaffected by growing the plants under different \text{ O}_2 concentrations. The inhibition was about 30\% regardless of preconditioning. The absolute rate of light-saturated \text{ CO}_2 uptake on a leaf area basis was higher in leaves developed in low \text{ O}_2 than in air. This was associated with a greater leaf thickness of the former.

As was mentioned in the preceding section of this report, \textit{Zea mays} (corn) differs from most other higher plant species in that it lacks an apparent inhibition of \text{ CO}_2 uptake by 21\% \text{ O}_2. It seemed of interest therefore to compare its response to different \text{ O}_2 concentrations during growth with that of \textit{Mimulus} and \textit{Phaseolus}. In a preliminary experiment, five matched pairs of corn seedlings, var. Ferry Morse Hybrid 901, were grown under 5\% and 21\% \text{ O}_2, with other conditions the same as those given for \textit{Mimulus}. As shown in Table 24, the enhancement of dry matter production in low \text{ O}_2, if any, was much less than in \textit{Mimulus} and \textit{Phaseolus}. Interestingly, however, the root-to-shoot ratio and the percentage of dry matter in the shoots (10.8\% in low \text{ O}_2, 6.3\% in air) were higher in the plants grown in low \text{ O}_2. This was also true of the other two species.

**References**


**TABLE 24. Effect of \text{ O}_2 Concentration on Dry Matter Production of Corn Seedlings**

<table>
<thead>
<tr>
<th></th>
<th>21% \text{ O}_2 grams</th>
<th>5% \text{ O}_2 grams</th>
<th>Ratio, 5% \text{ O}_2 to 21% \text{ O}_2</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Entire plant, final weight*</td>
<td>1.479 ± 0.123</td>
<td>1.683 ± 0.129</td>
<td>1.14</td>
</tr>
<tr>
<td>(2) Entire plant, original weight</td>
<td>0.210</td>
<td>0.210</td>
<td>...</td>
</tr>
<tr>
<td>(3) Growth in 10 days, (1) minus (2)</td>
<td>1.269</td>
<td>1.473</td>
<td>1.16</td>
</tr>
</tbody>
</table>

* Difference between means is scarcely significant: 0.3 < P < 0.4.
Hybridizations in Solidago
Malcolm A. Nobs, Olle Björkman, and William M. Hiesey

The exploratory crossings reported in Year Book 65, p. 471, between different forms of the Solidago virgaurea—S. multiradiata complex have yielded a number of F₁ hybrids now growing in the Stanford garden that show interesting and complex characteristics. Among these, for example, is a cross between the shade form of Solidago virgaurea from Hallands Väderö in southern Sweden, mentioned in earlier sections of this report, and a subalpine from S. multiradiata from near our Timberline transplant station in California. The success of this and other hybridizations has prompted us to follow a more extensive program this year utilizing other intercontinental combinations featuring interaltitudinal and interlatitudinal crosses between contrasting races of what appear to be members of the Solidago virgaurea—S. multiradiata species-complex that is widely distributed over the northern hemisphere.

Our first objective is to determine the degrees of biosystematic relationship between key ecological forms that have been referred to various named taxa, yet appear to be closely related to be capable of gene exchange.

A second objective is to compare the genetic constitution of parallel ecological races that are found in corresponding kinds of habitats in widely separated continents. Such races apparently were evolved independently over long periods of geologic time from a common ancestral stock.

The hybridizations that are being made include combinations between high-altitude forms from the Sierra Nevada of Spain and high-altitude forms from the Sierra Nevada of California, and between these two and lowland counterparts from the same two continents, all from about 39°N. latitude. In addition, races from approximately 70°N in Scandinavia and in Alaska are being crossed with each other, and also with the southern races just mentioned. Observations on the responses of parental races and their F₁ and F₂ progenies at the Stanford, Mather, and Timberline transplant stations are essential to interpret the genetic composition and potential of the parental races.

We are indebted to Dr. John Koranda and Dr. William Mitchell of the Alaska Experiment Station at Palmer for collections of living material of Alaskan forms.

Comparative Physiological Studies on Solanum dulcamara
Eckard Gauhl

The recent developments in plant ecological research aimed at uncovering internal physiological mechanisms that have evolved in plants native to different climates excite inquiries concerning species groups other than Solidago and Mimulus. The combined results from independent studies on a diversity of species are more likely to lead to the discovery of general principles than the study of only one or two species-complexes.

The choice of material to be used for this kind of work is of utmost importance because of the exacting practical experimental requirements for such investigations. Essential requirements are (1) that the species occur over a wide geographical range having a great diversity of habitats, (2) that its races be easy to grow and be propagated as clones in order to facilitate experiments with genetically identical material in various controlled environments, and (3) that its
leaf and stem structure be such as to facilitate measurements of gas exchange in intact leaves while still attached to the living plant. 

*Solanum dulcamara* L., the bittersweet, meets these requirements well. It is distributed in Europe from the Atlantic coast in France northward to central Norway, and south to northern Africa. It extends to India and Asia in the east and occurs in scattered areas throughout China and Japan. In central Europe it grows in shady swamps with alder (*Alnus glutinosa*), or in deep shade within stands of reed grass (*Phragmites communis*) along borders of lakes. Other races occupy exposed habitats on coastal sand dunes near sea level, or on rocky slopes of southern exposure in the alps up to altitudes of 1,700 m. The most contrasting ecological races are readily distinguishable morphologically.

A number of select clones have been collected in central and southern Europe, some of which have been brought to Stanford under a U.S. Department of Agriculture import permit. Current work is being confined mainly to three clones that represent extremes of habitat origin and of growth responses to controlled conditions. One clone originally from a reed grass marsh near Frankfurt grows better under low light intensity than under high. Another from a sand dune along the Baltic Sea in northern Germany that has thick, fleshy leaves and short internodes becomes prostrate when grown under strong light, and semi-erect under weak light. A third clone, originally from a dry, exposed rocky habitat near Rovinj along the northern Dalmatian coast of Yugoslavia, resembles in growth habit the inland marsh form, but grows better under strong than under weak light.

Comparative studies on rates of CO₂ uptake of intact leaves when grown under high and low light intensities have been started along lines described above for clones of *Solidago* and *Mimulus*. It is planned to pursue these studies in other directions also, with special consideration of interrelations between water metabolism and photosynthetic performance in contrasting climatic races of *Solanum*.

**Clusters of Tree Species on Both Sides of the Pacific**

*Jens Clausen*

Trees are suitable for use in gross surveys of the vegetations of the world. They are conspicuous enough to be easily seen and fairly good records of the limits of tree species are available.

Records and experimental data indicate that the greater number of the world's tree species are arranged in clusters of species, which experimental taxonomists call "cenospecies." Certain taxonomic sections of genera are on the size-order of species clusters. The species of such a cluster are closely enough related to permit occasional interchange between their heredities, either at present or in the past. The evolutionary significance of the species cluster has generally been overlooked.

Species clusters of this kind circle the earth within definite latitudinal belts. Modern taxonomists, however, constantly look for minor differences between the taxa within each cluster to apply different species names to them in various parts of the world. By splitting the species clusters, sometimes even into separate genera, sense has been lost of the deep-seated physiological and ecological characteristics that are built into the heredities of each cluster and that can be expressed in morphological characters. Heredity holds the natural clusters
subunits. The principle here discussed characterizes also herbaceous species complexes around the world.

Such facts suggest that genes that govern morphology and those governing physiology are correlated in their inheritance. Such correlations are based on the principle of interlocking heredity (coherence) that we have shown exists between climatic races of plants. Similar although much stronger correlations probably limit families and orders to their climatic zones.

Developing a sense of close relationship among the members of a species cluster brings a sense of perspective that is vital to the study of taxonomy. A study of the tree species clusters on both sides of the Pacific illustrates the principles involved in this kind of modern plant taxonomy.

Based on worldwide field experiences, Misao Tatewaki's comprehensive report (1958), supplemented by personal discussions with him on field tours in Hokkaido in 1966, have deeply influenced the report to follow.

*Trees of high latitudes.* On both sides of the Pacific approximately 12 clusters of tree species follow a wide belt, extending from about 70° to 35° North latitude (Table 25). The individual species of each of the 12 clusters are species of high latitudes and high altitudes. Interestingly enough, 6 of the 12 clusters belong to the pine family Pinaceae. Carolus Linnaeus (1753) included all of the 6 clusters in the genus *Pinus.*

The top cluster in Table 25 includes all species of the larch genus *Larix.* There are North American *L. laricina* Koch, the Japanese *L. leptolepis* Gord., the East Asiatic *L. kamtschatica* Carr., *L. gmelini* Gord., and *L. sibirica* Ledeb., and the type species of the cluster, the European *L. decidua* Mill. C. Syrach Larsen (1937) found that all these species are able to interbreed, and the species of the larch cluster are now being commercially intercrossed.

Two species clusters belong to the genus *Pinus,* namely a two-needle and a five-needle complex of species. Species belonging to different clusters do not interbreed. This hereditary separation enables the species of two clusters to maintain their identity when growing together in the same local area.

The two-needle cluster of species includes the North American lodge pole pines, *P. contorta* Doug., *P. Murrayana* Grev. et Balf., and *P. banksiana* Lamb., with the Japanese *P. thunbergiana* Franco, *P. densiflora* Sieb. et Succh., and with the scotch pines of Siberia to Europe, *P. silvestris* L., *P. montana* Mill., and the dwarf *P. mugho* Turra. *Pinus silvestris* is the type species of the cluster. Many natural hybrids have been suspected among the latter three and among the Japanese species. Righter and Stockwell (1949) described the fertile species hybrid *P. Murrayana X P. banksiana,* and intermediates are known where the two species meet in the wild. Like the larches, the two-needle pine cluster circles the earth and apparently shares a common gene pool.

The five-needle pine cluster in western North America is represented by the hardy whitebark pine *P. albicaulis* Engelm. On the Asiatic side of the Pacific are the species *P. pumila* Regel, *P. koraiensis* Sibth. et Succh. of Northeastern Asia to Japan, and *P. sibirica* du Tour. from the Altai to the Ural Mountains and Peninsula Kola. The type species of the cluster *P. cembra* L., occurring in the Carpathian Mountains to the Swiss Alps (Critchfield and Little, 1966), completes the circle. *P. cembra,* *P. sibirica,* and *P. koraiensis* are generally erect trees; *P. pumila* is always an elfinwood bush, but *P. albicaulis* is a tree in subalpine environments and
<table>
<thead>
<tr>
<th>Eastern Hemisphere</th>
<th>Pacific Ocean</th>
<th>Western Hemisphere</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PINACEAE, n = 12</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Larches</td>
<td>LARIX decidua</td>
<td>sibirica-kamtschatka</td>
</tr>
<tr>
<td></td>
<td></td>
<td>leptolepis</td>
</tr>
<tr>
<td>2. Scots pines</td>
<td>PINUS silvestris</td>
<td>sibirica-koreensis-pumila</td>
</tr>
<tr>
<td></td>
<td>montana</td>
<td></td>
</tr>
<tr>
<td>3. Cembras</td>
<td>PINUS cembra</td>
<td>sibirica-koreensis-pumila</td>
</tr>
<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>4. Spruces</td>
<td>PICEA excelsa</td>
<td>obovata-jezoensis-glehnii</td>
</tr>
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<td></td>
<td>smithii</td>
<td></td>
</tr>
<tr>
<td>5. Firs</td>
<td>ABIES alba</td>
<td>diversifolia-sieboldii</td>
</tr>
<tr>
<td></td>
<td></td>
<td>brunoniana</td>
</tr>
<tr>
<td>6. Hemlocks</td>
<td>TSUGA (extinct)</td>
<td>diversifolia-sieboldii</td>
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<td>sitchensis-canadensis</td>
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**CUPRESSACEAE, n = 11**

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<tr>
<td>7. Junipers</td>
<td>JUNIPERUS communis</td>
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<td>communis</td>
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**SALICACEAE, n = 19**

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<td>8. Aspens</td>
<td>POPULUS tremula</td>
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<td>davidiana</td>
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<td>tremuloides</td>
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<td>9. Willows</td>
<td>SALIX phylicifolia</td>
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<td>pyriform-pulchra</td>
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<td>pulchra-arbuscula</td>
<td></td>
</tr>
</tbody>
</table>

**BETULACEAE, x = 14**

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>10. Birches</td>
<td>BETULA tortuosa</td>
<td></td>
</tr>
<tr>
<td></td>
<td>cajanderi-ermanni</td>
<td></td>
</tr>
<tr>
<td>11. Alders</td>
<td>ALNUS incana</td>
<td></td>
</tr>
<tr>
<td></td>
<td>maximovics-inokumae-crispa</td>
<td></td>
</tr>
<tr>
<td></td>
<td>incana-crispa</td>
<td></td>
</tr>
</tbody>
</table>

**ROSACEAE, n = 17**

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>12. Mountain ashes</td>
<td>SORBUS aucuparia</td>
<td></td>
</tr>
<tr>
<td></td>
<td>amurensis-scopulina</td>
<td></td>
</tr>
<tr>
<td></td>
<td>commixta</td>
<td></td>
</tr>
<tr>
<td></td>
<td>sitchens-canadensis</td>
<td>decorra</td>
</tr>
</tbody>
</table>

Dots indicate that the particular species is continuous over the region. The second lines of the clusters indicate the geographic location of certain southern species.

An elfinwood cushion type above the tree line (Year Book 62, pp. 394–398; and Clausen, 1965). Members of this cluster of species extend to and beyond the tree line in North America and Eurasia, intermediates are recorded where the species meet. This cluster of species and the members of the two-needle cluster occur together in many populations but do not intercross.

As far as is known at present, the spruces, firs, and hemlocks constitute three other high latitude circumpolar clusters of trees, as shown in Table 25. The spruces (Picea), firs (Abies), and hemlocks (Tsuga) are composed of several geographic species that have many intermediates. Species within the Picea and Abies clusters are known to interbreed (Wright, 1953). Like the other members of the pine family, all appear to have 12 pairs of chromosomes, and they were included by Linnaeus as species of the genus Pinus. The presently recognized species represent geographic, climatic, and edaphic adaptations to many environmental niches within the belt covered by the three genera around the world. The species of a cluster share a common gene pool.
Juniperus communis L. is still recognized as one species around the world by conservative taxonomists. Its lowland and inland forms are of erect columnar shape, whereas forms from windy coasts and alpine habitats are low elfinwood cushions. In transplant experiments in Denmark, C. Syrach Larsen has shown that the differences in growth form are hereditary. The junipers of this species have 11 pairs of chromosomes and cover approximately the same wide belt as the six clusters of conifers previously discussed.

Five nonconiferous high-latitude clusters of tree species include the aspens, willows, birches, alders, and mountain ashes (Table 25). Each of these is composed of closely related species that together circle the earth at high to medium North latitudes. All are well known for their interspecific crossability; the willows, birches, alders, and mountain ashes cross irrespective of differences in number of chromosomes.

The quaking aspens constitute a circumboreal natural group of species, differing from other poplars by their ability to spread vegetatively through root shoots. A stand of 20 to 50 trees may consist of one individual. The aspens have 19 pairs of chromosomes, and the North American Populus tremuloides Michx. is being commercially intercrossed with the Asiatic-European P. tremula L. The hybrids are fertile and possess hybrid vigor. The Japanese aspens, P. jezoensis Nakai and P. davidiana Dode, have rhizomes like the other quaking aspens, and P. davidiana has been crossed with P. tremula (Chiba, 1966).

The North American–Eurasiatic alders, Alnus, were thought to follow a 14-series in chromosome number (Gram, Muhle Larsen, C. Syrach Larsen, and Westergaard, 1941). Chiba, however (1966: 155–156), found a diploid Japanese species, Alnus inokumae Mur. et Kus., having \( n = 7 \). Chiba was able to cross it with tetraploid forms of A. hirsuta Turcz., A. glutinosa Gaertn., and A. japonica Call., \( n = 14 \), and the hybrids have \( 2n = 21 \) chromosomes (Chiba, 1966: 1–16). The genus is commonly supposed to have many hybrids around the earth.

The birches, Betula, also intercross irrespective of differences in chromosome number and of differences in the appended species name. Helms and Jørgensen (1925) found that Betula alba, \( n = 28 \), growing in a wet Danish moor, crossed with B. verrucosa, Ehr., \( n = 14 \), along drier, sandy edges of the moor. Partially fertile hybrid progenies ranging from approximately 21 pairs to intermediate numbers between 21 and 27 were found along the borderline of the two populations, suggesting that possibly 7 is the basic chromosome number also in Betula. The variation in birch is so great that classification is very difficult, but Table 25 lists some of the names applied to its species around the high latitudes.

An equal number of species names have been applied to the Sorbus aucuparia cluster, the mountain ashes of the rose family, having 17 pairs of chromosomes. In North America are Sorbus americana March., S. decora Sarg., S. scopulina Greene, and S. sitchensis Roem; in Japan are S. sambucifolia Roem., S. matsumurana Koehne, and S. commixta Hedl.; in northeastern Asia is S. amurenensis Koehne, and in Europe, the type species S. aucuparia L. The growth forms vary greatly among the member species, but a member of this highly variable cluster can always be expected to occur at high latitudes and high altitudes.

The 12 cold-tolerant species clusters discussed here are on both sides of the Pacific, but the member species
within each cluster are difficult to dis-
tinguish because they share common
gene pools. Members of distinct clus-
ters, on the other hand, are clearly
separated from each other.

Within the southern hemisphere
there are no species clusters that in
tolerance to extremely cold climates
match the 12 uniquely cold-tolerant
northern hemisphere species clusters.

Clusters of tree species of medium
cold tolerance. A different and much
larger group of tree species occupies
medium latitudes from about 55° to
25°N, on both sides of the Pacific.
Some of these species and genus clus-
ters grow at comparable latitudes on
both sides of the Pacific in both the
northern and southern hemispheres.
The several thousand medium tolerant
tree species are less tolerant to cold
than the species native to the high
latitudes, but they are much more
tolerant than species native to the
low latitudes on both sides of the
equator. At latitudes where species
clusters of both high and medium
tolerance overlap, those of medium
tolerance grow at lower altitudes than
the species of the highly cold-tolerant
clusters.

Like the high latitude species,
those of medium tolerance are orga-
nized in evolutionary clusters of
closely related species. Sufficient ex-
perimental evidence is on hand to
indicate that the species of several of
these clusters share common gene
pools on both sides of the oceans.
Table 26 lists examples of tree species
clusters of medium tolerance on both
sides of the Pacific.

The top cluster is a belt of white
pines of medium tolerance having five
needles and elongated cones that con-
stitute the taxonomic section Strobus.
On the North American side this clus-
ter is marked by the type species, the
eastern white pine, P. strobus L., the
Great Basin limber pine, P. flexilis
James, the western white pine, P.
monticola Doug., and possibly the
sugar pine, P. lambertiana Doug. On
the western side of the Pacific are the
Armand pine, P. armandi Franch,
native to Taiwan, South China, and
Vietnam, the Indian blue pine, P.
griffithi McClelland of the Himalayas
to Kashmir, and the Balkan pine,
P. peuce Griseb., native to southeastern
Europe. Little and Righter (1965)
tercrossed these species in all direc-
tions, producing successful hybrids.
The white pines accordingly form an
evolutionary cluster that spans the
earth at medium latitudes.

Several genera of the Taxodiaceae-
Cupressaceae group circle the earth,
and they follow an 11-series in chro-
mosome numbers both north and
south of the equator and across the
oceans (Table 26). These genera con-
tain the cypresses, the incense cedars,
thujas, sequoias, and swamp cy-
presses. Specialists have split each of
these clusters into distinct genera by
calling attention to small differences,
but have neglected the gross simi-
larities and adaptations and chromo-
somal heredity that unite them across
the oceans and across the equator.

One of these clusters is the cy-
presses, Cupressus-Chamaecyparis.
Along the west coast of North Amer-
ica five to six Cupressus and two
Chamaecyparis species are recog-
nized. Osborn (1941) described, how-
ever, a hybrid between the Monte-
rey cypress, Cupressus macrocarpa
Hartw., native to the central Cali-
ifornia coast, and the Alaska cedar,
Chamaecyparis nootkatensis Spach.,
a mountain species growing from
northern California to Alaska. This
“intergeneric” hybrid is fertile, and
its progeny segregates the parental
characters, suggesting that at least
these two species belong to one genus
and that in this group the prevailing
taxonomy is misleading. In Japan
Chamaecyparis pisifera Lindl. was
previously considered a Thuja, and C.
**TABLE 26. Examples of Trees of Medium Tolerance, Latitudes 20°–55°, Both Hemispheres**

<table>
<thead>
<tr>
<th>Eastern Hemisphere</th>
<th>Pacific Ocean</th>
<th>Western Hemisphere</th>
</tr>
</thead>
<tbody>
<tr>
<td>PINACEAE, n = 12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>White pines: PINUS peuce-griffithii-armandi</td>
<td>monticola-strobus</td>
<td></td>
</tr>
<tr>
<td>TAXODIACEAE-CUPRESSACEAE, x = 11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Incense cedars: LIBOCEDRUS</td>
<td>Taiwan</td>
<td>Sierra Nevada</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chile</td>
</tr>
<tr>
<td>Sequoias: (extinct) METASEQUOIA</td>
<td></td>
<td>SEQUOIA-TAXODIUM</td>
</tr>
<tr>
<td>Cypresses: CHAMAECYPARIS obtusa</td>
<td></td>
<td>nootkatensis</td>
</tr>
<tr>
<td>CUPRESSUS sempervirens</td>
<td></td>
<td>macrocarpa</td>
</tr>
<tr>
<td>Thuja: THUJA orientalis</td>
<td></td>
<td>plicata</td>
</tr>
<tr>
<td>FAGACEAE, Northern Hemisphere, n = 12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>White oaks: QUERCUS robur</td>
<td>crenata</td>
<td>lobata-alba</td>
</tr>
<tr>
<td>Chestnuts: CASTANEA sativa</td>
<td></td>
<td>americana</td>
</tr>
<tr>
<td>CASTANOPSISinus</td>
<td></td>
<td>chrysophylla</td>
</tr>
<tr>
<td>Beeches: FAGUS silvatica</td>
<td>Taiwan</td>
<td>grandifolia</td>
</tr>
<tr>
<td>NOTHOFAGUS, n = 13, Tasmania, New Zealand</td>
<td></td>
<td>Chile, Patagonia</td>
</tr>
<tr>
<td>MAGNOLIACEAE, x = 19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAGNOLIA obovata</td>
<td></td>
<td>grandiflora</td>
</tr>
<tr>
<td>TILIACEAE, x = 41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basswood: TILIA cordata</td>
<td>amurensis-japonica</td>
<td>americana</td>
</tr>
<tr>
<td>ACERACEAE, x = 13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maples: ACER platanoides</td>
<td>mono</td>
<td>macrophylla-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>saccharum</td>
</tr>
</tbody>
</table>

obtusa was shifted from Cupressus to Chamaecyparis. The European species is referred to as Cupressus sempervirens L.

Excessively detailed taxonomic studies have been even more misleading in presenting relationships between the incense cedars, the old genus Libocedrus, which spans the Pacific both north and south of the equator (Table 26). Based on differences in the arrangements of stomata and leaves, and disregarding the remarkable gross similarities, the genus has been split into five. The chromosome number is 11 in L. bidwilli Hook. f., and L. plumosa Sarg. of New Zealand (Hair and Beuzenberg, 1958), and also n = 11 in the Chilean L. chilensis Endl. (Hunziker, 1961) on the other side of the Pacific, evidence that this odd number of chromosomes persisted through epochs of time and wide spatial isolation.

The Sequoia-Taxodium group (Table 26) represents another 11-chromosome conifer cluster. It is now split into the genera Cryptomeria and Metasequoia on the west side of the Pacific, and Sequoia, Sequoiadendron, and Taxodium on the east side. Miki (1965) showed that counterparts of Sequoia and Sequoiadendron are present in Tertiary beds in Japan. In past ages Sequoia relatives covered north latitudes between 34° and 58° around the earth (Florin, 1963: 201 and maps 28–30). Embryologies differ in this primitive conifer group but one senses great similarity between the assumed genera of this cluster although experimental evidence is lacking.
Among the broad-leaved trees of the medium latitude and altitude the members of the beech family, 
*Fagaceae*, stand out (Table 26). The oak genus, *Quercus*, circles the earth at medium northern latitudes, although a few species spill over to the vicinity of the equator and beyond at high altitudes in Central America and Indonesia (Oersted, 1871). Applying conservative classification, between 300 and 500 species are known in the genus, which is divided into six to eight subgenera, each having several subsections.

Hybridization is common between oak species around the world, but the genus contains many genetically separate species clusters. The clusters are deciduous in the northern parts of the oak belt, and evergreen in the south.

The chromosome number is uniformly \( n = 12 \) in about 58 species of *Quercus*, *Castanea*, *Castanopsis*, and *Fagus*, a situation similar to that of the pine species.

The white oaks in Table 26 exemplify one widely distributed deciduous cluster of oak species, namely *Quercus alba* L. of eastern North America, *Q. lobata* Née of California, *Q. crispula* Née of Japan, and *Q. robur* L. of Europe. Members of distinct oak clusters may grow adjacent without intercrossing.

The beeches, *Fagus*, have probably only one species cluster around the earth, but hybrids between the European *Fagus sylvestra* L. and the North American *F. grandifolia* Ehrh. are being produced by shipped pollen at the Hörsholm Arboretum in Denmark, which suggests a close genetic relationship.

The southern hemisphere genus *Nothofagus* is morphologically a close counterpart to *Fagus*. It crosses the Pacific between South America, New Zealand, and Australia. The southern hemisphere beeches differ from *Fagus* in having 13 instead of 12 pairs of chromosomes as seen in the genetically closely related red and mountain beeches *N. menziesii* (Armstrong and Wylie, 1963). *Nothofagus* possibly differentiated chromosomally from the northern beeches very long ago, but their evolutionary history is still unknown.

Other genera that cross the Pacific and have closely related species on both sides are the magnolias, the basswoods, and the maples. Their chromosome numbers follow unusual odd basic series that remain the same on both sides of the Pacific (Table 26). Such innate constancy, correlated with the generic morphologies and persistence within the latitudinal belt on both sides, testifies to coherence in heredity that persisted through geologic ages as plant families moved around the world.

**Low latitude tree species.** The greatest number of the world's recognized tree species, possibly some 60,000 to 80,000, have remained within a belt of some 50° in latitude on both sides of the equator. They are here because most of them are tropical and unable to tolerate even occasional mild frosts. This characteristic contrasts sharply with the trees of the high latitude group that exist in regions that are subjected to frosts eight to nine months of the year.

The low latitude tree species occupy the forests on both sides of the Pacific and the oceanic islands, extending through southeastern Asia to Africa and to both of the Americas, including the Caribbean Islands. Many families and orders are restricted to these latitudes. Those families that extend into the temperate zones have evolved genera of different kinds within the temperate zones.

Within the low latitudes the tree lines are at remarkably low altitudes (Clausen, 1963), and species native
there may show apparent damage even before the freezing point of water has been reached.

At the equator only very few tree species occur at altitudes beyond 3000 meters (Table 27). Such trees include certain Araucarias and Podocarps, Drimys, which is remotely related to the buttercups and magnolias; the so-called pepper tree, Schinus molle, of the Anacardiaceae; the camphor tree, Cinchona, of the Peruvian cloud forests; and Polylepis leptophylla, a tree-like plant of the rose family, throughout the drier Andes. The base of Table 27 indicates also that typical mid-latitude tree families miss the low latitudes completely.

The many thousands of tree species that occur only below the frost line suggest that there is no lack of materials from which trees capable of surviving at high altitude might have evolved. Characters used in plant classification are generally morphological. One must therefore conclude that physiological adjustments to climatic zones are somehow tied to the morphology of the major plant orders and families. These gross adjustments are retained as members of such families migrate across oceans.

The genus Eugenia of the myrtle family provides an illuminating example. It has evolved about 1,000 species that cover both of the Americas at low latitudes—besides 100 in the Caribbean Islands, 45 in the Pacific Islands, about 34 in Australia and Southeast Asia, 150 in the Indies, and 45 in Africa. It has been able to move across oceans, but has not extended

### Table 27. Examples of Trees of Low Tolerance, Low Latitudes, 25°N to 25°S
(Major Portion of World’s Trees, Possibly 50,000 to 70,000 Species)

<table>
<thead>
<tr>
<th>Eastern Hemisphere</th>
<th>Pacific Ocean</th>
<th>Western Hemisphere</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARAUCARIACEAE, n = 13</td>
<td></td>
<td>Peru, Brazil</td>
</tr>
<tr>
<td>ARAUCARIA species in Australia, New Zealand</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PODOCARPACEAE</td>
<td></td>
<td>Peru, Brazil</td>
</tr>
<tr>
<td>Podocarps: PODOCARPUS-DACRYDIUM, New Guinea to 3600 m, New Zealand</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RANALES, buttercup-magnolia relatives; DRIMYS, Tasmania, New Guinea to 4000 m</td>
<td></td>
<td>Peru, Brazil</td>
</tr>
<tr>
<td>ROSALES</td>
<td></td>
<td>Peru, Brazil</td>
</tr>
<tr>
<td>Cunonias: WEINMANNIA, New Zealand, Hawaii</td>
<td></td>
<td>Andes, Brazil</td>
</tr>
<tr>
<td>Rosaceae, POLYLEPIS leptophylla</td>
<td></td>
<td>dry Andean, 3000 m</td>
</tr>
<tr>
<td>Legumes, SOPHORA, New Zealand, Hawaii, 3000 m</td>
<td></td>
<td>high Andes</td>
</tr>
<tr>
<td>PARIETALES, camellia relatives</td>
<td></td>
<td>Brazil, tree line 1900 m</td>
</tr>
<tr>
<td>TERNSTROEMIA, CLUSIA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MYRTALES, myrtles, melastomes</td>
<td></td>
<td>Mexico, Andes, Brazil</td>
</tr>
<tr>
<td>EUGENIA, ca. 1000 species</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Africa, India, Indonesia, Australia, Pacific Islands</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RUBIALES, coffee and camphor relatives</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COFFEA, Congo, Ethiopia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CINCHONA</td>
<td></td>
<td>Peruvian Andes, 3700 m</td>
</tr>
</tbody>
</table>

Missing at low latitudes: willows, walnuts, birches, alders, oaks, beeches, chestnuts.
into cooler latitudes. The large number of species indicates a high degree of morphological variability, but this and many other genera appear to be trapped climatically.

In the discussion above it has been necessary to amplify somewhat the concept of vegetation in relation to latitudinal and altitudinal zones. Vegetations overlap, permitting members of the hardiest group to adjust to habitats within the medium latitude zone by occupying the higher altitudes or cooler local habitats. Similar overlapping is found between the medium and low latitude plants, so that certain species of a cluster may spill over to a different belt.

It is necessary in studies of this kind to apply a relatively conservative concept of species and genera. Modern tendencies to split species, genera, and families obscure the broad, deep-seated physiological and ecological relationships that are built into the various heredity patterns and hold the natural entities together even on their worldwide migrations.

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STAFF ACTIVITIES

Several botany classes from the University of California at Davis and at Santa Cruz, and from Stanford, visited the laboratory and the mountain stations during the year. Demonstrations and talks were given by the Experimental Taxonomy group. The Bay Area Biosystematists group met at the laboratory in January.

The greenhouses and the garden at the laboratory were used by Dr. Peter Raven of Stanford for his studies of the evening primrose family. Mrs. Mary Mantuani, a graduate student at Duke University, started a comparative study of water relations of ecological races of *Solidago* at Mather.

The Northern California Photobiology and Photochemistry Group visited the Department in January and heard short talks from each of the Photosynthesis group. Dr. Brown served as vice president of the Group this year.

Dr. Jens Clausen attended the Eleventh Pacific Science Congress in Tokyo. He also attended the International Symposium on Plant Biosystematics at Tokyo University, arranged by the International Organization of Plant Biosystematics. While visiting with friends and colleagues of up to 40 years' standing, Clausen was introduced to Japanese investigators in cytogenetics, ecology, taxonomy, and plant breeding at the universities in Tokyo, Kyoto, and Sapporo, and the National Institute of Genetics at Misima.

Studies of the Japanese forest vegetation were made possible by a special traveling symposium September 4 to 9 through the Japan Alps. The symposium subject was "The Ecological Basis of Nature Conservation in Alpine and Subalpine Zones." About 70 biologists from different countries participated in the symposium organized by Professor Makoto Numata of Chiba University. After the tour, Dr. Clausen and Professor Misao Tatewaki of the University of Hokkaido studied the forests of Hokkaido for about a week.

On September 17 and 18 Dr. Shigeru Chiba, Director of the Kuriyama Institute for Improvement in Forests, and Dr. Clausen discussed principles of forest genetics. Dr. Chiba showed the modern Institute and the extensive experiment grounds owned and operated by the Oji Paper Company.

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Anderson, Jan M., *see also* Fork, David C.


Fork, David C., *see also* Amesz, Jan.


French, C. S. (Inventor), and Charlton M. Lewis (Registered Patent Agent), Plane Table Plotter, a map making device, U.S. Patent #3,302,293, 1967.


French, C. S., *see also* Pickett, J. M.


Holmgren, Paul, *see also* Björkman, Olle.

de Kouchkovsky, Yaroslav, *see also* Fork, David C.

Lewis, Charlton M., *see also* French, C. S.


**SPEECHES**

Amesz, Jan, Spectrophotometric measurements of reactions of photosynthetic intermediates, Seminar, Department of Botany, University of Illinois, Urbana, Illinois, October 11, 1966.

Björkman, Olle, Photosynthetic adaptability to light in plants native to shaded and exposed environments, Seminar, Botany Department, University of California, Davis, California, December 6, 1966.

Björkman, Olle, Adaptation of photosynthesis to light intensity in sun and shade ecotypes of *Solidago virgaurea*, Seminar, Department of Biological Sciences, Simon Fraser University, Burnaby, B. C., Canada, January 26, 1967.

Björkman, Olle, Ecological adaptation of photosynthesis, Seminar, Department of Biology, University of California, Santa Cruz, California, May 17, 1967.

Brown, Jeanette S., Chlorophyll fluorescence emission of a diatom, Pacific Slope Biochemical Conference, University of California, Davis, California, June 17, 1967.


Clausen, Jens, Species clusters in tree vegetations around the Pacific, Eleventh Pacific Science Congress, Tokyo, Japan, August 27, 1966.

Fork, David C., Studies on components in the electron-transport chain of photosynthesis, Seminar, Botany Department, University of California, Davis, California, November 22, 1966.

French, C. S., Forms of chlorophyll in algae and mutants, Pacific Slope Biochemical Conference, University of California, Davis, California, June 17, 1967.

Hiesey, William M., Genetic vs. environmental effects on photosynthetic rate of clones of contrasting climatic races of *Mimulus*, American Society of Plant Physiologists, AIBS Meeting, College Park, Maryland, August 17, 1966.

Hiesey, William M., Cytogenetic and eco-physiological studies in *Mimulus*, Seminar, Department of Biology, California Institute of Technology, Pasadena, California, May 16, 1967.

Michel-Wolwertz, M. R., The different chlorophylls extracted from plants, Pacific Slope Biochemical Conference, University of California, Davis, California, June 17, 1967.

Nobs, Malcolm, Cytogenetics and climatic selection in *Mimulus*, Seminar, Department of Genetics, University of California, Davis, California, October 27, 1966.
PERSONNEL

Biochemical Investigations

Staff: C. Stacy French, Director; Jeanette S. Brown, David C. Fork; James H. C. Smith, Emeritus

Carnegie Corporation Fellows: Jan Amesz,1 Ulrich W. Heber2
Institution Research Fellows: Marie-Rose Michel-Wolwertz,3 Jean-Marie Michel,4 James M. Pickett5
Technical Assistants: Alan de Schweinitz,6 Marion A. Koerper,7 Mark C. Lawrence, Suzanne Parmelee,8 William T. Rhodes9
Part-time Laboratory Helpers: Christine M. Anderson,10 Stephen J. Fulder11

Experimental Taxonomy

Staff: Jens C. Clausen, Emeritus; Olle Björkman, William M. Hiesey, Malcolm A. Nobs
Institution Research Fellow: Eckard W. Gauhl12
Technical Assistants: Frank Nicholson, Pamela Radford13
Summer Research Assistants: Oakley Shields,14 Stephen G. Wood15
Clerical Assistant: Marylee Eldredge
Accountant-Secretaries: Richard F. Gill,22 Clara K. Baker23
General Department Secretary: Lena R. Barton
Mechanical Engineer: Richard W. Hart Custodian: Jan Kowalik

1 Through April 21, 1967. From Biophysical Laboratory, State University, Leiden, The Netherlands.
2 From May 3, 1967. From Botanisches Institut, Universität Düsseldorf, Bonn, Germany.
3 From Jan. 4, 1967. From Centre de Recherches de Gorse, Belgium.
4 From Jan. 4, 1967. From Centre de Recherches de Gorse, Belgium.
5 From Oct. 1, 1965. From Department of Zoology, University of Texas, Austin.
7 To September 28, 1966.
9 To September 30, 1966.
10 Through August 19, 1966.
PLATES
Plate 1. Growth of bean seedlings in air containing low versus normal oxygen concentration. Above: Plants at the beginning of a growth experiment (top row) and after 6 days in normal air with 21% O₂ (second row from the top). Below: Corresponding matched plants before and after growth in 2.5% O₂.
**Plate 1**

*Department of Plant Biology*

**Air**
- At start
- After 6 days

**Low O\(_2\)**
- At start
- After 6 days