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Department of Plant Biology

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Department of Plant Biology

Stanford, California

C. Stacy French
Director
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Carnegie Institution Year Book 67, 1967-1968
INTRODUCTION

The first International Congress of Photosynthesis Research was held in June 1968, in Germany. It gave an excellent opportunity to survey the present state of the subject and to see the relations between the work of this laboratory and that in progress elsewhere. This full-scale Congress developed from a smaller group previously called the Western European Conference on Photosynthesis that had met twice before.

The establishment of a Congress, to convene at three-year intervals, focuses the efforts of people from many of the traditional academic disciplines on a problem that requires investigation from widely divergent viewpoints. It also represents the evolutionary culmination of the long history of research in photosynthesis. A hundred years ago photosynthesis was primarily of interest to botanists, trained in the classification of plants. Slowly the field of plant physiology developed and the knowledge of how plants convert carbon dioxide into food with the energy of sunlight became one of its major subdivisions. As the basic questions about the process became more clearly formulated the more easily definable problems were taken up by chemists and physicists, usually with the expectation of a quick breakthrough from the application of their more sharply defined methods of experimentation and interpretation. The initial over-simplification characteristic of much of this phase in the history of our subject has, however, led to a sharper formulation of known facts and to a more precise delineation of some significant though poorly understood questions. We seem to have passed through the period of naïveté in the application of the more rigorous disciplines to biological problems. Rather than being considered a limitation on precise experimentation, the diversity of species and of physiological states of living plants is now being exploited. This is done by selection of species particularly well adapted to specific kinds of experimentation, by specially induced and selected mutants, and by controlled preconditioning of the plant material. Excellence of chemical and physical procedures combined with weak biology, or the converse situation, is no longer a characteristic of work in our field.

At this Congress, a major fraction of the work reported was on the nature of the electron transport chain whereby two consecutive photochemical reactions, driven by different forms of chlorophyll, release oxygen from water and form reducing power. The attempt to separate those parts of chloroplasts that are responsible for each of the two separate photochemical reactions is being widely pursued. This year at the Department, Drs. Michel and Dr. Michel-Wolwertz, Institution Research Fellows from Belgium, have improved the still primitive art of chloroplast fractionation. Their separation methods avoid the need of adding detergents which, though widely used, may damage the material. The relative activities for two photochemical reactions of their different chloroplast fractions contrasted greatly, and they found characteristic differences in the absorption spectra of comparable fractions from several species of plants.

The absorption spectra of chlorophyll complexes in fractions prepared from chloroplasts that were nearly specific for system 1 or system 2 activity differed greatly from each other in the region from 680 to 715 nm. The fraction giving high system 1 activity had high absorption in that part of the spectrum. A striking parallelism to this situation was found in isolated but not fractionated chloroplast particles from mutant leaves. Drs. Fork, Heber, and Michel-Wolwertz found far higher absorption from 680 to 715 nm in a mutant that gave only system 1 activity than in another mutant.
with only system 2 activity. The difference between these spectra presumably represents the absorbance of a specific system 1 pigment.

Eventually we may hope for improvements in the technique for measuring action spectra of specific reactions driven by the separated particles to a precision that is routine in recording absorption spectra. Then it will be possible to characterize definitely the specific forms of chlorophyll that are responsible for the activity of each system.

The function of the carotenoids is one of the persistent questions about photosynthesis that seem to have a variety of answers. All plants that do photosynthesis have these yellow pigments. Some but not all of the many carotenoids may act as light absorbers for the reaction. They are believed to work by passing on their absorbed energy to chlorophyll. Another recognized function is that of an internal light filter whereby carotenoids protect chlorophyll from bleaching by blue light. Furthermore, the reversible oxygenation of particular carotenoids is thought by some investigators to be a part of the oxygen-evolving step of photosynthesis. In last year's report Drs. Fork and Amesz described rapid changes of light absorption by carotenoids in red and in brown algae. This year Dr. Fork gives the results of a detailed study of carotenoid changes in a yellow-green alga that is particularly suitable because it lacks chlorophyll b. Activation of either of the two photochemical systems of this alga, *Botrydiopsis*, gave identical carotenoid changes. Since the changes were in the same direction when driven by either system, the carotenoid responsible for the spectral changes cannot be located between the two photochemical reactions. There are two types of absorbance changes, one slow, the other rapid; but since they have identical spectra, the same carotenoid pigment is activated by both of the photosystems.

Another part of photosynthesis extensively discussed at the Congress was the effect of light on CO₂ evolution by plants in an atmosphere with oxygen but devoid of CO₂. This photorespiration is another aspect of O₂ uptake by certain steps in the process of photosynthesis, long a subject of interest to our group.

The overall result of photosynthesis is evolution of oxygen from water followed by utilization of the remainder as a source of power for making reduced compounds and high-energy phosphate bonds. Nevertheless, oxygen is taken up at several different places in the photosynthetic system of plants and this utilization of some oxygen may be essential to the normal functioning of the mechanism. The uptake of oxygen may well have significance in adjusting the rates of some different steps by a feedback mechanism. The importance of this effect in understanding the details of the photosynthetic process is self-evident, and investigations of the oxygen uptake have been made from many different viewpoints. Two different aspects of the subject are discussed in this year's report. Professor Heber, a Visiting Investigator from Düsseldorf, describes the interaction of oxygen with the electron transport system at a point near photosystem 1 but located on the opposite side of that system from system 2 where oxygen is produced and where there are also other sites of oxygen uptake. The action spectra of light-induced oxygen consumption measured at the Department some years ago with different procedures by Dr. Vidaver and by Dr. Fork also related one site of oxygen uptake to system 1.

Studies on the inhibiting effect of oxygen on the rate of photosynthesis and growth of whole plants have been continued this year by Dr. Björkman. New experiments on the effects of oxygen concentration on the growth of plants in controlled environments confirm and extend findings reported last year on the enhancement of growth of some plants under low concentrations of oxygen (4% to 5%) as compared with normal air (21%). The new data also indicate that
there is an interaction between $O_2$ and $CO_2$ concentrations as measured by the degree of enhancement of growth of plants caused by low oxygen concentration.

An effect possibly of appreciable significance that is, however, not yet well understood was turned up this year by Dr. Brown. She found that small increases of temperature appreciably reduce the height of the absorption bands of chlorophyll in its natural complexes. If the temperature increase is small enough, the effect is reversible. A similar response, the one that led to these experiments, had been known for cytochrome, another protein complex. This high sensitivity to the temperature, fascinating in itself as a phenomenon, may eventually contribute to a better description of the way in which chlorophyll is bound to its carrier protein. The time course of the effect, the extent of this change for the various forms of chlorophyll, and the influence of temperature changes on the shape of the spectral bands offer promising areas for future work.

In the search for improved methods of chloroplast fractionation Dr. Michel-Wolwertz investigated the effects of enzymes that digest proteins or fats. The hope was to break some of the bonds between the protein carriers of the different forms of chlorophyll and thus to facilitate the separations. Molecular sieve resins were used for testing the separability of the partially hydrolyzed chloroplast fragments. No improvement in separability resulted. However, an enzyme, protease, was found to convert one form of chlorophyll $a$ to another. That such a conversion can take place by partial hydrolysis supports the idea that the longer wavelength forms of chlorophyll $a$ are aggregates of smaller chlorophyll protein complexes. The action spectra for various photochemical activities of chloroplast fragments partially digested by enzymes remain to be determined. Such experiments would tell whether the chlorophyll complex, whose absorption spectra change, also changes its activity.

One of the basic experiments leading to the realization that two separate photochemical reactions are used in photosynthesis, and that these reactions are driven by different forms of chlorophyll, was the demonstration of the enhancement effect. This effect, discovered by Robert Emerson, was that photosynthesis with two beams of light of different wavelengths is greater than the sum of photosynthesis for the two beams given separately. Dr. Eckhard Loos, a Research Fellow from Munich, had found, before coming to the laboratory, that the enhancement effect at high light intensity became smaller than the value predicted from measurements at low intensity if studied by the uptake of radioactive carbon dioxide. This year the experiments were repeated by measuring oxygen evolution instead of carbon dioxide uptake. In the recent oxygen experiments, however, the decline of activity at high intensity in different algae was attributable solely to the normal limitations of photosynthesis by non-photochemical reactions that approach a saturating rate at high light intensity.

A cable was installed this year connecting the laboratory to two of Stanford's large computer systems. One of these, ACME at the Medical School, is still an experimental enterprise. ACME operates as an on-line computer accepting data and programs and returning the computed results on an electrical typewriter in the laboratory. It is also intended to accept electrical data signals, either analogue or digital, directly from experimental equipment in the laboratory and perform on-line calculations or control operations. Thus measured data will be computed and plotted in the desired form on our own recorders during the experiments. The electrical data input systems for ACME are nearly completed for use with the measurement of light-induced absorption changes in photo-
synthetic plants and for digitizing recorded curves. Furthermore, the typewriter can be connected to Stanford's WYLBUR system for program and data input with convenient revision facilities to the large IBM 360/67 system. WYLBUR output computations and the graphs are produced at the Computation Center close to our laboratory. Much use of the WYLBUR system has been made this year for analysis of absorption spectra of the different types of chlorophyll.

The gift of a suitable program from the Shell Development Laboratory for use with the computer has made it possible to do many more and greatly improved analyses of absorption spectra. By such analysis of particularly significant new measurements we are continuing the attempt to determine the spectra of the naturally occurring forms of chlorophyll in chloroplast fractions and in certain algae. The objective is to find out how many such forms of chlorophyll exist, which of them are part of each of the two photochemical systems of photosynthesis, and to describe in detail the curves for each separate form of chlorophyll. Some progress has been made and it now appears that different forms of chlorophyll with the same wavelength peak may vary remarkably in the width of their absorption bands.

Continuing emphasis on the basic mechanisms underlying the evolution of higher plants has characterized the work of the Experimental Taxonomy group during the current year. The analysis and integration of the extensive data from the long-term cytogenetic, transplant, and physiological investigations on the Erythranthe section of *Mimulus*, begun in 1947, is progressing with the preparation for publication as Volume V of the Institution's monograph series *Experimental Studies on the Nature of Species*. This book is scheduled for completion in the coming year.

Dr. Björkman has developed a rapid method of measuring photosynthetic rates with an oxygen electrode in green tissues of liverworts. This method makes possible the study of rapid transient effects on photosynthesis and respiration during alternating light and dark periods, and of the effects of rapid changes in temperature, CO₂ and O₂ concentrations, and light intensities. It is also an effective instrument for comparative studies on ecological races.

In *Solanum dulcamara*, a European species of nightshade, Mr. Eckard Gaul has established the occurrence of distinct obligate shade forms of the species in contrast with other races that are capable of utilizing high light intensities efficiently in photosynthesis. This finding parallels the discovery by Björkman and Holmgren some years ago on *Solidago virgaurea*, the common European goldenrod. Mr. Gaul has also found that races of *Solanum* that occur in marshy sites in central Germany are strongly inhibited in photosynthetic rate when subjected to mild water stress in contrast with races native to dry areas along the Dalmatian coast in southeastern Europe whose photosynthetic rate is unaffected when grown under the same conditions.

Dr. J. H. Silsbury of the Waite Agricultural Research Institute at Adelaide, South Australia, spent six months at the Stanford laboratory as a Carnegie Fellow. His major objective was to study techniques of measurement of photosynthesis and to compare the photosynthetic characteristics of an agronomically important form of *Lolium rigidum*, an annual ryegrass from the Mediterranean region, with a form of the closely related *Lolium perenne*, a perennial grass from Algeria. Dr. Silsbury was on sabbatical leave from the Waite Institute and, after visiting other laboratories in the United States and in Europe, returned to Adelaide to pursue further comparative studies under controlled conditions of various agronomic strains.
GROWTH OF *Mimulus*, *Marchantia*, AND *Zea* UNDER DIFFERENT OXYGEN AND CARBON DIOXIDE LEVELS

Olle Björkman, Eckard Gauhl, William M. Hiesey, Frank Nicholson, and Malcolm A. Nobs

Last year we reported that the production of dry matter in *Mimulus cardinalis* and *Phaseolus vulgaris* was markedly enhanced when the oxygen concentration of the air surrounding the green parts of the plants was reduced from the normal level of 21% to only a few percent. These comparative experiments were made at a CO₂ concentration of normal air (approx. 0.03%). This year we have studied the effect of oxygen concentration on growth at lower and higher CO₂ concentrations as well. Clone 7211-4 of *Mimulus cardinalis*, TO1-10 of the liverwort *Marchantia polymorpha*, and corn seedlings *Zea mays*, Ferry-Morse hybrid 901, were used in these studies. As in the previous work, the roots of the higher plants were aerated in nutrient solution with normal air (21% O₂, 0.03% CO₂) so that only the composition of the atmosphere surrounding the shoots was varied. With *Marchantia* the entire plants were exposed to the different O₂ and CO₂ concentrations. The temperature in all cases was 25°C.

As reported elsewhere (*Year Book 65*, pp. 446–454, *Year Book 66*, pp. 220–228, *Year Book 67*, pp. 479–482) the rate of photosynthetic CO₂ uptake in *Mimulus* and *Marchantia* is markedly enhanced when the oxygen concentration of the air is reduced from 21% to a few percent, whereas in corn oxygen has little, if any, effect. Similarly, the carbon dioxide compensation point for photosynthesis (i.e., the CO₂ concentration at which there is no net uptake or release of CO₂ in the light) has a relatively high value in *Mimulus* and *Marchantia* (0.005–0.01% CO₂) in 21% O₂, whereas at an O₂ concentration of a few percent the CO₂ compensation point approaches zero. In corn the CO₂ compensation point is close to zero also in 21% O₂.

On the basis of this information one would predict that if *Mimulus* or *Marchantia* were kept at a CO₂ concentration approaching the CO₂ compensation point for photosynthesis under normal O₂ concentration, growth would be stopped. Reducing the O₂ concentration while maintaining the CO₂ concentration at the same low level would increase photosynthesis to a positive value and should thus enable the plants to grow. Corn, on the other hand, would have a positive photosynthetic rate even at 21% O₂ and at a low CO₂ concentration, and one would expect that it would grow also under these conditions.

As shown in Table 1 and Plates 1 and 2, the results of actual growth experiments support these predictions, based on photosynthetic characteristics. Under 21% O₂ and 0.011% CO₂ growth in *Mimulus* and *Marchantia* is almost entirely inhibited, whereas at this same CO₂ concentration but at 4% O₂, the plants are able to grow at a moderate rate. The growth rate of corn at 0.011% CO₂ was unaffected by the O₂ concentration: the increase in dry matter was the same at 21% and 4% O₂ and was close to the value for *Mimulus* under 4% O₂.

When CO₂ concentration is increased beyond the CO₂ compensation point for photosynthesis, dry matter production in *Mimulus* and *Marchantia* increases both under 21% and 4% O₂. At approximately normal air CO₂ concentration (0.032%), dry matter yield is about 90% and 50% greater in 4% O₂ than in 21% O₂ for *Mimulus* and *Marchantia*, respectively. When the CO₂ concentration is increased to about twice that of normal air (0.064%), dry matter production increases both under 21% and 4% O₂, but the increase is relatively greater under 21%. This results in a lower degree of
TABLE 1. Effect of O₂ Concentration on Dry Matter Production at Different CO₂ Concentrations

<table>
<thead>
<tr>
<th>% CO₂ Concentration</th>
<th>Dry Weight Increase in 10 Days, mg/plant</th>
<th>% Increase of Growth in 4% O₂ over 21%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4% O₂</td>
<td>21% O₂</td>
</tr>
<tr>
<td>Mimulus cardinalis, Jacksonville</td>
<td>150¹</td>
<td>&lt;10¹</td>
</tr>
<tr>
<td>0.011</td>
<td>1076²</td>
<td>565²</td>
</tr>
<tr>
<td>0.032</td>
<td>1144²</td>
<td>804²</td>
</tr>
<tr>
<td>Marchantia polymorpha, TO1-10</td>
<td>24</td>
<td>7</td>
</tr>
<tr>
<td>0.011</td>
<td>83</td>
<td>55</td>
</tr>
<tr>
<td>0.034</td>
<td>101</td>
<td>78</td>
</tr>
<tr>
<td>Zea mays, Ferry-Morse hybrid 901</td>
<td>196⁴</td>
<td>216⁴</td>
</tr>
<tr>
<td>0.011</td>
<td>1473⁵</td>
<td>1269⁵</td>
</tr>
</tbody>
</table>

¹ Difference between means, very highly significant: P < 0.01.
² Difference between means, highly significant: P < 0.05.
³ Difference between means, scarcely significant: 0.3 < P < 0.4.
⁴ Difference between means, not significant: P > 0.5.

enhancement by low O₂ concentration at high CO₂ concentration. Nevertheless, even at 0.064% CO₂, dry matter production in Mimulus and Marchantia is substantially higher when O₂ concentration is kept at 4% as compared with 21%.

Possibly the lower degree of enhancement of dry matter production by low O₂ at high as compared with normal CO₂ concentration reflects a decreasing effect of oxygen concentration on the rate of photosynthetic CO₂ uptake as the CO₂ level is increased. It should be pointed out, however, that these experiments were made under continuous light, a high CO₂ concentration, and at a temperature near the optimum for photosynthesis. Total photosynthesis is, therefore, very high, probably many times higher than in natural habitats. Under such conditions the capacity of growth processes other than photosynthesis may partially limit the rate of growth. If this is the case, an additional increase in the rate of photosynthesis would only be partially expressed in an increased dry matter production.

Comparative Physiological and Biochemical Studies on Marchantia from Habitats with Contrasting Temperatures

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

In our comparative studies on physiological and biochemical characteristics of contrasting ecological races of the same or related species we began work this year on the liverwort Marchantia polymorpha L. This species has characteristics that make it an especially effective experimental subject in conjunction with current work on species of higher plants. Our principal immediate objective is to compare clones of Marchantia from widely contrasting latitudes in a critical comparative analysis of their photosynthetic and respiratory responses as a function of temperature. The wealth of background information already available from earlier investigations that include taxonomy, cytology, and controlled growth studies facilitates our effort.

Marchantia polymorpha provides certain experimental advantages over species of higher green plants that make possible a wider choice of experimental
techniques to probe in depth with increased precision steps that underlie such complex functions as photosynthesis and respiration in genetically and ecologically diverse races. This species occurs from arctic polar regions to the equatorial tropics. The anatomical structure of *Marchantia*, especially the absence of stomata and complex vascular tissues and its compact growth of thallus tissues, simplifies kinetic studies of photosynthesis in vivo. Genetically identical material can easily be grown in quantity through vegetative propagation of single clones, either through gemmae or small pieces of thalli. The absence of protein-precipitating substances, and apparently also of other compounds that commonly act as enzyme inhibitors in plant extracts, is of great importance in biochemical work. Finally, close genetic manipulation of experimental materials can be realized since the thalli are haploid, have a relatively low chromosome number \((n=8)\), and male and female gametes are formed on different thalli.

During the summer of 1968 we plan to collect living *Marchantia* clones from the Point Barrow area in northernmost Alaska (71° N.L.) as well as from the equatorial Galapagos Islands. These clones together with those from near Point Reyes Peninsula (north of San Francisco, California, 38° N.L.) are to be used as materials in the contemplated work. This would include studies of the response of growth to a number of different temperature regimes in the laboratory, kinetic studies of photosynthesis and respiration, and studies on the extent to which photosynthetic and respiratory properties of the diverse clones can be modified by growing them under different temperature regimes. Some of the early work on the response of photosynthesis to temperature, light intensity and oxygen concentration of clone TO 1-10 from the Samuel P. Taylor State Park near the Point Reyes Peninsula in central coastal California, and on the effect on these characteristics of growing this clone under different temperatures, is reported below.

We plan also to compare the activities and kinetic properties of some selected enzymes that are known to be of key importance in the metabolism of green plants among the clones from the different habitats and experimental growing conditions. The enzymes chosen for these studies are carboxydismutase (ribulose-1, 5-diphosphate carboxylase), phosphoribulokinase, phospho (enol) pyruvate carboxylase and citrate synthase (condensing enzyme). Methods for the preparation of extracts of these enzymes from *Marchantia* tissues and various assay procedures are currently being tested.

**Effect of Temperature and Oxygen Concentration on Photosynthesis in Marchantia polymorpha**

*Olle Björkman and Eckard Gauhl*

As a first step in our comparative work on adaptation to temperature among *Marchantia polymorpha* clones from habitats with contrasting temperatures we have studied the effect of temperature on photosynthesis in clone TO1-10 originally from the Samuel P. Taylor State Park near the Point Reyes Peninsula in central coastal California. Photosynthesis measurements were made in the temperature range from 6° to 34°C on ramets previously grown at 10°, 20°, and 30°C.

As is also the case in the majority of higher plants investigated, the rate of photosynthetic CO₂ uptake in the liverwort *Marchantia polymorpha* is markedly inhibited by the oxygen in normal air (Fig. 1). There is evidence that the inhibition is caused by a reoxidation of one or several photosynthetic intermediates by molecular oxygen ("photorespiration"). The photosynthetic responses to temperature of the *Marchantia* clone were therefore also measured under an atmosphere with an oxygen content of 1% to 2%.
Fig. 1. Rate of apparent photosynthetic CO₂ uptake as a function of oxygen concentration in *Marchantia polymorpha* T01-10. [CO₂]: 0.032%; 22°C. The clone was grown at 4% oxygen (broken line) and 21% oxygen (solid line). The temperature for growth was 20°C.

The lower part of Fig. 2 shows the temperature curve for light-saturated photosynthesis in normal air (21% O₂, 0.032% CO₂). In this and in the following graphs the rate of CO₂ evolution in the dark has been added to the apparent rate of CO₂ uptake in the light. The rate of light-saturated photosynthesis shows a strong dependence on temperature and increases in a nearly linear fashion from 6° to 20°C. The temperature optimum occurs in the range 27° to 30°C regardless of the temperature at which the clone was previously grown.

The rate of photosynthesis on the basis of unit dry weight or thallus area is somewhat lower at all temperatures when the clone is grown at 10° as compared with 30°C. Expressed on the basis of soluble protein the rate at all temperatures is highest when the plant is grown at 30°, and lowest when grown at 10°C.

The apparent lack of a photosynthetic acclimation to low temperature in this *Marchantia* clone is consistent with its slow growth at 10°C, but contrasts with the results found in the higher plant species *Eucalyptus californica* and *Polygonum bistortoides* by Mooney and Shropshire, 1967. The latter species appear to possess great phenotypic plasticity in the photosynthetic characteristics since preconditioning to different temperatures for 24 hours greatly changes the temperature...
dependence of light-saturated photosynthesis.

The temperature dependence of light-saturated photosynthesis at 2% O₂ for the Marchantia clone TO1-10 is shown in the upper part of Fig. 2. The response differs considerably from that found in Solidago virgaurea and Mimulus cardinalis (Year Book 66, p. 222). In Marchantia the rate of photosynthesis shows a much stronger decrease in the range from 20°C down to 6°C than do the higher plants. Moreover, the enhancement by low oxygen is small, or even absent, at temperatures below 10°C in Marchantia. It does not reach its maximum value until the temperature exceeds 20°C, in contrast with Solidago and Mimulus which show little change in enhancement by low O₂ with temperature change. It is noteworthy, however, that the optimum temperature for the light-saturated rate of CO₂ uptake in Marchantia is not markedly affected by O₂ concentration but lies in the range 28°C to 31°C.

Fig. 2. Temperature dependence of light-saturated photosynthesis at low and normal oxygen in Marchantia polymorpha TO1-10 grown at 10°C, 20°C, and 30°C. [CO₂]: 0.032%.

Fig. 3. Lower part of curves for photosynthesis as a function of light intensity (665 nm) at low and normal oxygen for different temperatures. Marchantia polymorpha TO1-10 grown at 20°C.
both at 21% and 2% O₂. Also, low O₂ enhances the maximum rate of CO₂ uptake to about the same degree regardless of the temperature under which the clone was previously grown even though the temperature at which enhancement occurs appears to increase with increasing temperature for growth.

Possibly, the temperature dependence of the light-saturated rate of CO₂ uptake in Marchantia reflects an increasing rate of reoxidation of photosynthetic products with increasing temperature. This reoxidation would then be negligible at 6°C, and increase with temperature faster than does photosynthesis in the range 6°C to 20°C. This hypothesis, however, is weakened by the finding that the enhancement by low O₂ becomes less dependent on temperature as the light intensity is decreased (Figs. 3 and 4). In the range where photosynthesis is linearly dependent on light intensity, the enhancement of CO₂ uptake that takes place when oxygen concentration is reduced from 21% to 1% seems to be independent of temperature. In the temperature range investigated (6°C to 26°C), the apparent quantum yield was found to be 0.068 mole CO₂ per absorbed einstein in 21% O₂ and 0.094 in 1% O₂. Another interesting observation is that the rise in the rate of CO₂ uptake with time upon exposure to light following darkness is considerably faster in 1% than in 21% O₂ even at low temperature where the photosynthetic rate at steady-state is the same at both oxygen concentrations (Fig. 5).

One interpretation of these results is that oxygen acts as an electron acceptor in the dark reaction chain of photosynthesis at a site before the thermal step that determines the overall rate of light-saturated photosynthesis at low temperatures. Electrons would then be drained from the chain to oxygen even at low temperatures, but this would not affect the rate of CO₂ uptake so long as the rate of flow to the step limiting the overall rate exceeds the capacity of this step.

Reference

**Differential Photosynthetic Performance of Solanum Dulcamara Ecotypes from Shaded and Exposed Habitats**

Eckard Gaulh

*Solanum dulcamara* L. is widely distributed in many contrasting natural environments. Since it is found in open as
well as in densely shaded habitats, it is a suitable species for experimental studies on the differentiation of the photosynthetic machinery among plants native to contrasting light climates.

Several clones isolated from selected populations in exposed and shaded habitats were used in a comparative study of growth responses and photosynthetic characteristics under two different light intensities (11 x 10^4 and 24 x 10^5 ergs cm^-2 sec^-1, 400-700 nm). Two clones representing extremes with respect to natural habitats and also in responses to preconditioning to different light intensities were later chosen for more detailed studies.

Clone Fe 2 was originally collected from an open sand dune on Fehmarn Island off the Baltic coast of Germany. The leaves of this plant are twice as thick when grown in strong light, and have three layers of palisade cells as compared with one layer when grown in weak light. Clone Mb 1 originates from a reed-grass marsh near Münchbruch, south of Frankfurt. Its stems wind around stalks of Phragmites communis which form a dense, shady stand with only a few other species. Leaves of this clone are only slightly thicker when grown in strong as compared with weak light and develop only one or two layers of palisade cells.

Fig. 6 shows typical curves of photosynthetic rate as a function of light intensity for leaves of both clones grown under high and low light intensities. Leaves of clone Fe 2, when developed under strong light, are capable of markedly higher rates of CO_2 uptake at high light intensities than when developed under weak light. They also require higher light intensities to saturate photosynthesis. In contrast, the light-saturated photosynthetic rates of Mb 1 leaves are about the same regardless of whether the plant has been grown in strong or weak light. The somewhat less steep initial slope of the curve for leaves developed under strong light indicates a reduced efficiency of utilization of light of low intensities.

To compare further the capacity of these two clones to adjust to contrasting light intensities, plants were grown under a low light intensity and subsequently exposed to a higher intensity for different lengths of time. Only mature leaves, already fully expanded at the time of transfer to the high intensity growth chamber, were used for measurements of photosynthesis and of the content of chlorophyll and soluble protein. Light-saturated photosynthesis was measured in white light of 3 x 10^5 ergs cm^-2 sec^-1 (400-700 nm). At this intensity the rate in leaves of clone Mb 1 often declined with time during measurement, presumably as a result of photoinhibition.

To determine the photochemical capacity of the leaves, the rate of photosynthesis was measured in monochromatic light of low intensities in the range where photosynthesis is a linear function of light intensity. Monochromatic light was isolated by an interference filter (665 nm). The fractional absorption of light by the leaf was determined in an Ulbricht integrating sphere using the same interference filter. Quantum requirements were then calculated as einsteins absorbed per mole CO_2 taken up by the leaf. Following the photosynthesis measurements, the chlorophyll and protein contents of the same leaf were determined. Tables 2 and 3 show results from one experimental series with plants of both clones grown first under weak light and then transferred to strong light for periods ranging from one to six days.

As can be seen from Table 2, the chlorophyll content per unit leaf area of leaves of clone Mb 1 decreases considerably after transfer to strong light. On the other hand, no such decrease is found in leaves of clone Fe 2. Both clones increased in leaf thickness during the six days in strong light but to different degrees, i.e., about 25% in Mb 1 and 75% in Fe 2. This increase in thickness
Fig. 6. Apparent rate of photosynthesis as a function of light intensity of a shade (Mb 1) and a sun (Fe 2) clone of *Solanum dulcamara* grown under a high light intensity of \(11 \times 10^4\) ergs cm\(^{-2}\) sec\(^{-1}\) (solid circles) and a low intensity of \(24 \times 10^4\) ergs cm\(^{-2}\) sec\(^{-1}\) (open triangles).

<table>
<thead>
<tr>
<th>Light intensity, ergs cm(^{-2}) sec(^{-1}) x10(^{-3})</th>
<th>0</th>
<th>50</th>
<th>100</th>
<th>150</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clone Mb 1</strong></td>
<td><img src="image1.png" alt="Graph of Clone Mb 1" /></td>
<td><img src="image2.png" alt="Graph of Clone Mb 1" /></td>
<td><img src="image3.png" alt="Graph of Clone Mb 1" /></td>
<td><img src="image4.png" alt="Graph of Clone Mb 1" /></td>
</tr>
<tr>
<td><strong>Clone Fe 2</strong></td>
<td><img src="image1.png" alt="Graph of Clone Fe 2" /></td>
<td><img src="image2.png" alt="Graph of Clone Fe 2" /></td>
<td><img src="image3.png" alt="Graph of Clone Fe 2" /></td>
<td><img src="image4.png" alt="Graph of Clone Fe 2" /></td>
</tr>
</tbody>
</table>

TABLE 2. Quantum Requirement and Chlorophyll Content of Leaves of Two Clones of *Solanum dulcamara* Grown in Weak Light and After Transfer to Strong Light for One to Six Days

<table>
<thead>
<tr>
<th>Clone Mb 1 from Shaded Habitat</th>
<th>Clone Fe 2 from Exposed Habitat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorophyll mg dm(^{-2})</td>
<td>Quantum Requirement Einst. abs. per mole CO(_2)</td>
</tr>
<tr>
<td>From start</td>
<td>6.1</td>
</tr>
<tr>
<td>Days in strong light</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>5.5</td>
</tr>
<tr>
<td>2</td>
<td>5.1</td>
</tr>
<tr>
<td>3</td>
<td>4.5</td>
</tr>
<tr>
<td>4</td>
<td>4.4</td>
</tr>
<tr>
<td>5</td>
<td>3.6</td>
</tr>
<tr>
<td>6</td>
<td>3.4</td>
</tr>
</tbody>
</table>
could account for the slight increase in chlorophyll content per unit leaf area in Fe 2 leaves.

Leaves of clone Mb 1 show a continuing increase in quantum requirement for CO₂ fixation during the six-day period. This can be interpreted to be a result of inactivation of photochemical partial reactions within these leaves. Substantial damage to the photochemical apparatus already is evident the first day after transfer to the higher light intensity. This early increase in quantum requirement is accompanied by only a slight decrease in chlorophyll content which indicates that the chlorophyll destruction observed later is not a primary effect of photoinhibition. In contrast, no significant change in quantum requirement occurred in Fe 2 leaves during the same period.

Leaves of clone Fe 2 continuously increase their light-saturated photosynthesis to almost twice the original rate during six days in strong light (Table 3). Simultaneously, the concentration of soluble protein increases greatly. This suggests that the increase in light-saturated CO₂ uptake during the experimental period is caused by a synthesis of one or several photosynthetic enzymes. The light-saturated photosynthesis of clone Mb 1 leaves, by contrast, does not increase during exposure to strong light.

These leaves show no consistent increase in protein content.

These results demonstrate that within the species Solanum dulcamara, ecotypes have evolved that have specific physiological mechanisms to cope with the light climate prevailing in their different natural habitats.

In practically all known instances, shaded habitats of Solanum dulcamara are moist and some are wet all year. In contrast, some exposed habitats are very dry as, for example, the gravel mound in the Mediterranean maqui near Rovinj, Yugoslavia, where clone Yu 5 was found to grow actively during the dry summer season. In early experiments it was found that clone Sh 2, which grows in a shaded Alnus glutinosa swamp near Frankfurt-Schwanheim, Germany, showed no signs of photoinhibition when grown under high light intensity. Moreover, it was able to increase its light-saturated photosynthetic rate when exposed to high light intensity during growth.

In order to determine whether there is an interaction between light intensity and water stress in influencing the distribution of Solanum dulcamara ecotypes, comparative growth experiments were made with clones Sh 2 and Yu 5. The plants were grown outdoors in full sunlight and with identical nutrients. Some propagules of both clones were

| TABLE 3. Protein Content and Light-Saturated Photosynthetic Rate of Leaves of Two Clones of Solanum dulcamara Grown in Weak Light and After Transfer to Strong Light for One to Six Days |
|--------------------------------------------------|--------------------------------------------------|
| Clone Mb 1 from Shaded Habitat | Clone Fe 2 from Exposed Habitat |
| Max. CO₂ Uptake μmole dm⁻² min⁻¹ | Sol. Protein mg dm⁻² | Max. CO₂ Uptake μmole dm⁻² min⁻¹ | Sol. Protein mg dm⁻² |
| At start | | |
| 7.0 | 39 | 7.0 | 32 |
| 1 | 4.6 | 46 | 7.0 | 34 |
| 2 | 6.8 | 55 | 10.2 | 43 |
| 3 | 5.3 | 45 | 10.4 | 50 |
| 4 | 6.0 | 49 | 10.7 | 54 |
| 5 | 5.7 | 45 | 11.5 | 59 |
| 6 | 5.3 | 37 | 13.0 | 69 |

* Light intensity for photosynthesis measurements: 3 × 10⁶ ergs cm⁻² sec⁻¹ (400-700 nm); temperature: 24°C; [CO₂]: 0.03%.
given only one third of the normal water supply, just enough to prevent wilting.

Fig. 7 shows curves for photosynthetic rate as a function of light intensity for leaves of the two clones grown under dry and moist conditions. Although clone Yu 5 grows more slowly under dry than under moist conditions, its photosynthetic capacity is not affected by the water stress. It should be noted, however, that the photosynthetic rate is not very high for this clone under any condition.

Fig. 7. Apparent rate of photosynthesis as a function of light intensity for two clones of Solanum dulcamara grown in strong light. Water supply normal (solid circles) and one-third of normal (open triangles).
On the other hand, the growth of clone Sh 2 is extremely retarded under the same water stress, and its light-saturated photosynthetic rate is much lower as compared with propagules given an ample water supply.

The greatly reduced initial slope of the curve for the Sh 2 leaf grown under dry conditions and high light intensity indicates that severe damage has taken place in the photochemical system. Clone Sh 2 thus appears to be photolabile only under water stress, since it is highly efficient even when grown under strong light when sufficient water is available. Clone Yu 5, on the other hand, shows only a moderate photosynthetic rate in saturating light, but it is capable of maintaining this rate even under drought.

**Carboxydismutase Activity in Shade-Adapted and Sun-Adapted Species of Higher Plants**

*Olle Björkman*

The findings that light intensity for growth markedly influences the activity of the photosynthetic enzyme carboxydismutase in the same individual plant and that *Solidago virgaurea* ecotypes from sunny and shaded habitats differ in the activity of this enzyme when grown at a high light intensity prompted us to investigate the carboxydismutase activity among a number of sun and shade species grown in their natural habitats. A full account of this investigation has been published (Björkman, 1968).

The various species used in the investigation are listed in Table 4. The shade species are limited in natural distribution exclusively to the shaded floors of dense forests, the sun species to moist sunny locations. All are natives of California except *Plantago lanceolata*, which is a naturalized introduction from Europe. The habitats of all the plants used in the present study are situated within 40 km of the laboratory at Stanford. The shade species were collected on the floors of two redwood forests; the light intensity at plant level in these habitats on the average was only 1 to 4 percent of full sunlight.

Cell-free leaf extracts of the plants from sunny habitats that are capable of much higher rates of photosynthetic CO₂ fixation in vivo clearly have much higher activities of carboxydismutase than those of plants growing in deep shade. The enzyme activity expressed on the basis of unit leaf area is of the order of ten times higher in the sun than in the shade

<table>
<thead>
<tr>
<th>Species</th>
<th>Shade Species</th>
<th>Sun Species</th>
<th>Per dm² Leaf Area</th>
<th>Per g Fresh Tissue</th>
<th>Per mg Chlorophyll</th>
<th>Per mg Soluble Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenocaulon bicolor</td>
<td>2.0</td>
<td>23.0</td>
<td>2.0</td>
<td>7.6</td>
<td>2.38</td>
<td>0.43</td>
</tr>
<tr>
<td>Aralia californica</td>
<td>2.0</td>
<td>15.0</td>
<td>2.3</td>
<td>7.6</td>
<td>3.28</td>
<td>0.24</td>
</tr>
<tr>
<td>Disporum smithii</td>
<td>1.0</td>
<td>17.0</td>
<td>1.1</td>
<td>7.6</td>
<td>3.28</td>
<td>0.25</td>
</tr>
<tr>
<td>Trillium ovatum</td>
<td>2.0</td>
<td>10.0</td>
<td>1.7</td>
<td>4.1</td>
<td>1.89</td>
<td>0.26</td>
</tr>
<tr>
<td>Viola glabella</td>
<td>1.0</td>
<td>12.0</td>
<td>1.2</td>
<td>4.9</td>
<td>2.81</td>
<td>0.23</td>
</tr>
</tbody>
</table>

* [HCO₃] = 5 × 10⁻⁴ M. [RuDP] = 3 × 10⁻⁴ M. 23°C.
species. Since the sun species have a greater ratio of volume to area (almost 50%) than the shade species, the difference between the two groups becomes somewhat smaller when the activity is expressed on the basis of leaf volume or fresh weight. Even when fresh weight is used as the basis for comparison, however, the plants show a carboxydismutase activity several times higher than the shade plants, and there is a strikingly parallel variation in the light-saturated rate of CO$_2$ fixation in vivo and the rate of CO$_2$ fixation by the enzyme in vitro among the species for which both were measured.

The chlorophyll content per unit weight of fresh leaves is higher in the shade species than in the sun species, whereas the opposite is true of the soluble protein content. Consequently, the difference in carboxydismutase activity between the two groups becomes greater when expressed on the basis of chlorophyll than it is when expressed on the basis of fresh weight, but considerably smaller when expressed on the basis of soluble protein. It is noteworthy in this connection that carboxydismutase probably accounts for a large fraction of the total soluble protein. This would, of course, tend to reduce differences in the activity of this enzyme when soluble protein is used as the basis for comparison rather than, for example, fresh weight.

The higher chlorophyll content but lower carboxydismutase activity in the shade as compared with the sun plants is of particular interest from an ecological viewpoint since it lends support to the idea that the fraction of the available chemical energy used for the synthesis of components determining the efficiency of light absorption in relation to the fraction used for the synthesis of components that determine the capacity of enzymic steps is larger among the shade plants than among the sun plants.

It seems likely that the low carboxydismutase activity in the shade plants is at least partly responsible for their low light-saturated rates of photosynthesis. Although it can be expected on theoretical grounds that shade plants have a low activity of other enzymes in addition to carboxydismutase, no direct measurements of enzyme activity supporting this supposition have as yet been made. There is, however, evidence that shade species have greater resistance to gas diffusion than do sun plants. This also would be expected to result in a lower light-saturated photosynthetic rate in the shade plants.

Hatch and co-workers have provided evidence for the operation of a C$_4$-dicarboxylic acid pathway for photosynthesis in certain monocotyledons, including many tropical grasses, but not in several dicotyledons (Hatch, Slack, and Johnson, 1967; Slack and Hatch, 1967). The species in which this pathway was found to be operative had low carboxydismutase activity but high levels of phosphopyruvate carboxylase. Very recently, Johnson and Hatch (1968) reported that the pathway operates also in the dicotyledonous genera Amaranthus and Atriplex. It is very interesting that the Atriplex species used by Johnson and Hatch, A. semibaccata, showed a high level of phosphopyruvate carboxylase but a very low level of carboxydismutase, whereas the Atriplex species used in the present study, A. patula var. hastata had a high carboxydismutase activity, indeed the highest among the species investigated. Apparently, therefore, differentiation in photosynthetic pathway has taken place within the same genus.

References

PHOTOSYNTHESIS OF AN AMPHIPLOID Mimulus IN COMPARISON WITH ITS PROGENITORS

William M. Hie.sey, Malcolm A. Nobs and Olle Björkman

The important evolutionary role played by amphiploidy in the synthesis of new species from ancestral forms of restricted geographical and ecological distribution has been recognized for some time (Clausen, Keck, and Hiesey, 1945). Frequently amphiploids are successful in inhabiting new environments not occupied by their parent species. The physiological basis for the evolutionary success of such amphiploids has been a matter of speculation.

A unique opportunity for comparing the photosynthetic performance of an interspecific tetraploid hybrid derivative with its markedly distinct diploid parent species was afforded by the production of an amphiploid between M. lewisii and M. nelsonii described in Year Book 65, pp. 468-471. The F1 hybrid between these species is normally diploid, like both parents, which have 8 pairs of chromosomes. This hybrid is highly sterile, but a tetraploid sector of one F1 individual having 16 pairs of chromosomes yielded viable seeds giving rise to a vigorous, fertile, self-perpetuating amphiploid population.

Clones of the original parental plants and of the diploid and tetraploid sectors of the F1, together with an amphiploid F2 individual, were grown as vegetative propagules under identical conditions in growth cabinets. During the growth period prior to the photosynthetic measurements, the light intensity was maintained at approximately 100,000 ergs cm⁻² sec⁻¹, the photoperiod was held at 16 hours, and day and night temperatures at 22° and 15°C, respectively. The plants were grown in perlite supplied with nutrient solution.

Light saturation curves were determined on attached leaves of intact plants with the apparatus and methods described in Year Book 63, pp. 430-431, and Year Book 65, pp. 461-468. The curve for the parental M. lewisii, clone 7405-4, originally from near our Timberline station at 10,500 feet elevation in the Sierra Nevada of California, is shown in Fig. 8. It is compared with that of M. nelsonii, clone 7422-12, originally from near El Salto, in the highlands of Central Mexico, and of the F2 amphiploid clone 7606-8.

All three clones have essentially identical curves at low light intensities in the nearly linear range 0 to 30,000 ergs cm⁻² sec⁻¹. As light-saturating intensities are approached, the curves diverge and show differences both in the light intensity required for saturation and in the maximum light-saturated rate attained. The M. lewisii clone saturates at a lower light intensity than the clone of M. nelsonii or the amphiploid. The amphiploid has a significantly higher light-saturated rate than either parent, although the F1 progenitor on both the diploid and tetraploid levels have rates that are intermediate between the parents (Table 5). The enhanced vigor of growth

| TABLE 5. Light-Saturated Rate of Photosynthesis of an Amphiploid Mimulus and of its Progenitors at Three Different Temperatures |
|-----------------|-----------------|-----------------|
|                 | Approximate Light-Saturation | Light-Saturated Photosynthetic Rate at 22°C, mg CO₂ dm⁻² hr⁻¹ | Photosynthetic Rate, % of Maximum |
|                 | Intensity, ergs cm⁻² sec⁻¹ |               | 10°C | 22°C | 38°C |
| 7405-4, M. lewisii P₁, n = 8 | 225,000 | 26.3 | 83.0 | 100.0 | 73.8 |
| 7422-12, M. nelsonii P₁, n = 8 | 325,000 | 31.3 | 82.5 | 93.6 | 78.8 |
| 7540-2-2x, F₁ Hybrid, n = 8 | 325,000 | 26.4 | 76.5 | 95.0 | 45.0 |
| 7540-2-4x, F₁ Hybrid, n = 16 | 325,000 | 30.0 | 85.3 | 91.0 | 37.4 |
| 7606-8, Amphiploid, n = 16 | 325,000 | 33.4 | 85.0 | 98.5 | 23.4 |

* [CO₂]: 0.03%.
Fig. 8. Photosynthetic rates of *M. lewisii*, *M. nelsonii*, and their tetraploid amphiploid derivative as a function of light intensity (600-700 nm).

of the amphiploid, as compared with the parents when all are grown under identical conditions, may therefore be due at least in part to the higher light-saturated photosynthetic rate.

The temperature dependence of net photosynthetic rate was likewise determined at a constant light intensity of 175,000 ergs cm⁻² sec⁻¹ in the wavelength range between 600 and 700 nm. Leaf temperatures were monitored during the measurements with miniature thermocouples in direct contact with the under leaf surfaces. Fig. 9 shows curves for the same three clones depicted in Fig. 8.

The results from these measurements reveal (1) that the temperature dependence curves for the ecologically and morphologically diverse parental clones *M. lewisii* and *M. nelsonii* are remarkably similar and surprisingly flat over the range 10° to 38°C, showing a high degree of tolerance over this range; and (2) that the amphiploid derivative, which has a higher photosynthetic rate than either parent over the cooler temperature ranges, is much more sharply inhibited in rate at temperatures above 25°C than is either parent. Curves of a general shape similar to the amphiploid were determined at both the diploid and tetraploid levels. The relative photosynthetic rates listed in the right-hand columns of Table 5 show the drop at high temperatures of the hybrid derivatives in comparison with the parental species.

To our knowledge, these are the first data on comparative photosynthetic rates of an amphiploid with its precisely
known parental individuals and the immediate progenitor individual plant on both the diploid and tetraploid levels. The measurements reflect the interaction of genomes of two taxonomically and ecologically distinct species on the functioning of a complex physiological character.

These data on *Mimulus* support the conclusion that the process of genome addition of two species through amphiploidy may alter basic physiological functions significantly. Such interactions at present cannot be explained in simple terms.

**References**


**VARIATION AMONG RYEGRASSES IN RATES OF APPARENT PHOTOSYNTHESIS**

J. H. Silsbury

The variation in rates of photosynthesis among individual plants and among plant populations is of considerable interest from the viewpoint of both ecology and plant productivity. Studies on the basic photosynthetic characteristics of ecological races of *Mimulus* and *Solidago* reported in this and previous Year Books show not only that such races differ in photosynthetic rates, but also that this attribute is inherited and probably has ecological significance.

Currently agricultural scientists are paying increased attention to the utilization of light energy by crop plants. Knowledge of basic differences in photosynthetic ability between species and cultivars could be of considerable value in improving crop production. Although
it is known that widely contrasting crop plants such as corn, wheat, sugar cane, and cotton differ in light-saturated rates of photosynthesis, attempts to demonstrate such differences among closely related species or varieties of cultivated plants have met only partial success.

*Lolium rigidum* is an annual ryegrass of predominantly Mediterranean distribution. *L. perenne* var. *Medea* is a perennial ryegrass derived from a summer-dormant form of *L. perenne* from Algeria. *L. rigidum* and *L. perenne* var. *Medea* were chosen for study because they are known to differ in both their absolute and relative growth rates. Rates of photosynthesis were determined with the techniques described in *Year Book* 63, pp. 430-431 and *Year Book* 65, pp. 461-468. In these measurements, portions of attached leaves 5.5 cm in length were used. Responses to different light intensities (0–3 × 10⁵ ergs cm⁻² sec⁻¹) and temperatures (10°–40°C) were studied for several leaves of individual plants in order to evaluate the range of performance of that plant as a whole and in relation to different plants within the population. It was found that light-saturated rates of photosynthesis decreased when leaves began to senesce and were lower in the proximal as compared with the distal portions of the leaf. Poor mineral nutrition also resulted in reduced rates.

Plants were grown in pots with a favorable nutrient regime both outdoors and in a glasshouse under a range of light environments. The photosynthesis–light intensity curves of both species reflect the light regime under which the plants had been grown. Leaves grown under high light (about 3 × 10⁵ ergs cm⁻² sec⁻¹) had higher light-saturated rates than leaves grown under low light intensity (about 2 × 10⁴ ergs cm⁻² sec⁻¹). However, the differences in *Lolium* are small in comparison with similar effects shown by other species.

In *Lolium rigidum* there was evidence that leaves grown under low light utilize low light intensities more efficiently than leaves grown under high light. Also it appears that high-light-grown leaves of *L. perenne* are more efficient at low light intensities than are those of *L. rigidum*.

The above results indicate that light-saturated rates of photosynthesis are quite comparable in the two species. However, it was noted that the rates varied from leaf to leaf, a greater rate generally being observed the smaller the specific leaf area (leaf area per unit leaf dry weight). The light-saturated rates of photosynthesis of a mature leaf of a number of different plants of each species grown under a range of light intensities were therefore determined, and the rate plotted against the specific leaf area (S.L.A.) of that portion enclosed in the leaf chamber. The results show that for each species, a decline in light-saturated photosynthesis is associated with an increase in S.L.A.; there is some residual variation about a regression line linking the two attributes. For a given S.L.A., *Lolium rigidum* tended to have a slightly higher photosynthetic rate than *L. perenne*.

An attempt was made to resolve these differences more clearly by measuring the light-saturated rates of the fifth leaf on the main stem of six seedling plants of each species grown under comparable conditions in the greenhouse. The average rate per unit leaf area of the six leaves of *L. perenne* was higher than that of *L. rigidum*, but the S.L.A. of the latter was lower. Plots of photosynthesis against S.L.A. again show a tendency for leaves of *L. rigidum* to have a higher rate of photosynthesis for the same S.L.A.

A situation similar to the above was found within *L. perenne* for two plants (Nos. 18 and 27) thought to differ in light-saturated rates. Plots of photosynthetic rate against S.L.A. for leaves of a number of clones grown under different light regimes show divergence between the two plants.

In conclusion, the above results show that the basic photosynthetic character-
ists of \textit{L. rigidum} and \textit{L. perenne} are very similar. Both tend to behave as “sun” plants in that when grown under low light intensity they retain a capacity for response to high light intensities. Maximum photosynthetic capacities are similar, but it is probable that the light-saturated rate of \textit{L. rigidum} is slightly greater than that of \textit{L. perenne}.

The temperature responses of the two species at a constant light energy level of $2 \times 10^5$ ergs cm$^{-2}$ sec$^{-1}$ show apparent photosynthesis to have a wide tolerance to this factor. The optimum temperature is 18°C–24°C for each species, but it is clear that the tolerance of \textit{L. rigidum} for high temperature is much greater than that of \textit{L. perenne}. This is in keeping with the summer-dormant behavior of the latter.

\textbf{Genetic Recombination and Transplant Responses among F$_3$ Progenies of \textit{Mimulus}}

Malcolm A. Nobs and William M. Hiesey

A logical follow-up of studies on the performance of contrasting races and species of \textit{Mimulus} and their F$_1$ and F$_2$ hybrids at the Stanford, Mather, and Timberline transplant stations (Year Book 66, pp. 208–214) is a corresponding analysis of selected F$_3$ progenies. Data gained from this additional step strongly reinforce evidence from F$_2$ progenies with respect to genetic coherence. They also throw new light on natural selection among genetically heterogenous populations.

The studies on F$_3$ populations of \textit{Mimulus} have concentrated on progenies resulting from a single cross between the altitudinally and morphologically contrasting forms of \textit{M. cardinalis} from near sea-level in coastal central California and subalpine \textit{M. lewisii} from 3,200 meters elevation in the Sierra Nevada. The extensive recombination of the parental characters in F$_2$ populations of this cross, and the diverse responses of individual plants at the Stanford, Mather, and Timberline transplant stations have been described in part in earlier reports (Year Book 60, pp. 381–384; and Year Book 62, pp. 387–392).

\textit{Segregation within F$_3$ progenies derived from contrasting F$_2$ individuals.} The patterns of segregation in F$_3$ populations differ widely, depending on the genetic constitution of the individual F$_2$ parent. This is illustrated in Fig. 10 where the frequencies of individuals falling into different morphological classes, as expressed by index values, are plotted as histograms. The index value of a given individual is determined by the summed expression of 11 essentially nonmodifiable characters that distinguish the parents. The scoring of each character is made on the basis of a scale of 9 on independent observations at Stanford, Mather and Timberline during a period of three years. Plants resembling \textit{M. lewisii} for a given character are given a rating of 1, and those resembling \textit{M. cardinalis}, a value of 9. The sum of characters of an individual falling into the \textit{M. lewisii} category could, therefore, have a minimum value of 11 in contrast with one of \textit{M. cardinalis} with a possible maximum value of 99. Since progenies resulting from self-pollination of either parent vary within an appreciable range, these idealized minimal and maximal index values are rarely found.

The histograms along the top line of Fig. 10 indicate the range of variation within populations of the self-pollinated \textit{M. lewisii} parent (left), the selfed \textit{M. cardinalis} (right), and their F$_1$ progeny (center). All these data were obtained from populations grown at Stanford. On the basis of the 11 characters used in determining the index values, the F$_1$ population tends to resemble \textit{M. lewisii} more than \textit{M. cardinalis} although the F$_1$ hybrids may be regarded as essentially intermediate between the parents.

The histogram immediately below the topmost line in Fig. 10 shows the frequency of character distribution within
Fig. 10. Ranges of segregation of characters in parental, first, second, and third generation progenies in the cross *Mimulus lewisii* × *M. cardinalis* as observed at Stanford. The index values are based on scored values of 11 characters of individual plants. Arrows point to individuals giving rise to the progenies depicted. Spontaneous seedlings established at Timberline were brought to Stanford for maturing.
a population of 310 $F_2$ individuals. The broad spread reflects the wide array of character-combinations of the parental plants found in the second generation. Even within this relatively small $F_2$ population sample, individuals occur that fall within the ranges of the progenies of both self-pollinated parents, but do not exceed their limits.

The middle histograms of Fig. 10 show the ranges of segregation among four $F_2$ progenies, each consisting of 200 individuals. Each population is the product of a self-pollinated $F_2$ plant having characters of the index class indicated above each histogram. The populations are distinctive in having peak frequencies at different values along the index scale. The population 7541, for example, which stems from 7111-16, a plant hardly distinguishable from the $M. lewisii$ parent, clusters on the left side of the index scale. There is, nevertheless, an appreciable range of segregation although much less than in the $F_2$ population. In contrast, the population 7565 from the $M. cardinalis$-like $F_2$ plant 7135-35 clusters far to the right on the index scale, and the majority fall within the morphological limits of the population derived from the selfed $M. cardinalis$ parent. Populations 7566 and 7543, the progeny of plants 7111-17 and 7112-55, respectively, which fall within the morphological range of $F_2$ hybrids, segregate about as widely as the original $F_2$ population.

The $F_3$ progeny often show marked transgressive segregation with respect to genetic segregation of a single character distinguishing the parents. The inheritance of pistil length, described in Year Book 83, pp. 432-438, may be cited as an example. Even when the aggregate combination of 11 characters is pooled in terms of index value, the segregation in some $F_3$ populations may extend beyond the mean index value of the self-pollinated parental progenies. An example is seen in population 7565 (Fig. 10) in which some individuals surpass the mean of selfed progeny of $M. cardinalis$.

The current season is the third during which the $F_3$ populations 7541, 7565, and 7566 have been tested as cloned individuals at the Stanford, Mather, and Timberline stations. It is now evident that striking differences in the response-pattern of 7541 in contrast with 7565 are being realized. Population 7541 has a markedly higher record for survival and dry weight production at Timberline than 7565, whereas at Stanford the reverse is true. Individual plants that are exceptions occur in both populations, but their frequency is low. In the $F_3$ population 7566 there is considerably greater diversity in response among individual plants at all three stations.

At Mather, where neither subalpine $M. lewisii$ nor coastal $M. cardinalis$ survive, the $F_3$ progeny of the $cardinalis$-like population 7565 has a higher percentage of nonsurvivors than the $F_3$ of the more $lewisii$-like population 7541. In population 7566 the plants vary from vigorous survivors to weak nonsurvivors; the vigorous plants invariably are morphologically similar to the $F_2$ hybrids. All the evidence from the $F_3$ populations therefore strongly supports the conclusion that morphological characters are strongly linked genetically with physiological qualities that are of importance for survival in different kinds of climates.

**Spontaneous “weedlings” at the transplant stations.** Additional evidence indicating that the type of coherence mentioned above is of importance in natural selection is afforded by a study of spontaneous seedlings of *Mimulus* that become established from seed of open-pollinated $F_2$ populations growing in the transplant gardens of Stanford, Mather and Timberline. Random samples of such “weedling” populations were taken from the gardens at each station during the spring of 1967 and grown in the nursery at Stanford to study their morphological characters. The lowermost two histograms in Fig. 10 show the frequency dis-
distribution of plants according to index values of samples taken from Stanford and Timberline. The separation of the clusters of frequencies at the two contrasting altitudinal stations is striking: Timberline favors the establishment of plants having a preponderance of lewisii-like characters in contrast with Stanford where only cardinalis-like seedlings establish.

**BIOCHEMICAL INVESTIGATIONS**

**LIGHT-INDUCED REACTIONS OF CAROTENOIDS IN THE YELLOW-GREEN ALGA *Botrydiopsis***

David C. Fork

A light-induced shift in the absorption spectrum of carotenoids was reported last year (*Year Book* 66, p. 160; Fork and Amesz, 1967) for a number of variously colored algae and for a barley mutant lacking chlorophyll b. One of the requirements for clear observation of the carotenoid changes is that the plant used does not contain chlorophyll b, since this pigment produces prominent light-induced changes of absorbance near 475 and 515 nm which effectively mask changes produced by carotenoids. This shift toward longer wavelengths of the absorption of a carotenoid was seen particularly clearly in the yellow-green alga *Botrydiopsis alpina* (Xanthophyceae) which contains no chlorophyll b.

Further studies on the carotenoid shift have been made with this alga. Action spectra for *Botrydiopsis* confirm the suggestion made earlier, based on studies of the red alga *Iridaea*, that excitation of each of the photosystems produces a carotenoid shift. Detailed measurements of difference spectra indicate that the same carotenoid is apparently participat-

![Fig. 11. Light-induced changes of absorbance in *Botrydiopsis alpina* induced by a broad band of red actinic light (2.8 × 10⁵ ergs cm⁻² sec⁻¹ between 650 and 800 nm). The magnitude of these initial deflections, the second slow changes, as well as the off deflections all depended strongly upon the schedule used. The traces shown here are those which resulted after the cells had been adapted to a schedule of 2 seconds light and 6 seconds dark. Halfband width of the measuring beam, 1.5 nm.](image)
ing in systems 1 and 2. The kinetic behavior of the carotenoid shift as well as the effect of a number of inhibitors of photosynthesis show many similarities between this change and that produced at 515 nm mentioned earlier.

Since both chlorophyll b and carotenoids produce positive absorbance changes at 515 nm it is clear that care should be exercised in the interpretation of results which are based upon studies of absorbance changes at this wavelength.

*Kinetics and difference spectra.* Kinetics of absorbance changes at 450, 467, 482, 497 and 517 nm produced by illumination of *Botrydiopsis* with a broad band of red light are shown in Fig. 11. In each case an initial rapid change was seen which was followed by a second larger, but slower change. The rise time of the initial change was measured at 518 nm and found to be as fast as the rise time (50 μsec) of the flash used. A rapid deflection also resulted upon darkening.

Fig. 12 shows difference spectra obtained when cells were illuminated with high intensity red light like that used to produce the traces for Fig. 11. The spectrum for the initial change had maxima at 451, 482, and 517 nm and minima at 467 and 497 nm. The off deflections shown in Fig. 11 are in each case larger than the on deflections. The difference spectrum of Fig. 12 for the off deflection is just reversed and has its peaks located at the same wavelengths as for the on deflection. The intensity of the light used had no effect on the difference spectra. The same spectra were found when low actinic intensity was used. Integration of the difference spectra given

![Fig. 12. Difference spectra of *Botrydiopsis* produced by red actinic light as described for Fig. 11 (8.7 × 10^4 ergs cm^-2 sec^-1). The kinetics of absorbance changes at maxima and minima are given in Fig. 11.](image)
in Fig. 12 suggests that these spectra are most likely produced by a shift toward longer wavelengths of the absorption band of a carotenoid originally having maxima at or somewhat below 440, 473 and 506 nm.

The effect of DCMU. The effect of the inhibitor DCMU [3-(3,4-dichloro-phenyl)-1,1-dimethylurea] is shown in Fig. 13. Trace a shows the light-induced absorbance change produced at 482 nm before adding DCMU. After addition of this inhibitor, the initial positive deflection remained (trace b). Its height was dependent upon the length of the dark intervals between exposures and is discussed below. DCMU completely inhibited the slower but larger absorbance increase following the initial deflection. After the cells had been in the light for several seconds a very small positive change persisted in the presence of DCMU. The effect of DCMU on the kinetics of the absorbance changes at those wavelengths shown in Fig. 11 was the same: positive transients persisted at 450 and 517 nm, negative ones at 467 and 497 nm. The difference spectrum for cells which had been treated with DCMU is given in Fig. 14. Maxima were again found at 450, 482, and 516 nm and minima at 466 and 497 nm. The spectrum is very similar to those shown in Fig. 12.

The height of the transient absorbance change persisting in cells inhibited with DCMU is strongly dependent on the length of the dark intervals given between light exposures. Fig. 15 shows the height of the initial transient (in this case measured at 515 nm) as a function of dark intervals between light exposures. Half of the maximum change was regenerated in about 4 seconds.

The effect of DCMU on the absorbance change at 482 nm was greatly altered after the addition of the donor system DAD (2, 3, 5, 6-tetramethyl-p-phenylenediamine) and sodium ascorbate. This couple is known from the work of Trebst and co-workers (1965; 1966) to be an effective donor of electrons which enter the electron-transport sequence very near system 1. Trace c of Fig. 13 shows the effect on the absorbance change at 482 nm of adding these compounds to whole cells of *Botrydiopsis*. Addition of only ascorbate to DCMU-inhibited cells did not produce an effect. TMPD (N,N,N',N'-tetramethyl-p-phenylenediamine) and ascorbate gave only a very small regeneration of this change. Difference spectra (Fig. 16) for the on and off deflections regenerated by DAD and ascorbate also have their maxima and minima at the same wavelengths as those spectra given in Figs. 12 and 14.

The effect of other inhibitors on the carotenoid shift. The uncoupler of phos-
Fig. 14. Difference spectrum for the magnitude of the initial absorbance change (see trace b, Fig. 13) produced by Botrydiopsis in 10^-5 M DCMU upon exposure to red light as described in Fig. 11 (7.9 x 10^4 ergs cm^-2 sec^-1).

Fig. 15. Height (regeneration) of the fast, initial transient of the 515-nm absorbance change in Botrydiopsis (as in trace b of Fig. 13) as a function of varied dark intervals between 6-second exposures to actinic light of 672 nm (5.1 x 10^4 ergs cm^-2 sec^-1). DCMU, 10^-6 M.
Fig. 16. Difference spectra for the magnitude of the absorbance changes of Botrydiopsis in DCMU, DAD, and ascorbate (as in trace c, Fig. 13) produced by a band of far-red actinic light (6.3 X $10^4$ ergs cm$^{-2}$ sec$^{-1}$ between 690 and 750 nm). DCMU, 5 X $10^{-4}$M; DAD, 6.6 X $10^{-4}$M, sodium ascorbate, about 2 X $10^{-3}$M. Halfband width of the measuring beam, 2 nm.

phorylation CCCP (carbonyl cyanide, m-chlorophenylhydrazone) at 10$^{-3}$M had an effect after cells had been incubated for about 30 minutes which was similar to that produced by DCMU. The initial on deflection persisted and the second, slow and larger change was inhibited. Quinacrine (atebrin) at 10$^{-2}$M inhibited both the initial transient and second wave. Vitamin K$_3$, which has been shown (Amesz and Fork, 1967) to poison reaction centers of system 2, inhibited the slow increase at 482 nm already at a concentration of 10$^{-3}$M but did not affect the initial transient. Hydroxylamine (2 X 10$^{-2}$M) acted like DCMU and inhibited the second, slow increase of the absorbance change at 482 nm but did not affect the initial on transient.

Action spectra for the production of the carotenoid shift. Action spectra, using equal numbers of incident quanta, were determined for production of the initial transient at 482 nm persisting after the addition of DCMU and for the slow increase of absorbance which occurred upon illumination of cells exposed to a continuous far-red background light. For the determination of the action spectrum of the initial transient remaining after the addition of DCMU, sufficiently low actinic intensities were used so that the rate of the initial change was linear with light intensity. The curve in Fig. 17 with a broken line shows a peak for this action spectrum near 681 nm and activity extending well beyond 700 nm. An action spectrum was also determined for the production of the 482-nm change in cells treated with DCMU, DAD and ascor-
Fig. 17. Action spectra for the production of the 482-nm absorbance change in Botrydiopsis. Solid circles: action spectrum for the rate of the slow absorbance increase determined in the presence of far-red background light (690-750 nm; $1.1 \times 10^4$ ergs cm$^{-2}$ sec$^{-1}$). The incident actinic intensities had halfband widths of about 9 nm and were equivalent to $1.32 \times 10^9$ einstein cm$^{-2}$ sec$^{-1}$ at 673 nm. Open circles: action spectrum in $10^{-4}$M DCMU (without far-red background light) for the rate of the rapid absorbance increase corresponding to trace b of Fig. 13. The incident actinic intensities used were equivalent to $0.77 \times 10^4$ einstein cm$^{-2}$ sec$^{-1}$ at 683 nm. The absorption of the cell suspension was measured in an integrating sphere with the same interference filters used for the action spectra. A scattering correction of about 6% was used. The absorption spectrum and action spectra were all measured using one sample of cell suspension.

bate (see trace c of Fig. 13). This spectrum was like the action spectrum for the production of the initial transient in DCMU-treated cells and had a peak near 681 nm.

In cells not treated with DCMU but exposed to far-red background light, the rapid initial change upon illumination was absent. Instead, a slow increase of absorbance occurred in the light and a fast off deflection followed upon darken-
shift. The action spectra for the transient carotenoid change persisting in DCMU (trace b, Fig. 13) as well as that measured after the addition of DAD and ascorbate show that system 1 alone participates in this reaction, since these spectra had peaks at 681 nm and high activity extending beyond 700 nm. They are very similar to the action spectrum measured (Fork et al., 1967) in the green alga Ulva for the oxidation of the f-type cytochrome, a well-known system 1 reaction. These spectra are also similar to the system 1 action spectra measured by Joliot et al. (1968) for the reduction of methyl viologen by chloroplasts poisoned with DCMU and supplied with DAD and ascorbate.

Measurements made last year (Year Book 66, p. 160) on the red alga Iridaea have also shown that each of the two photochemical systems produced a carotenoid shift. This distinction was possible because the absorption of the pigments associated with systems 1 and 2 is widely separated spectrally. Thus 684 nm, absorbed largely by chlorophyll of system 1 produced a time course for the carotenoid shift which was different from that produced by 622-nm light, absorbed predominantly by phycocyanin of system 2. Moreover, the ratios of the activity of 684- to 622-nm light in bringing about the carotenoid shift in this alga were the same as for cytochrome oxidation.

If electron flow is interrupted with DCMU in Botrydiopsis the excitation of system 1 produces a rapid shift of carotenoid absorption toward longer wavelengths which reverses again in the light. This same transient shift occurs in red algae excited with red light (but not treated with DCMU) because in these algae red light is unable alone to sustain complete photosynthesis efficiently.

The slower, but larger, shift of carotenoid absorption apparently accompanies sustained electron flow. The reestablishment of partial electron transport after addition of DAD and ascorbate to DCMU-treated algae produced a lasting carotenoid shift in the light, sensitized by system 1.

System 2 also produced a sustained carotenoid shift in the light. This was seen (Fig. 17) in the action spectrum for the slowly occurring shift measured in the presence of far-red background light. This action spectrum with a peak near 674 nm and activity dropping to zero around 700 nm reflects participation by system 2. In these respects it resembles other action spectra for system 2 (Müller et al., 1963) and the spectrum for system 2 measured as O₂ evolution in chloroplasts by Joliot et al. (1968); however, the spectrum of Botrydiopsis is not asymmetric, since this alga contains no chlorophyll b.

Vitamin K₃, among other substances, suppresses electron flow probably by acting as "artificial" traps in system 2 (Amesz and Fork, 1967). This compound, like DCMU, also selectively stopped the slow phase of the carotenoid shift. A similar effect was seen with the system 2 inhibitor, hydroxylamine. Explanation of the results obtained with the uncouplers CCCP and quinacrine on the basis of electron flow is not apparent and needs more study.

So far, there is no evidence to suggest that a different carotenoid participates in the absorption shifts taking place upon excitation of systems 1 and 2. Detailed measurements of difference spectra for the transient shift persisting in DCMU as well as those sustained by DAD and ascorbate (system 1) were similar to each other and to difference spectra obtained before poisoning. Also, difference spectra for the slow shift taking place in the presence of background light (system 2) were the same as the above-mentioned spectra.

The shifts in carotenoid absorption reported (Year Book 66, p. 160) for plants lacking chlorophyll b as well as those described here have many similarities in common with the 475-, 415-, 650-nm change seen in green algae and...
higher plants containing chlorophyll $b$. The latter change apparently does not result from an oxidation-reduction reaction but rather seems to be caused by an alteration in the environment of chlorophyll $b$ which produces a small change in its absorption spectrum. Junge and Witt (1968) have recently suggested that this change is an indicator for proton translocation across a membrane.

Both the carotenoid shift and the chlorophyll $b$ changes begin with a fast, initial transient sensitized by system 1. The initial, fast carotenoid shift, like the chlorophyll $b$ change, is dependent upon the preceding dark time and increases up to a certain maximum with increasing dark time. DCMU in each case only inhibits the slow, second increase of both changes. This slow change for each is sensitized by system 2.

All the similarities between the carotenoid shift, which has one of its maxima near 515 nm, and the 515-nm change, which is produced by chlorophyll $b$, make it necessary to proceed with caution in a description of what role chlorophyll $b$ plays in photosynthesis. Since the carotenoid shifts were seen in a barley mutant lacking chlorophyll $b$ (Year Book 66, p. 165) it is not unreasonable to assume that these shifts also occur in other higher plants as well as in green algae containing chlorophyll $b$, and that light-induced absorbance changes caused by both compounds are visible at 515 nm. Since absorbance changes attributable to carotenoids are near zero at 473 nm in Botrydiopsis and at 480 nm in mutant barley while the change caused by chlorophyll $b$ has a negative maximum near these wavelengths, it may be possible by appropriate choice of wavelengths to observe the chlorophyll $b$ change without interference from the carotenoid change.

References


STUDIES ON THE PHOTOSYNTHESIS OF PLASTOME MUTANTS OF Oenothera

David C. Fork, Ulrich W. Heber and Marie-Rose Michel-Wolwertz

We have previously investigated (Fork and Heber, 1968) electron-transport reactions in 5 mutants of the higher plant Oenothera hookeri and O. suaveolens that are known to have deficiencies in their plastomes (the genetic material residing in the plastids). We are grateful to Prof. W. Stubbe for these mutants. Since all of these mutants also have defects in photosynthesis, we investigated their fluorescence characteristics and light-induced absorbance changes in an attempt to localize the site of the block in photosynthesis and to relate mutational changes of the plastome to specific biochemical events.

Examination of 4 of the mutants (designated Ia, I8, Iy and IIy) revealed that photosystem 2 was largely, or completely, nonfunctional. In all of these mutants the excitation of system 2 was largely, or completely, nonfunctional. In all of these mutants the excitation of system 2 did not cause reduction of oxidized cytochrome $f$. Moreover, the system-2 dependent absorbance change at 518 nm seen in normal leaves was absent. Also, these mutants had a high initial fluores-
cence in the presence or absence of DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea] which did not change during illumination, indicating that the reaction centers of system 2 were affected by the mutations. Photosystem 1 appeared to function normally.

A fifth mutant (IIα) had an impairment in photosystem 1. Even high intensity far-red light did not lead to an accumulation of oxidized cytochrome f as seen in normal plants. Photosystem 2 was functioning as shown by the fast reduction of the primary system-2 oxidant (Duysens' "Q") and by the characteristics of the 518-nm absorbance change.

The studies mentioned above suggested that the reaction centers of photosystem 1 or 2 were nonfunctional or perhaps missing altogether in the mutants. We therefore measured low temperature spectra of chloroplast particles isolated from normal leaves, from a mutant deficient in photosystem 1 (IIα) and from a mutant deficient in photosystem 2 (IIγ). These particles were prepared by grinding leaves in an isotonic mannitol buffer; the larger particles were removed by filtration and low-speed centrifugation.

Chloroplast particles were collected by centrifugation at 8000 × g and resuspended in 0.025 M tris buffer, pH 7.8. Samples were cooled with liquid N₂ and absorption spectra measured with the spectrophotometer designed to minimize scattering as described in Year Book 66, p. 175.

Fig. 18 shows significant differences in the absorption spectra of particles derived from mutants IIα and IIγ particularly in the region between 677 and 720 nm. The spectrum of particles obtained from normal leaves (not shown) follows closely that of mutant IIγ up to 677 nm and from there on it falls between the curves of mutant IIγ and IIα. The spectrum for chloroplast particles derived from mutant IIα, without system-1 activity, showed a steeper decline from about 677 nm toward longer wavelengths when compared with particles obtained from mutant IIγ or normal chloroplasts. At about 700 nm the absorption of these particles approached zero. By contrast, particles from mutant IIγ, lacking system-2 activity, when compared to normal or mutant IIα had absorption well beyond 677 nm and approached zero only around 720 nm.

![Fig. 18. Absorption spectra measured at 77° K for chloroplast particles isolated from Oenothera mutants IIα (having system 2 but no system 1 activity) and IIγ (system 1 but no system 2 activity).](image-url)
These findings should indeed be expected if some specialized forms of chlorophyll necessary for the conversion of light energy into biochemical energy are missing. It is known that chlorophyll a forms, absorbing maximally around 700 nm (Kok's P700 and Butler's C700), comprise part of the reaction centers of system 1. The low absorption of mutant IIα in this region suggests that these pigments may be for the most part missing in this mutant. Conversely, the mutant (IIγ) which has system-1 but no system-2 activity has pronounced absorption around 700 nm suggesting the presence of those specialized chlorophylls associated with system-1 reaction centers. The fact that the absorption of this mutant is greater than that of the normal plant beyond 677 nm may be caused by the absence of other specialized chlorophyll(s) associated with system 2 absorbing maximally at shorter wavelengths.

In addition to showing that the site of the genetic block of photosynthesis is at or close to the reaction centers of either photosystems 1 or 2, these results have a bearing on the function of the plastome. Since the genetic defect of these mutants is located in the plastome that is contained in the plastids, these experiments suggest that this genetic entity controls electron transport reactions in the lamellar structure of chloroplasts.

Reference

Effect of Enzymatic Digestion of Chloroplast Lamellae on Chlorophyll Absorption

Marie-Rose Michel-Wolwertz

Currently much effort is being spent in attempts to separate different forms of chlorophyll by fractionating chloroplast preparations. Hydrolytic enzymes have proved to be useful. Greenblatt et al. (1960), using lipase, separated the chloroplast lamellae from one another, whereas with trypsin he disrupted the internal structure of the lamellae. By treatment of Chromatium chromatophores with proteolytic enzymes in the presence of Triton X-100, Vernon and Garcia (1967) isolated three distinct pigment-protein complexes.

We have investigated the effect of treatment with proteases and a lipase on the absorption spectra of fragmented chloroplasts. In one organism, Euglena, a selective conversion of longer to shorter wavelength forms of chlorophyll was found. The finding that modification of the carrier protein can change one of the native chlorophyll complexes into another spectroscopic type is of considerable significance to the basic problem of the nature of the structural differences between the different forms.

Euglena gracilis (Indiana Culture Col. No. 752) and Chlorella pyrenoidosa (Indiana Culture Col. No. 1230) were used. Euglena, in addition to the two absorbing forms Ca 670 and Ca 680 that are present in most plants, contains a large amount of a third form, Ca 695, when grown in dim light. Chlorella contains relatively more Ca 680 and Cb 650 in addition to Ca 670.

The cells were broken by forcing them three times through the needle-valve homogenizer. Chloroplast fragments were separated from soluble cellular material by centrifugation and were resuspended in buffer (0.35 M NaCl, 0.02 M tris-HCl, pH 7.4 and 0.01 M EDTA).

Enzymatic digestions were carried out at 33°C under nitrogen for 1 hour. After incubation, absorption spectra were measured for treated samples as well as for controls without enzyme.

When chloroplast fragments from Euglena or Chlorella were treated with papain or pancreatic lipase, they were immediately precipitated. Therefore we will report further only the results obtained with protease (from Streptomyces...
griseus), with trypsin and with wheat lipase.

**Enzymatic degradation of Euglena chloroplast fragments.** Fig. 19 shows the absorption spectra, measured at room temperature, of *Euglena* chloroplast fragments treated with protease (1 μg protease/μg chl) and of a control sample. Protease preferentially destroyed Ca 695; at the same time, an increase in absorption of “Ca 670” was observed. The spectrum of the difference measured at liquid nitrogen temperature between the control and the protease-treated sample (protease minus control) is given in Fig. 20. This spectrum clearly shows that by protease degradation, Ca 695 was transformed into “Ca 670.” From a series of calculated difference spectra for these preparations the “Ca 695” was found to have a wavelength peak of 693 nm and a half width of about 24 nm (French et al., 1968).

Longer incubation time (3–4 hours) or higher enzyme concentration (20 μg protease/μg chl) resulted in partial destruction of Ca 680 and Ca 670; the Ca 680 was the first to disappear. Similar results were obtained by trypsin degradation. No modifications of the spectrum were observed when *Euglena* chloroplast fragments were treated with wheat lipase.

**Enzymatic degradation of Chlorella fragments.** Absorption spectra, measured at −196°C, of chloroplast fragments of *Chlorella* treated for 1 hour by protease, by wheat lipase and the control sample are shown in Fig. 21. The three curves were adjusted to the same height at 678 nm. Protease preferentially destroyed the “Ca 680,” lipase the “Ca 670.” No change in the total chlorophyll following enzymatic degradation was detected by spectroscopic analysis of acetone extracts from enzyme-treated material.

**Fractionation attempts by gel filtration.** Unfortunately, attempts to separate the chlorophyll-lipoprotein complexes of

![Absorption spectra](image-url)

**Fig. 19.** Absorption spectra, at room temperature, for *Euglena* chloroplast fragments treated with 1 μg protease/μg total chlorophyll. The enzymatic digestion and the control were incubated at 33°C for 1 hour under nitrogen.
enzyme-treated chloroplast fragments by gel filtration were unsuccessful. Although the various agarose gels used offered a large range of exclusion molecular weight (from 40,000 to 150,000,000) poor resolution was obtained with all samples tested. When the sample was layered on the column it either passed through in a large band without separation, or it separated into different bands which were elutable only by use of detergents.

Fig. 20. The difference spectrum (protease minus control) measured at $-196^\circ$C, between a sample of *Euglena* chloroplast fragments treated with protease and a control. Conditions same as for Fig. 19.

References


Figure 21. Absorption spectra measured at $-196^\circ C$ for Chlorella fragments treated with wheat lipase and a control. The enzyme concentration was 5 µg/µg total chlorophyll. The enzymatic treatments and the control were incubated at 33°C for 1 hour under nitrogen.

Fractionation of the Photosynthetic Apparatus from Broken Spinach Chloroplasts by Sucrose Density-Gradient Centrifugation

Jean-Marie Michel and Marie-Rose Michel-Wolwertz

In recent years, many attempts were made to fractionate the photosynthetic apparatus into particles containing mainly one or the other of the two photosystems. Detergents have been widely used for this purpose. The complexes usually released with detergents had often partly or completely lost their photochemical activities. The goal of the present study was to find a method of chloroplast disintegration and fractionation without use of detergents. We succeeded in separating fractions of spinach, Chlorella and Euglena. This section reports the separation from broken spinach chloroplasts of three bands by centrifugation in a continuous sucrose gradient, without detergents.

The spectral properties and the photochemical activities of the three bands were studied. The results indicate that band 1 (low density) contained particles enriched in photosystem 1, whereas bands 2 and 3 (high density) contained particles enriched in photosystem 2. These particles, like the digitonin particles, are much reduced in their photochemical activities.

Fractionation method. Chloroplasts were isolated from spinach leaves purchased at the local market and suspended in 0.05 M Tricine-NaOH buffer adjusted to pH 7.8 containing 0.15 M KCl. The fresh chloroplast suspension was forced three times through a needle-valve press.
operating at maximum pressure (12,500 lb/in²). The broken material, hereafter called homogenate, was fractionated on a linear sucrose density-gradient (12.5%-50%) by centrifugation at 60,000 g for 45 minutes. All the operations were carried out at 4°C. After centrifugation the different bands were collected separately. Their pigment contents and some of their photochemical activities were measured.

Centrifugation pattern. By centrifugation on a continuous sucrose gradient, the homogenate of broken chloroplasts separated into three green distinct bands (Fig. 22) and a few large particles sedimented on the bottom of the centrifuge tube. The three bands had about the same width. We called them band 1, band 2, and band 3, starting from the top of the gradient.

Distribution of chlorophyll a and b in the bands. The homogenate or the fractions were dialyzed and extracted with 80% acetone. Chlorophyll contents were determined spectrophotometrically according to Mackinney.

Table 6 shows the relative content of chlorophylls a and b in the homogenate and in the different bands for three different experiments. The chlorophyll content in the three bands was nearly the same. From 20 to 30% of the chlorophyll layered on the top of the gradient was found in each band and 14 to 30% of the total was lost in the sediment and between the bands. The a/b ratio was much higher in band 1 than in bands 2 and 3 or in the homogenate. Compared to the original homogenate, band 3 and also band 2 were enriched in chlorophyll b.

It should be pointed out, however, that if the chloroplasts were isolated and broken in a buffer of low ionic strength (0.05 M Tricine-NaOH at pH 7.8 containing 0.001 M MgCl₂, 0.005 M NaCl and 0.001 M KH₂PO₄) instead of one of high ionic strength (Tricine containing 0.15 M KCl) the three bands were found to have almost the same a/b ratio.

**TABLE 6. Chlorophyll Distribution in the Bands Separated from Spinach Chloroplasts Broken in 0.05 M Tricine-NaOH, 0.15 M KCl pH 7.8**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Percent of Total Chlorophyll in the Different Fractions</th>
<th>Chlorophyll Ratio a/b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expt 1</td>
<td>Expt 2</td>
</tr>
<tr>
<td>Broken chloroplasts</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Band 1 (low-density)</td>
<td>20.2</td>
<td>27.2</td>
</tr>
<tr>
<td>Band 2 (medium-density)</td>
<td>27.7</td>
<td>29.0</td>
</tr>
<tr>
<td>Band 3 (high-density)</td>
<td>21.5</td>
<td>29.5</td>
</tr>
<tr>
<td>Sediment + material between bands</td>
<td>30.4</td>
<td>14.1</td>
</tr>
</tbody>
</table>
Fig. 23. Absorption spectra of the spinach chloroplast homogenate and of the bands separated from this homogenate. The bands correspond to those in Fig. 22 and were dialyzed overnight against buffer before recording the spectra; band 2 had an absorption spectrum similar to band 3.

Fig. 24. Absorption spectra for the homogenate of broken spinach chloroplasts and of band 1 and band 3 after dialysis overnight against the buffer.
Absorption and fluorescence spectra. The absorption and fluorescence emission spectra were recorded at 20° and -196°C. The spectrofluorometer and the spectrophotometer used were described in Year Book 65, p. 483, and Year Book 66, p. 175. Fig. 23 shows the absorption spectra, at room temperature, of the homogenate and of bands 1 and 3. The curves were adjusted to the same height at the maximum in the red. The absorption band of chlorophyll b, located near 650 nm, was clearly visible in band 3, whereas it appeared only as a shoulder in band 1. Again it is important to note that almost no differences were observed in the spectra of band 1 and band 3 when chloroplast material was broken in buffer of low ionic strength. Near 700 nm, band 1 absorbed more than band 3 and even more than the homogenate. Also in the 450-500 nm region, band 1 showed a greater absorbance than band 3.

Spectra of these samples measured at -196°C and matched to the same height at the maximum in the red are given in Fig. 24. At low temperature, the main peak of both the homogenate and the bands appeared as a double structure: a well-defined peak near 678 nm and a shoulder at about 670 nm. The relative heights of these two spectral bands were not the same in all the fractions. In band 1, the absorbance at 670 nm was almost equal to the absorbance at 678 nm, whereas in band 3, the peak located near 678 nm was higher than the shoulder situated at 672 nm. Identical results were obtained by Briantais (1967) for fractions separated by centrifugation from Triton X-100 treated chloroplasts.

Fig. 25 compares the fluorescence emission spectra at -196°C, of the homogenate of broken spinach chloroplasts and of the bands. Excitation at 435 nm.
sion spectra at −196°C of the homogenate and of the three bands that were isolated. All the samples showed an emission peak at 681 to 682 nm (F 682); a second broad emission band was present at 730 nm (F 730). However, the F 730/F 682 ratio was greater in band 1 than in bands 2, 3 or the homogenate. In bands 2 and 3 a supplementary emission peak was visible at 700 nm whereas this peak was absent from band 1 and the homogenate.

Photochemical Activities

NADP reduction. The rate of NADP reduction was calculated from the increase in absorbance at 340 nm using an extinction coefficient of 6.22 × 10⁶ cm² M⁻¹. We used diaminodiurol (DAD) and Na ascorbate as electron donors. Table 7 shows that band 1 had a rate of NADP reduction close to the rate of broken chloroplasts whereas the ability of band 2 and especially of band 3 to reduce the NADP was much lower on the same chlorophyll basis.

DCPIP reduction. Hill activity was measured using 2,6-dichlorophenol-indophenol (DCPIP) as electron acceptor by following the decrease in absorbance at 620 nm. The rate of DCPIP reduction was calculated using an extinction coefficient of 20.6 × 10⁶ cm² M⁻¹. Fig. 26 shows the absorbance changes observed at 620 nm when broken chloroplasts and the fractions were illuminated with blue actinic light. After turning on the light, a decrease of the absorbance at 620 nm was observed with broken chloroplasts and bands 2 and 3 while almost no decrease was seen with band 1. The rate of DCPIP reduction was obtained from

![Diagram](image)

**Fig. 26.** Absorbance changes recorded for a reaction mixture containing 2.5 ml of a sample fraction with a chlorophyll (a+b) content of 50 µg and 0.08 µmoles of DCPIP; light: 430 nm (Wₐₜ = 70 nm), 1.5 × 10⁶ ergs cm⁻² sec⁻¹.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Rate of NADP Photoreduction, µmoles NADP (mg chlor.)⁻¹ hr⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broken chloroplasts</td>
<td>9.40</td>
</tr>
<tr>
<td>Band 1 (low-density)</td>
<td>7.40</td>
</tr>
<tr>
<td>Band 2 (medium-density)</td>
<td>1.11</td>
</tr>
<tr>
<td>Band 3 (high-density)</td>
<td>0.39</td>
</tr>
</tbody>
</table>

*The reaction mixture, 2.5 ml, contained the sample with a chlorophyll (a+b) content of 33 µg and (in µmoles) DCPIP, 0.05; Na Ascorbate, 2; Tricine, 33; ADP, 1.5; KH₂PO₄, 1.3; NADP, 0.5; NaCl, 20; MgCl₂, 23; and 0.6 ml of a crude preparation of ferredoxin and NADP reductase (obtained from broken spinach chloroplasts); light: 660 nm (W = 7.5 nm) 1.9 × 10⁶ ergs cm⁻² sec⁻¹.
the slope of the slow decrease of absorbance which was measured after the light had been on for several seconds. Band 1 hardly reduced any DCPIP whereas bands 2 and 3 had a rate of DCPIP reduction almost two times higher than the homogenate.

**P700 content.** The relative amount of P700 in the different samples was estimated by measuring the light-induced bleaching of P700 at 700 nm. The photomultiplier was located at about 30 cm below the sample in order to minimize the effect of chlorophyll fluorescence. The signal of the P700 change was corrected for these fluorescence changes. The extinction coefficient for P700 was assumed to be the same as for chlorophyll a in acetone at 663 nm. The results are given in Table 8. It appears that band 1 contained more P700 in proportion to the chlorophyll content than the two other fractions. These results were confirmed by the measurement of light-induced optical density changes between 400 and 450 nm in the presence of DAD-Na ascorbate as an electron donor system. These difference spectra are given in Fig. 27. The importance of the change around 430 nm, generally attributed to P700, was three times higher in band 1 than in bands 2 or 3.

**Fluorescence measurements.** The time course of fluorescence was measured at 684 nm with the photomultiplier at an angle of 45° with respect to the blue actinic beam. The time response was 1 msec. Chlorophyll concentrations for the different samples were adjusted to the same value by dilution; the apparatus setting and the intensity of the blue actinic light were kept the same for the different measurements to permit a comparison of the relative fluorescence yield of the different samples. The fluorescence was measured after a dark period of 60 sec. Fig. 28 shows that the relative yield of fluorescence and the amount of variable fluorescence (defined in *Year Book 66*, p. 166) were both much higher in bands 2 and 3 than in the chloroplast homogenate or in band 1. The addition of DCMU to the sample slightly increased the total fluorescence but decreased the variable fluorescence.

**TABLE 8. The P700 Content of Different Fractions Obtained from Spinach Chloroplasts**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Moles Chl (a + b)/Moles P700</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expt 1</td>
</tr>
<tr>
<td>Broken chloroplasts</td>
<td>535</td>
</tr>
<tr>
<td>Band 1</td>
<td>350</td>
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<tr>
<td>Band 2</td>
<td>950</td>
</tr>
<tr>
<td>Band 3</td>
<td>825</td>
</tr>
</tbody>
</table>

![Fig. 27. Light-minus-dark difference spectra of the bands obtained from broken spinach chloroplasts. Actinic light, red 3.18×10⁵ ergs cm⁻² sec⁻¹.](image)
Fig. 28. Time course of fluorescence for the three bands after 60 sec in darkness. All the samples had the same chlorophyll \((a + b)\) content.

**Summary**

Our experiments show that without the use of detergent, spinach chloroplasts can be broken into particles that separate into three distinct bands when centrifuged in a sucrose gradient. The low-density particles (band 1) are different from the high-density particles (bands 2 and 3) with respect to pigment content and photochemical activities. Band 1 particles are enriched in chlorophyll \(a\) and reduce NADP, whereas particles of bands 2 and 3 do not reduce NADP but exhibit the Hill reaction and are relatively enriched in chlorophyll \(b\). Moreover, particles of band 2 show an enhanced yield and greater variable fluorescence than those of band 1.

Our lighter particles are similar in their spectra and photochemical activities to the small particles obtained by fractionation of detergent-treated chloroplasts, showing the properties associated with photosystem 1. Our heavier particles are similar to the large particles obtained from detergent-treated chloroplasts, showing almost exclusively the characteristics of photosystem 2.

**Reference**


**Absorption Spectra of Fractions Obtained by Sucrose Gradient Centrifugation from Chlorella Pyrenoidosa**

Marie-Rose Michel-Wolfertz and Jean-Marie Michel

The previous report has shown that spinach chloroplasts, fragmented by three passages through the needle-valve homogenizer, may be separated without use of detergents into three distinct bands by centrifugation in a sucrose gradient. These bands were different with respect to their photochemical activities and pigment content. Band 1 (low-density particles) was enriched in photosystem 1, and the heavier bands 2 and 3 (high-density particles), in system 2.

The aim of this study was to investigate the absorption properties of fractions obtained from another green plant by the method described for spinach. We chose *Chlorella pyrenoidosa* (Indiana Culture Col. No. 1230) and compared the absorption spectra, at low temperature, of the different bands with the absorption spectrum of the homogenate of broken *Chlorella*, and also with the absorption spectra, at room temperature, of acetone extracts of bands 1 and 3.
Results

After centrifugation in the sucrose gradient, the pigmented material was located in three distinct bands of the same width. The bands were numbered 1, 2, and 3 starting from the top of the gradient tube. The distribution of chlorophyll in the different bands was similar to that in the fractionation of spinach chloroplasts.

Absorption spectra at low temperature.
The upper part of Fig. 29 gives the absorption spectrum, at $-196^\circ$C, of the original homogenate of broken Chlorella cells. Chlorophyll a showed a distinct peak at 678 nm and a shoulder near 670 nm. The absorption band of chlorophyll b at about 648 nm was clearly visible. In the blue region, the main peak was located at 438 nm with a shoulder at 417 nm. Between 450 and 500 nm, three small maxima were distinguishable: near 477 nm (due to chlorophyll b) and near 486 and 495 nm (the carotenoid region). The lower part of Fig. 29 compares the absorption spectra, at $-196^\circ$C, of bands 1 and 3. The two spectra were adjusted at the time of recording to the same height at 678 nm. In the red part of the spectrum, all peaks and shoulders of bands 1 and 3 were located at the same wavelengths: 678, 670, and 648 nm; however, the relative heights of the maxima were different in the two bands. The absorbance of chlorophyll b at 648 nm appeared as a well-defined peak in band 3 and only as a shoulder in band 1. Band 1, on the contrary, absorbed relatively more at 670 nm and on the long-wavelength side of the 678-nm peak than did band 3. This suggests that the long wavelength absorption band of the light particles has a different shape and greater width than a band with a similar 678-nm maximum in the heavier particles.

In the Soret region, band 3 absorbed less at 437–438 nm and at 417 nm than band 1 whereas its absorption between

Fig. 29. Absorption spectra of the homogenate of broken Chlorella cells (upper) and of band 1 and band 3 separated from the homogenate by sucrose density-gradient centrifugation (lower). The spectra were adjusted to the same height at 678 nm.
460 and 500 nm was greater than that of band 1, especially near 476 where chlorophyll b absorbs.

**Absorption spectra of acetone extracts.**

The differences in pigment composition in the fractions observed in the absorption spectra at low temperature were also evident from the absorption spectra of acetone extracts shown in Fig. 30. The two curves were adjusted to give an equal absorption at 664 nm. The acetone extract of band 3 as compared with that of band 1 had a greater absorbance near 645 nm where chlorophyll b absorbs in solution. In the blue region, band 3 also absorbed relatively more, especially between 450 and 480 nm. This difference in the 450 to 480 nm region proves that the chlorophyll b and the carotenoid content of band 1 and band 3 were different.

Measurements of photochemical activity showed that band 1 obtained from *Chlorella* cells was enriched in photosystem 1 whereas bands 2 and 3 had higher activity for photosystem 2. These results obtained with *Chlorella* were exactly the same as those obtained with fractions separated from broken spinach chloroplasts.

These results with *Chlorella* parallel those with spinach fractions: photosystem 1 particles (band 1), separated by the fractionation method described above, have relatively greater absorbance between 680 and 700 nm whereas photosystem 2 particles (bands 2 and 3) are relatively enriched in Cb 650.

**Absorption and Fluorescence of Fractions from Several Plants**

*J. S. Brown and L. Prager*

After the successful fractionation of spinach chloroplasts and *Chlorella* on a sucrose gradient, described above, a series of similar experiments was performed on other plants. We have examined some aspects of the procedure itself and have noted differences and similarities that exist between widely different plants fractionated in the same way.

Our four criteria for determining a successful fractionation or separation were that the lighter fraction (1) have a higher chlorophyll a/b ratio than the heavier fraction or the homogenate; (2) have a higher absorbance in the 680 to 710 nm region than the heavier fraction; (3) have a lower ratio of F680 to F725 fluorescence and (4) have a lower fluorescence yield.

![Absorption spectra](image)

**Fig. 30.** Absorption spectra, at room temperature, of acetone extracts of band 1 and band 3 separated from the homogenate of broken *Chlorella* cells. The two spectra were adjusted to the same height at 665 nm.
Procedure

The algae were centrifuged from their growth medium, rinsed with distilled water, and resuspended in buffer for breaking. With the higher plants and the liverwort, about 40 g of leafy material was blended in a sorbitol buffer and filtered through cheese cloth. The chloroplasts in the filtrate were sedimented by centrifugation, washed with distilled water, resuspended in Tricine buffer and forced through the needle-valve press three times. This was essentially the same procedure followed by the Michels. A modification added by us was to centrifuge the broken mixture for 10 min at 3,000 g and to discard the sediment in order to have a relatively clear homogenate that could be compared spectroscopically with the fractions.

With spinach we attempted to determine what steps of the procedure were essential for a successful fractionation. Aliquots of washed chloroplasts were suspended in 0.05 M Tricine, pH 8 containing 0.45 M, 0.15 M or 0.05 M KCl and also in 0.05 M Tris, pH 8 containing 0.45 M, 0.15 M or no KCl before passing through the press. The sucrose solution contained 0.05 M Tricine, pH 8, and 0.15 M KCl in all six cases.

Following centrifugation in linear sucrose gradients for 30–45 min at 60,000 g, three green bands were observed in each case. As judged by criteria 1 and 2 above, the three fractions in each buffer-KCl combination corresponded to the system 1 (top fraction) and system 2 (both of the heavier fractions) particles in 0.15 M KCl-Tricine as described above by the Michels (Year Book 67, pp. 508–514). The KCl concentration influenced the amount of chlorophyll found in each fraction after a given period of centrifugation. Without KCl, only a little material reached the lower level after one hour whereas with 0.45 M KCl more than 50% of the chlorophyll was in the denser layer after 15 min. Possibly KCl causes the system 2 particles to contract and become denser, thereby facilitating their separation from system 1 particles.

Since fractions 2 and 3 from the linear gradient were alike according to the above criteria and also in NADP reduction and Hill activity as reported by the Michels, a sucrose step gradient was sought that would retain system 1 particles near the top and system 2 particles in a single lower band. A three-step gradient of 12%, 30% and 50% sucrose was found to achieve this purpose. Of course the fact that system 2 particles of two different densities do exist should not be forgotten. Eventually differences between them in other respects than density may be found.

The relative fluorescence yield was measured for the original homogenate and dialyzed fractions from the sucrose gradient. A drop of diluted sample was placed in a 0.2-mm deep slot of an aluminum strip and covered with a glass coverslip. Fluorescence emission spectra were recorded at room temperature as described in Year Book 65, p. 494. At least two dilutions were measured to be sure that self-absorption was minimal. The relative yield was calculated from the peak height near 680 nm, corrected for the response of photomultiplier at the voltage used and divided by the total chlorophyll concentration. The latter was determined in acetone extracts according to Mackinney.

Results

The results with each kind of plant are described below, and the relative fluorescence yields presented in Table 9.

Marchantia polymorpha. Three fractions were observed in the linear gradient. The lighter fraction could be distinguished from the lower two fractions by the four criteria stated above. Fig 31 (A) shows absorption spectra of the light and heavy fractions measured at −196°C.

Hordeum vulgare. Normal barley and a mutant lacking chlorophyll b (Boardman and Highkin, 1960) were fraction-
TABLE 9. The Relative Fluorescence Yield on a Chlorophyll Basis of the Homogenate and Fractions from Sucrose Gradients

<table>
<thead>
<tr>
<th>Material</th>
<th>Original Homogenate</th>
<th>Fractions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Light</td>
</tr>
<tr>
<td>Spinach</td>
<td>3.8</td>
<td>1.8</td>
</tr>
<tr>
<td>Marchantia polymorpha</td>
<td>1.9</td>
<td>0.4</td>
</tr>
<tr>
<td>Barley, wild type</td>
<td>1.68</td>
<td>0.59</td>
</tr>
<tr>
<td>Barley, mutant lacking chlorophyll b</td>
<td>0.98</td>
<td>0.68</td>
</tr>
<tr>
<td>Stichococcus</td>
<td>2.5</td>
<td>1.35</td>
</tr>
<tr>
<td>Tribonema</td>
<td>2.7</td>
<td>0.83</td>
</tr>
<tr>
<td>Euglena-Hutner's</td>
<td>1.23</td>
<td>1.75</td>
</tr>
<tr>
<td>Euglena-CM-citrate</td>
<td>3.16</td>
<td>2.75</td>
</tr>
<tr>
<td>Euglena-CM-EDTA</td>
<td>2.0</td>
<td>1.4</td>
</tr>
</tbody>
</table>

The homogenate from normal barley separated into three fractions in the linear sucrose gradient. Spectra of these fractions showed a good separation of fraction 1 and 2 (and 3) similar to that illustrated for Marchantia in Fig. 31(A). On the other hand, the b-less mutant separated into only two fractions, a broad band at the top and narrow band just above the bottom, having identical absorption spectra at \(-196^\circ\)C.

However, the emission spectra measured at either 20° or \(-190^\circ\)C were very different, the long wavelength emission maximum near 720 nm being enhanced in the lighter fraction. The heavier fraction from both normal and mutant plants had a higher fluorescence yield than the lighter fraction of the original homogenate (Table 9). Boardman and Thorne, 1968, treated this same mutant barley with digitonin and found no differences in the fluorescence yields of the centrifugal fractions. Apparently chloroplast particles from the mutant are more susceptible to damage by detergents than are normal plants.

We conclude that two kinds of particles were obtained from the mutant as well as the normal plants, but that the enhancement of long wavelength absorption usually observed in the lighter (criterion 2) did not occur in the mutant particles.

Stichococcus bacillaris. Two bands differing in absorption and fluorescence, as shown in Figs. 31(B) and 32, were observed near the top and bottom of a linear sucrose gradient. This alga, Indiana Culture Col. No. 419, has an unusually large proportion of chlorophyll b \((a/b = 1.6)\) that may be correlated with proportionally low level of Ca 670.

The fluorescence spectra in Fig. 32 illustrate the relatively greater emission at longer wavelengths from the lighter fraction 1. This higher ratio of long to shorter wavelength fluorescence in fraction 1 has been seen in all the plants that have been successfully fractionated. But when homogenates or fractions from different plants are compared, the relative proportion and peak positions of the long wavelength emission bands seen at \(-190^\circ\)C vary considerably. The same unknown reason may account for the variation in shape of the red absorption band of chlorophyll in vivo as well as for the differences in the emission bands.

Tribonema sp. A successful fractionation of this alga, Indiana Culture Col. No. 639, has not been achieved according to the absorption and fluorescence criteria. Two bands were observed in the linear sucrose gradient but they had very similar absorption and emission spectra. The component that absorbed the longer wavelengths was unusually labile in the broken cell homogenate, probably because of a destructive factor in the mixture since it was stable in the fractions from the gradient.

Euglena gracilis. When Euglena, Indiana Culture Col. No. 752, are cultured under light-limiting conditions, their proportion of chlorophyll b decreases and
that of chlorophyll a 695 increases with time. Therefore, we fractionated cells grown for different periods of time to see whether the different relative proportions of the chlorophyll forms in the homogenates would be reflected in the absorption spectra of the fractions.

In each of four separate experiments two fractions were observed in the linear gradient, but these had nearly identical spectra and relative fluorescence yields.

For these experiments, the *Euglena* were grown in the neutral medium devised by Cramer and Myers in which EDTA had been substituted for citrate as a chelating agent in order to decrease possible bacterial and fungal contamination. Since J.-M. Michel observed that particles prepared from *Euglena* grown with EDTA had little or no photochemical activity compared to those from cells grown in the citrate medium, we fractionated *Euglena* grown in the C-M citrate medium and also in an acidic medium devised by Hutner.

Again the fractions had the same absorption spectra, but their fluorescence properties differed. Not only were the relative yields different (Table 9) but also the lighter fraction had relatively more long wavelength emission.

*Euglena* previously have been fractionated with the aid of deoxycholate or
digitonin (Year Book 64, pp. 374–379) into particles of different densities. By that procedure the heavier particles were enriched in chlorophyll b and Ca 695 compared to the original homogenate. The lighter fraction, showing a single absorption band at 672 nm, may have been a detergent-solubilized form of chlorophyll. Since only derivative absorption spectra were measured, the experiment with deoxycholate has been repeated, and absorption spectra of the original homogenate and centrifugal fractions recorded at −196°C (Fig. 31C).

These results with detergent-treated *Euglena* in which the heavier fraction has relatively more long wavelength-absorbing chlorophyll are different from those of either detergent or the Michels’ fractions of other algae and leaves. Perhaps a unique molecular arrangement of chlorophyll b and Ca 695 in *Euglena* prevents the usual separation of particles having different chlorophyll forms.

Thus, in *Euglena*, attempts to obtain fractions with different proportions of chlorophyll b and forms of chlorophyll a have been unsuccessful unless a detergent is added. However, fractions with different fluorescence characteristics can be obtained on a sucrose gradient without detergent, providing the cells are grown in a medium without EDTA.

**Summary**

When leaves of barley and *Marchantia* were homogenized and then centrifuged in a linear sucrose gradient, three bands appeared that were similar to those observed previously in spinach. With several algae and a barley mutant lacking chlorophyll b, only two bands were found in the linear gradient. The heavier of these two bands corresponded to fractions 2 and 3 from spinach.

The fractions prepared from either *Tribonema* or the barley mutant had nearly identical absorption spectra. The expected relative increase in long wavelength absorption in the lighter fraction 1 did not occur in these plants lacking chlorophyll b. The fluorescence emission spectra and yields were also alike in *Tribonema* fractions. But the emission from the mutant barley fractions differed in the same way as it did in wild-type barley. *Euglena* fractions had the same absorption, but differences in emission similar to those in higher plants were observed provided the cells had not been cultured in a medium containing EDTA.

These results demonstrate that the fractionation procedure devised by the Michels may be successfully applied to several kinds of plants. In some plants, notably those in which chlorophyll b is lacking or variable in amount, the fractions can apparently have similar absorption spectra but different fluorescence spectra and yields. We presently have no explanation for this result unless the existence of additional, as yet undetected, absorbing forms of chlorophyll is postulated.

**References**


**EMERSON ENHANCEMENT AT DIFFERENT INTENSITIES AND RATIOS OF TWO LIGHT BEAMS**

Eckhard Loos

In a recent investigation (Loos, 1967) the Emerson enhancement effect was studied by measuring 14CO2 uptake. Constant ratios of short-wavelength to far-red light were used while the intensity was varied. Those experiments gave lower enhancement at higher light intensities even within the linear range, a finding contrary to the results of Bannister and Vrooman (1964). Recently the variation of enhancement with light intensity has been studied by measuring oxygen evolution in *Chlorella* and in *Porphyridium* to compare with previous measurements of CO2 uptake.
Material and methods. *Porphyridium cruentum* was grown in artificial sea water in shaken flasks aerated with 3% CO$_2$ in air. Cool-white fluorescent tubes provided a light intensity of 3500 Lux at the bottoms of the flasks. The extinction of the suspension at 515 nm, determined in a Beckman DU spectrophotometer with a 1-cm cuvette, was used as a measure of algal density. Cultures were started with an extinction of about 0.3 and in two to four days they had grown from 5 to 8 times the concentration of the inoculum. They were centrifuged and resuspended in enough fresh nutrient medium to give an extinction of 0.5 in a 1:10 dilution. When a suspension of this density or lower was used in the electrode compartment, the rate of O$_2$ evolution was proportional to the density, which shows that there was no trouble from mutual shading of the cells.

*Chlorella vulgaris* was grown at 6,500 Lux, whereas the other conditions, except for the medium, were the same as with *Porphyridium*. Most *Chlorella* experiments were done with four-day-old cells having an extinction of 0.6 at 515 nm in a 1:10 dilution. With this density mutual shading was probably small, for it did not occur with *Chlorella pyrenoidosa* cells showing in a 1:10 dilution at 515 nm an extinction of 0.47 or less.

Relative rates of oxygen evolution were measured with the polarized platinum electrode covered with Teflon described by Pickett and French (1967). The electrical signal obtained from the electrode was amplified and recorded. The flow system of the electrode assembly contained nutrient medium that was equilibrated with 5% CO$_2$ in air. Two 150 W quartz-iodine projection lamps were used with a lens system. Light intensity was varied by adjusting the lamp voltages. To obtain the desired spectral bands the light beams were filtered through 4 cm of water, heat reflecting filters and interference filters with halfband widths of 20 nm or less. A beamsplitter divided and mixed the two beams by transmission and reflection in such a way that part of each one impinged on the algae and part on an RCA vacuum photodiode (S-1 response), the photodiode calibrated at the desired wavelengths with a calibrated thermopile in place of the algae.

Measuring procedure. Relative rates of oxygen evolution were taken from the values reached five minutes after the onset of illumination when a fairly constant rate had been established (Fig. 33). This time was chosen because it is just beyond the time of the initial rise of rate yet does not include much of the further rate increase that continued for a longer period as indicated by the dotted line in Fig. 33. Every new illumination period was separated from the preceding one by a dark interval to achieve a stable baseline rate. For a better comparison

![Fig. 33. Time course of oxygen exchange in Chlorella. Wavelength 574 nm, light intensity: 5480 ergs cm$^{-2}$ sec$^{-1}$. Broken line indicates time course if light had not been turned off.](image)
Fig. 34. Dependence of oxygen exchange on light intensity in Chlorella at two wavelengths. (A): 716 nm (triangles); (B): 648 nm (triangles). The solid circles indicate calculated rates of $P_{1+2}$ for the maximum values of $E$ found for each ratio at lower intensities.
of photosynthetic rates from different experiments, rates of O₂ evolution were expressed as fractions of the light saturated value, which was determined with short-wavelength light in every experiment.

Calculation of enhancement. Enhancement, E, was calculated as

\[ E = \frac{P_{1+2} - P_2}{P_1} \]

where \( P_1 \) is the rate of O₂ evolution with the longer wavelength light primarily activating photosystem 1, \( P_2 \) is the rate for the shorter wavelength system 2 light and \( P_{1+2} \) is the rate with both beams on together.

Results: Chlorella. With low light intensities the rate of photosynthesis was generally linear (Figs. 34 and 35). No evidence was found for the "Kok effect," an increase in the slope of the curve at low intensity, that could have led to a lowering of enhancement values as pointed out by Myers (1963).

The result of an enhancement experiment with 648 and 716 nm for short-wavelength and far-red light, respectively, are listed in Table 10. It is seen that when the ratio \( P_2/P_1 \) is kept approximately constant, the enhancement factor \( E \) becomes smaller as the rates \( P_2 \) and \( P_1 \) increase. Comparison with the light curve of photosynthesis (obtained in the same experiment, Fig. 34B) suggests that the lower values of \( E \) might be

<table>
<thead>
<tr>
<th>( P_2/P_1 )</th>
<th>( E )</th>
<th>( P_{1+2} )</th>
<th>( P_2 )</th>
<th>( P_1 )</th>
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<tr>
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<td>0.235</td>
<td>0.273</td>
<td>0.0247</td>
</tr>
<tr>
<td>10.9</td>
<td>1.32</td>
<td>0.474</td>
<td>0.530</td>
<td>0.0434</td>
</tr>
</tbody>
</table>

* 648 nm
† 648 + 716 nm
‡ 716 nm

Fig. 35. Dependence of oxygen exchange on intensity in 674-nm light in Porphyridium (triangles). The circles have the same meaning as in Fig. 34(B).
caused by rates of $P_{1+2}$, which are in the bending part of the light curve. To test this the following procedure was adopted: For those cases showing low $E$ values for a certain ratio $P_2/P_1$, rates of $P_{1+2}$ that are equivalent to the maximum enhancement factor for that ratio were calculated. If the measured rates of $P_{1+2}$ were in the bending part of the light curve, thereby lowering the enhancement factor, the calculated $P_{1+2}$ rate should lie on the extrapolation of the linear part of the light curve. Fig. 34 (B) shows that the calculated $P_{1+2}$ rates do coincide well with the linear part of the light curve and its extrapolation. This indicates that any decrease of $E$ at the higher rates of $P_2$ and $P_1$ can be ascribed to the bending of the light curve of photosynthesis. Two other experiments gave results consistent with this finding.

Porphyridium. In far-red light a change in slope in the light curves indicating a "Kok effect" was occasionally observed; in four experiments on the behavior of enhancement at higher light intensities, the "Kok effect" interfered in only one case with the interpretation of the enhancement values. With green light (574 nm) no "Kok effect" was seen.

The results of the two best enhancement experiments are given in Table 11.

### TABLE 11. Enhancement in Porphyridium

<table>
<thead>
<tr>
<th>$P_2/P_1$</th>
<th>$E$</th>
<th>$P_2^*$</th>
<th>$P_{1+2}^*$</th>
<th>$P_1^+$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.625</td>
<td>1.25</td>
<td>0.088</td>
<td>0.20</td>
<td>0.11</td>
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<tr>
<td>0.70</td>
<td>1.25</td>
<td>0.20</td>
<td>0.57</td>
<td>0.29</td>
</tr>
<tr>
<td>1.06</td>
<td>1.31</td>
<td>0.083</td>
<td>0.19</td>
<td>0.079</td>
</tr>
<tr>
<td>1.09</td>
<td>1.45</td>
<td>0.20</td>
<td>0.47</td>
<td>0.19</td>
</tr>
<tr>
<td>1.04</td>
<td>1.32</td>
<td>0.49</td>
<td>0.53</td>
<td>0.47</td>
</tr>
<tr>
<td>1.81</td>
<td>1.81</td>
<td>0.09</td>
<td>0.18</td>
<td>0.05</td>
</tr>
<tr>
<td>1.93</td>
<td>1.65</td>
<td>0.20</td>
<td>0.37</td>
<td>0.103</td>
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<tr>
<td>2.03</td>
<td>1.24</td>
<td>0.48</td>
<td>0.78</td>
<td>0.24</td>
</tr>
<tr>
<td>Expt 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.80</td>
<td>1.95</td>
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<td>0.254</td>
<td>0.0536</td>
</tr>
<tr>
<td>2.68</td>
<td>1.94</td>
<td>0.37</td>
<td>0.634</td>
<td>0.137</td>
</tr>
</tbody>
</table>

* 574 nm
† Expt. 1: 574 + 684 nm; Expt. 2: 574 + 703 nm.
‡ Expt. 1: 684 nm; Expt. 2: 703 nm.

With constant ratios of $P_2$ to $P_1$ at different absolute values of $P_2$ and $P_1$, very similar enhancement factors are obtained, except for $P_2=0.5$ where the value of $E$ is severely diminished. In this case, however, the corresponding rates for $P_{1+2}$ (0.88 and 0.78) lie in the bending part of the light-photosynthesis curve as shown in Fig. 35. As previously described for Chlorella, $P_{1+2}$ values were calculated for Porphyridium on the basis of the maximum enhancement at lower intensities and plotted. Again the calculated values of $P_{1+2}$ are found on the extrapolation of the linear part of the light curve (Fig. 35).

From these experiments with Chlorella and Porphyridium the conclusion is drawn that light saturation of photosynthesis is sufficient to explain a decline in enhancement when the rates $P_2$ and $P_1$ are increased. Bannister and Vrooman (1964) also arrived at the same conclusion in their study of enhancement of oxygen evolution under different illumination conditions with Chlorella.

Discussion. The discrepancy between the results presented here and the findings for $^{14}$CO$_2$-assimilation does not seem easily explainable at first sight. One has, however, to consider the way in which the results for $^{14}$CO$_2$-assimilation were derived. $E$ was determined at different values of $P_2/P_1$ obtained by changing $P_1$ for two different rates of $P_2$. With a large ratio, $P_2/P_1$, (about 12) and large rate of $P_2$, a drop of $E$ was observed. This did not occur with a smaller ratio, $P_2/P_1$. It was argued that light saturation of photosynthesis could not be the reason for this because such a drop in $E$ should have occurred instead with the smaller ratio $P_2/P_1$ corresponding to the bigger $P_1$ rate.

This argument was based on the behavior of $E$ with different rates of $P_2$ and a fairly large ratio $P_2/P_1$. For a large ratio $P_2/P_1$, when there is a small rate $P_1$, the values of $P_{1+2}$ and $P_2$ are relatively large and their difference is small; thus minor variations of $P_{1+2}$ or $P_2$ may
lead to considerable changes of the value of $E$. (Compare also in Fig. 34B the relatively small corrections needed for $P_{1+2}$ to obtain full reconstitution of enhancement factors). In view of this consideration, a reinvestigation of enhancement for $CO_2$-assimilation at higher light intensities seems desirable.

References

INHIBITION OF CYCLIC ELECTRON TRANSFER IN VIVO BY RED LIGHT AND BY OXYGEN

Ulrich Heber

Light-induced electron transport is known to be accompanied by ion and water transport across the membranes of the thylakoid system in isolated chloroplasts. The ensuing shrinkage of the chloroplasts changes the light-scattering properties of the preparation. We therefore used light-scattering changes to study cyclic electron flow in vivo with two different wavelengths of light at various intensities and with different oxygen concentrations. The experiments were carried out with intact leaves.

Light-scattering changes were detected in the leaf by the attenuation of a measuring beam of 530 nm with the photodetector put in line with the measuring beam. Changes in scattering appear in this arrangement as changes in absorbance. They are distinguished from true absorbance changes by their kinetics, their magnitude, sensitivity to observation in the Ulbricht sphere and sensitivity to certain uncouplers. Fig. 36 gives the intensity dependence of changes that have been tentatively identified by these criteria as photoinduced shrinkage of chloroplasts in leaves.

Fig. 36. The extent of photo-induced shrinkage of a *Mimulus verbenaceus* leaf in CO$_2$-free air and in nitrogen as a function of the intensity of the exciting red or far-red light. Shrinkage is indicated by slow increase in the apparent absorbance of the leaf at 530 nm. Red: a broad band from about 620 to 820 nm; half-band width 125 nm, from 635 to 760 nm. Far-red: a band from about 690 to 820 nm; half-band width 60 nm, from 700 to 760 nm.

Under nitrogen, illumination with far-red light of low intensity that predominantly excites photosystem 1 causes shrinkage. Red light, exciting both photosystems 1 and 2, is also very effective at low intensities. At higher intensities of red light, however, shrinkage is progressively inhibited. In CO$_2$-free air, the shrinkage in red light follows a saturation curve. With far-red light no response is observed at low intensities but shrinkage does occur at high intensities.

**Interpretation of Fig. 36.** As in other experiments, not described here, the action spectrum of shrinkage in nitrogen as compared with that in air was found to be shifted toward longer wavelengths. Shrinkage in far-red light under nitrogen was assumed to represent a cyclic electron flow mediated by photosystem 1. Likewise low-intensity red light in nitrogen permits cyclic electron flow to occur. At higher intensities, however, photosys-
system 2 becomes sufficiently excited to reduce electron carriers between photosystem 1 and 2 thus rendering them ineffective as electron acceptors for cyclic electron flow. Consequently cyclic electron transport becomes inhibited and shrinkage no longer occurs.

Far-red, of an intensity sufficient to produce large shrinkage in nitrogen, is ineffective in CO₂-free air indicating that oxygen reacts with an electron carrier connected to photosystem 1 and drains electrons from the cyclic pathway. The large increase of shrinkage seen on admission of oxygen to a leaf in nitrogen that has been inhibited by high-intensity red light marks the onset of electron flow from water to oxygen. Accordingly, the action spectrum of shrinkage in the presence of oxygen is that of a reaction involving both photosystems.

Comparison of shrinkage with cytochrome f reduction. The redox state of electron carriers involved in cyclic electron flow should also reflect the proposed inhibition of cyclic electron transport by oxygen and by strong excitation of photosystem 2 in nitrogen. Cytochrome f donates electrons to the reaction centers of photosystem 1 and should in turn become reduced by carriers of the cyclic pathway during cyclic electron transfer. Its reduction should be seen in the dark immediately following a light period. If the initial rate of dark-reduction of cytochrome f is plotted as a function of the light intensity of a preceding illumination period the results depicted in Fig. 37 are obtained. It is obvious that the pattern of cytochrome reduction is similar to that of the photo-induced shrinkage of Fig. 36. In agreement with the interpretation of shrinkage, the dark reduction of cytochrome after exposure to far-red light is much faster in nitrogen than in CO₂-free air, which indicates the functioning of cyclic electron flow in nitrogen and its inhibition by oxygen. The very slow dark reduction of cytochrome f after illumination with high-intensity red light in nitrogen demonstrates the inhibition of cyclic electron flow under these conditions.

The inhibition of cyclic electron flow by oxygen, although seen only under far-red or low-intensity red illumination in

![Diagram](image-url)

**Fig. 37.** The rate of dark-reduction of photo-oxidized cytochrome f measured at 420 nm in a leaf of *Mimulus verbenaceus* as a function of the previous illumination with red or far-red light. The rate of dark-reduction was calculated from the slope of the initial decay of the signal after darkening.
leaves, suggests that cyclic electron transport reaches significant proportions only under anaerobiosis.

The intensity dependence of shrinkage in leaves shows the interdependence of the two photosystems. The experimental results have been explained on the basis of the series formulation of photosynthesis. It appears difficult, if not impossible, to accommodate the data in a model of photosynthesis with two separate photo-reactions having independent electron transport pathways.

Dependence of shrinkage on oxygen concentration. Fig. 38 shows changes in shrinkage of a spinach leaf caused by establishing different oxygen concentrations in the previously anaerobic system.

In low-intensity red as well as in far-red light, one notices with increasing oxygen concentration a large swelling which then levels off. As outlined above, this swelling is due to a deletion of cyclic electron flow with oxygen acting as an electron trap. In strong red light, admission of oxygen again causes a large increase in scattering indicating electron flow from water to oxygen. For medium intensities of red and relatively high intensities of far-red light, two effects show up with increasing oxygen concentration: First, a large shrinkage which settles to a constant value in the range between 1% and 5% O₂. The interpretation of this shrinkage is the same as for that of strong red light. The far-red intensities

![Fig. 38. Extent and direction of shrinkage or swelling induced in a spinach leaf under continuous illumination as a function of the concentration of oxygen and of light intensity. Positive changes in the optical density at 530 nm denote shrinkage; negative changes denote swelling. The reference level is the shrinkage condition produced in nitrogen.](image-url)
seem now to be strong enough to provide electrons from water by excitation of pigment system 2. Second, for higher O₂ concentrations the degree of shrinkage becomes gradually smaller and when changing from nitrogen to 21% oxygen even swelling is observed. An explanation for this phenomenon is not yet at hand.

Under the simplifying assumption that the light scattering response caused by low oxygen concentrations of less than 5% is a measure of the rate of a reaction of oxygen with the electron transport chain, the affinity of the system for oxygen was determined. The half-maximal response of shrinkage or swelling was observed at an oxygen concentration of 1.4 µM or approximately 1100 ppm of oxygen in nitrogen.

Effect of CO₂. Admission of CO₂ to a leaf kept in nitrogen and illuminated with far-red or low-intensity red light also resulted in a drastic inhibition of shrinkage. The half-maximal response to CO₂ in nitrogen was at approximately 10 ppm. Carbon dioxide is assumed, similar to oxygen, to drain electrons from the cyclic pathway and thereby to interrupt it. The high effectiveness of CO₂ in this reaction is in accord with the role of CO₂ as the main, though indirect, electron acceptor in photosynthesis.

Because O₂ and CO₂ inhibit shrinkage caused by excitation of photosystem 1 (low-intensity far-red or a monochromatic beam of 709 nm), the interaction of both O₂ and CO₂ with the electron transport chain should occur directly or via intermediates at the reducing end of photosystem 1. Preliminary experiments with isolated chloroplasts also agree with this interpretation. Isolated and washed chloroplasts, incapable of performing cyclic electron transport, still take up oxygen by a light dependent reaction even after electron flow photosystem 2 has been blocked by DCMU and a donor system is available to feed electrons to photosystem 1. The rate may be as high as 30 µmoles per mg of chlorophyll per hour. As ferredoxin is largely missing from these preparations, interaction with oxygen seems to be at the level of the first reductant generated by photosystem 1.

Part of the work described in this report has been published in more detail (Heber and French, 1968).

Reference

FACTORS AFFECTING ABSORPTION AND FLUORESCENCE SPECTRA OF NATURAL CHLOROPHYLL COMPLEXES

J. S. Brown

Although several forms of chlorophyll have been observed as peaks and shoulders in spectra for many years, their physical form is unknown. So far these chlorophyll forms have eluded our attempts to isolate them in pure form. Consequently to study the physical characteristics of chlorophyll in vivo we are restricted mainly to spectroscopic measurements.

A series of experiments in which algae or chloroplast particles have been subjected to various physical treatments has yielded some information about the natural state of chlorophyll. We have studied the effect of chlorophyll concentration within the cells, heat treatments and ultraviolet irradiation on the absorption and in some cases also the fluorescence of several organisms.

The absorption and fluorescence spectra of chloroplast fractions from a variety of plants is reported in another section.

Effect of Concentration on the Spectra of Chlorophyll in Chlorella

The shape of absorption and fluorescence emission spectra of intact algae may be distorted by a high chlorophyll concentration. Since our knowledge of the absorbing forms of chlorophyll a depends
upon the interpretation of complex spectra, the effect of varying the concentration of chlorophyll within the cells was investigated.

French et al. (Year Book 65, p. 492 and Year Book 66, p. 177) compared spectra of a series of Claes Chlorella mutants. Mutant No. 520 contained very little chlorophyll and nearly all of it as one form when grown in the dark. Spectra of these cells showed a single absorption maximum near 670 nm, no chlorophyll b and a single fluorescence emission maximum near 680 nm. (We wish to thank Mr. Norio Murata for pointing out the error in plotting Fig. 30B of Year Book 65, p. 495. The whole curve should have been put 10 nm toward longer wavelengths). The spectrum of Mutant 520 contrasts with that of normal green Chlorella that has an absorption peak at 677 nm and a shoulder at 670 nm indicating at least two forms of chlorophyll a. The normal fluorescence emission spectrum of Chlorella measured at low temperature shows two maxima, one near 680 nm and another broad band near 730 nm.

To determine whether the unusual spectra of Mutant 520 were due to a pigment mutation or to low chlorophyll concentration, a comparison was made with absorption and fluorescence spectra of Chlorella protothecoides (Indiana Culture Col. No. 25) in which the amount of chlorophyll formed is directly proportional to the nitrogen-carbon ratio in the growth medium (Shihira-Ishikawa and Hase, 1964). If this alga is grown on a glucose medium containing a minimal amount of nitrogen, very little chlorophyll is formed.

When grown in a medium containing 0.5% glucose and 0.05% urea, C. protothecoides was green, but in 1.0% glucose and 0.01% urea, the culture appeared about half as green as the usual Chlorella culture. An inoculum of cells from this pale green culture transferred to a medium containing 1.0% glucose and no nitrogen grew for several days, and the resulting suspension was white. However, chlorophyll was present as shown by the absorption spectrum of a dense cell suspension in Fig. 39; also shown

Fig. 39. Absorption spectra of Chlorella protothecoides measured at -196°C. Green culture grown on 0.55% glucose and 0.05% urea. White culture, measured on a much higher cell concentration, was grown on 1.0% glucose without an added nitrogen source.
is the spectrum of a thin suspension of green Chlorella.

Although the exact shape and relative heights of the chlorophyll a and b peaks are distorted by the high ratio of scattered to absorbed light in the cells containing very little chlorophyll, the spectra are very similar in shape and in their proportions of the chlorophyll forms. A comparison of these spectra in the blue region shows that the proportion of carotenoid to chlorophyll is greater in the cells grown in the lower nitrogen concentration.

The fluorescence emission spectra of cells from the green and the white cultures are shown in Fig. 40. The cells containing very little chlorophyll show much less long-wavelength fluorescence emission near 730 nm. The relatively enhanced long-wavelength emission seen in the green cells may be an artifact due to reabsorption of fluorescence near 680 nm by chlorophyll (Goedheer, 1964), or the normal green cells may contain a small amount of a highly fluorescent pigment that is absent in the cells grown in low nitrogen.

Absorption spectra of green Chlorella protothecoides, of two species of C. pyrenoidosa (Indiana Culture Col. Nos. 252 and 1230), and of aqueous extracts of the latter all have similar red bands.

The peak near 680 nm is always higher than the shoulder at shorter wavelengths. Algae from different genera may have different proportions of the chlorophyll a forms and therefore exhibit spectra of different shapes, but within specific groups, the shapes of the spectra are much alike.

We conclude that the doublet structure of the red absorption band in Chlorella is not caused or affected by the chlorophyll concentration within the cell although the long wavelength fluorescence emission maximum may be artificially enhanced. Assuming that chlorophylls a and b are normally attached to a lipoprotein framework, an explanation for the difference between the mutant and C. protothecoides may be as follows: C. protothecoides grown on low nitrogen may be able to synthesize only a small amount of this lipoprotein framework, but what it does make is normal, and the attached chlorophyll exhibits the usual spectrum. The mutant may be unable to form the basic structure, and therefore the small amount of chlorophyll formed is not attached in the normal way and displays only a single absorption band.

**Effect of Temperature on Absorbance of Chloroplast Particles**

Schejter and George (1964) observed that the 695-nm absorption band of ferricytochrome c was reversibly decreased by heating the solution from 10° to 60°C, and that this effect was probably caused by a conformational change in the protein portion of the hemoprotein. These results suggested that information about the nature of chlorophyll-lipoprotein complexes might be gained from similar heating experiments. In the present study, changes in absorption with changes in temperature from 15° to 70°C were measured in chloroplast particles of different sizes. In contrast to the hemoproteins, chlorophyll-lipoprotein complexes are insoluble.

Chloroplasts or algae were broken in
the needle-valve press and centrifuged to obtain fractions of different densities. Also spinach fractions or bands from the sucrose gradient, described by the Michels, were tested. Samples were placed in a jacketed cuvette in the recording spectrophotometer and heated or cooled by flowing water. Five to ten minutes were required to change the temperature.

Two types of decreases in apparent absorption with increasing temperature were observed. One type illustrated in Fig. 41 (A), could result from a decreased light scattering by the chloroplast particles when these are heated. The larger the particles, the greater was this type of change. It occurred throughout the measured spectrum and was completely reversible up to 40°C. This change may be similar to light-induced conformational changes.

The second type, illustrated by Fig. 41 (B), appears to be a true decrease in absorption. This change takes place with very small green particles of Chlorella, and it is only partially reversible. Chlorophyll a 680 is more easily bleached by heat than are the shorter wavelength absorbing forms. The reversible part of the change may be attributed to a decrease in light scattering discussed above.

Since the different forms of chlorophyll bleach at different rates with heating, two spinach fractions from a sucrose gradient, top fraction 1 enriched in long wavelength chlorophyll, and lowest fraction 2 in short wavelength chlorophyll a and chlorophyll b, were heated. Each fraction, heated to 40°C, showed a reversible bleaching pattern similar to that in Fig. 41 (B) for Chlorella particles. When each fraction was subsequently heated to 70°C and returned again to 15°C, the original peak position shifted 6-8 nm towards shorter wavelengths. Fig. 42 shows the plotted difference of absorbance of each fraction measured at 15°C before and after heating to 70°C for several minutes. The bands with maxima at 683 nm in fraction 1 and 682 nm in fraction 2, represent the chlorophyll complex destroyed by heat.

If these fractions had contained the same chlorophyll forms in different proportions, the difference spectra would be similar in peak position and shape, and they are not. The small difference in the heights is not significant because the original peak absorbance of fraction 2 was 0.06 greater than fraction 1.

The difference spectra were dissimilar in bandwidths and peak positions. This suggests that the fractions representing photosystem 1 and 2 activities contain separate forms of chlorophyll, both of which show peak absorption near 680 nm. We arrived at a similar tentative conclusion from curve analysis of spectra of the fractions. This work is reported in another section.

Effect of Ultraviolet Irradiation on Chlorophyll Absorption and Fluorescence in Phaeodactylum

Irradiation of algae or chloroplasts with ultraviolet light preferentially destroys photosystem 2 activity (Mantai and Bishop, 1967). Since the long wavelength absorption and fluorescence bands of the diatom, Phaeodactylum tricornutum, have been attributed to photosystem 2 (Brown, 1967), it was of interest to see if ultraviolet irradiation would specifically destroy these bands.

The algae, suspended in a thin layer of culture medium in a petri dish, were exposed to a sterilamp. Absorption and fluorescence emission spectra of the cells measured before and after exposure are presented in Figs. 43 and 44.

The absorption bands near 705 and 685 nm were both diminished by the ultraviolet light. The decrease in fluorescence at 710 nm is probably correlated with the loss of the 705-nm absorption band because chlorophyll a 685, observed in most plants, does not fluoresce at room temperature. The total relative fluorescence, measured as the area under the
Fig. 41. (A) Absorption spectra of spinach chloroplast fragments suspended in 0.01 M Tris, pH 8.5 measured at 15°, 40°, and again at 15°C. (B) Absorption spectra of supernatant from broken Chlorella, centrifuged at 10,000 g for 15 min, measured at 10°, 50°, and again at 10°C.
Fig. 42. Difference in absorbance between spectra measured at 15°C before and after heating to 70°C. Spinach fraction 1 from top of sucrose gradient and fraction 2 from lower part of gradient. Original absorbance approximately 0.6 at 678 nm. Fractionation carried out in 0.45 M KCl, 0.05 M Tricine at pH 8.0. The fractions were dialyzed to remove the sucrose before measuring the spectra.

Fig. 43. Absorption spectra of Phaeodactylum tricornutum at -196°C before and after irradiation with ultraviolet light, 253.7 nm, for 10 min.
Fig. 44. Fluorescence emission spectra of *Phaeodactylum tricornutum* before and after irradiation with ultraviolet light, 253.7 nm, 10 min; measured at 20°C, excitation at 436 nm.

curves, was also decreased by the ultraviolet irradiation.

The long wavelength absorption bands are easily destroyed by several mild treatments such as heating to 40° for 10 min, freezing and thawing, or exposure to 10% ethanol for several hours (Brown, 1967). Following each of these previously studied treatments the chloroplast within the cells disintegrated, and the suspension changed color from brown to green simultaneously with the disappearance of the long wavelength fluorescence band. Following ultraviolet irradiation, no immediate change in the cell structure corresponding to the change in fluorescence could be observed, although after several hours the cell interior did break up, indicating the lethality of the irradiation. Thus the loss of long wavelength fluorescence need not be correlated with gross morphological change.

These results are consistent with the hypothesis that the absorption band near 704 nm that fluoresces at 710 nm in *Phaeodactylum* is associated with photosystem 2.

**Phaeophytin Formation in Ochromonas**

In Year Book 66, p. 196, it was reported that mild heating or freezing and thawing of *Ochromonas danica* caused a change in its chlorophyll absorption spectrum from three maxima between 665 and 695 nm to a single band at 670 nm. Subsequent experiments have shown that this change results from the conversion of chlorophyll *a* to phaeophytin or phaeophorbide within the cell. Even in cells from a culture only just beyond the logarithmic growth phase, considerable phaeophytin was detected. Thus, the spectrum of the damaged cells may represent phaeophytin rather than an altered form of chlorophyll *a*.

This conclusion also changes the previous interpretation of the fluorescence spectra. In *Ochromonas* it appears that the main fluorescence emission band may be shifted from 680–685 to 690 nm by self-absorption.

No method has yet been found to extract pigment lipoprotein particles from *Ochromonas* without converting chlorophyll to phaeophytin.

**References**


**Connection of the Laboratory to the Computation Center of Stanford University**

David C. Fork

We have recently installed a cable connection between this laboratory and two facilities of the Computation Center at Stanford University.

One of the facilities now available for our use, called ACME for Advanced Computer for Medical Research, is an on-line computer system for data collection and control of experiments which is sponsored by the Macy Foundation and the National Institutes of Health.
The main computer of the ACME system is an IBM 360 Model 50. The ACME system also uses an IBM 1800 computer linked by a special channel to the Model 50. We have access to the computer "on line" on a time-sharing basis by means of an IBM 2741 typewriter terminal located in the laboratory. In addition to using the typewriter terminal, data can be sent to the computer as analogue signals (6 inputs) or digital signals (1 input), both via the 1800. The system will also accept direct input into the 360 for high speed experiments or data from small computers via high speed parallel data links.

Results of experiments may be obtained in the laboratory on the same typewriter terminal used to put in data or via the 1800 as analogue (4 outputs) or digital output (1 output) signals to drive various display equipment. The input and output system includes file handling and retrieval facilities.

One of the uses planned for ACME is to facilitate the analysis of large amounts of data which can be generated in a short time during studies of light-induced absorbance changes or the polarographic determination of $O_2$ exchange.

The other facility on the Stanford campus which can be used from our terminal contains an IBM 360 Model 67 computer. The terminal may be used for text editing, and computation jobs may be submitted through the terminal for standard batch processing.

The program for curve analysis described in another section of this report, for example, was stored on the disk file of this computer, the data for curves to be analyzed was sent to the computer via our terminal, and the results printed out on the high-speed printer and plotted on the Calcomp plotter at the Computation Center.

A CURVE DIGITIZER

C. S. French, R. W. Hart and Mark Lawrence

Direct digital recording of absorption spectra for data handling by computer is being developed by instrument manufacturers and appears to be on its way in for future routine use. We have considered, but at least temporarily abandoned, the thought of digitizing our spectrophotometer output through the ACME computer. The reasons for not taking this step are: the limited times of day that ACME is operational, the need to keep the ACME connection free for more pressing uses, and the cost.

There are many useful spectra on hand that we wish to put through the digital curve analysis procedure. Therefore, we have made a device to convert plotted curves into tabulated numbers for the computer input. This device moves a table carrying the plotted curve by a definite distance each time a knee switch is closed. This step distance on the x-axis is selected by a double set of change gears in wavelength steps of 0.5, 1.0, 2.0, 3.0, 4.0, or 5.0 nm. Our usual wavelength scales are $\frac{1}{4}$ inch $= 10$ nm for absorbance or $\frac{1}{2}$ inch $= 10$ nm for fluorescence spectra. A cursor observed under magnification is set by a hand wheel on the curve at each wavelength step. The counter, presently in use, reads 0.00842 inch per unit giving a precision of about 0.1% f.s. for the original record of a spectrum 8 inches high. A typical spectrum covering 160 nm can be converted in about 20 minutes to a table of heights at 1-nm intervals by reading a digital counter. A shaft angle encoder is being installed to provide a direct connection to the on-line ACME computer for more rapid read-in. The manual cursor setting will be retained since it provides a convenient way to average visually the minor irregularities of the record. The range on the x-scale is 16 $\frac{1}{2}$ inches and on the y-scale 15 $\frac{1}{2}$ inches.

The unfortunate custom of publishing absorbance spectra on too short a wavelength scale makes it very difficult, even with this device, to make adequately accurate measurements of absorbance values at particular wavelengths from most of the spectra in the literature. To
make important data retrievable from published curves the wavelength scale needs to be adjusted at the time of recording or of plotting so the maximum slope on the side of a steep band is less than 80°. Few published spectra meet this criterion. As digital methods become more widely used in spectroscopy, and as the shape, as well as the peak position of the curves, becomes of interest to more workers, we may hope for editors' encouragement of the publication of data tables as well as curves in the routine reporting of absorption spectra. When digital recording of spectra combined with curve analysis becomes routine, complicated spectra can be reported in complete detail with high accuracy as a table of parameters for the component curves. Then curve fitting, even where it does not necessarily further theoretical understanding, can be used to record spectral measurements in a compact and accurately retrievable fashion.

**ANALYSIS OF SPECTRA OF NATURAL CHLOROPHYLL COMPLEXES**

*C. S. French, J. S. Brown, Lillian Prager, and Mark Lawrence*

We are continuing the attempts to define the absorption spectra of the various naturally occurring chlorophyll complexes so often discussed in this *Year Book*.

What is the significance of this concern about the spectra of the separate forms of chlorophyll in plants? In the first place comes the simple description of the spectroscopy of the most obvious colored substance on earth. Secondly, precise knowledge of the spectra of the separate forms should not only show the number of existing natural chlorophyll complexes but also may lead to some understanding of the structural relations of the chlorophyll molecules to each other and to the remainder of the protein complex. Thirdly, there is the pressing need, for use in kinetic studies of photosynthesis, to know from analyses of absorption spectra what fraction of the incident light of a particular wavelength is absorbed by each photochemical system. Now, that information can be obtained only from action spectra for the two photochemical effects.

The problem we face is illustrated in Fig. 45 where the absorption spectrum of extracted and purified chlorophyll *a* is compared with the absorption spectrum of chloroplast fragments from *Euglena* at liquid nitrogen temperature. In the *Euglena* spectrum the small shoulder at 649 nm is due to chlorophyll *b* while the other humps at longer wavelengths show the presence of four different kinds of chlorophyll *a* complexes. The purpose of the work in progress is to derive the individual absorption spectra of each of these and of other naturally occurring forms of chlorophyll.

One theory about the natural chlorophyll *a* complexes is that there is a comparatively small number of definite and universally distributed forms of chlorophyll *a* each with its own definite and constant absorption spectrum. Accordingly the variation in the shape of measured absorption spectra for different algae and for fractions prepared from chloroplasts is attributed to variations in the relative proportions of these few specific compounds. For many years this concept has been implicit in the work of this laboratory. Another theory, apparently of greater validity, is that the forms of chlorophyll are pigments with absorption maxima of slightly different wavelength positions and widths depending on the source or treatment of the material. A third concept is that there may be such a large number of chlorophyll *a* complexes that their absorption peaks may be found more or less at random from 662 to beyond 715 nm. We expect that the work in progress may clarify the relative merits of these three contrasting ideas.

The effect of anomalous dispersion on the measurements of absorption spectra of chlorophyll complexes is an ever re-
Fig. 45. The spectrum of the alga *Euglena* shows bands due to four different forms of chlorophyll $a$ at 672, 681, 693 and 706 nm. Each of these forms has its own characteristic spectrum presumably somewhat similar to that for isolated chlorophyll $a$ (broken line) but shifted in wavelength. The small band at 649 nm is due to chlorophyll $b$.

To take an extremely sceptical point of view one may ask if the absorption band near 680 nm is a real absorption band from which the presence of a particular form of chlorophyll "Ca 680" may be identified or if this observed peak is at least partially due to increased scattering on the long wavelength side of the 670-nm absorption peak. In fact, the extreme sharpness of the "680" peak in some chloroplast fractions studied this year may eventually be attributed more to scattering by anomalous dispersion than to absorption. The studies in progress on the shapes of absorption spectra of chlorophyll complexes bear directly on the question. The application of theory, such as it is, to experimental measurements of mixed absorption and scattering phenomena (Latimer, *Year Book* 56, p. 265) is not a simple matter although such an attempt has been made elsewhere in the past year (Hagemeister, 1968) with intact *Chlorella* cells.
Some of the different chlorophyll \( a \) complexes are known from action spectra to be associated with two different photochemical reactions in photosynthesis. By comparison with the precision of absorption spectrophotometry, the determination of action spectra is a crude operation at best. Furthermore, the fractionated chloroplast particles whose pigment systems are of particular interest may have very low activities for the measurable photochemical reactions, which makes it more difficult to get action spectra for the fractions than for whole chloroplasts.

Action spectra must of necessity be measured at room temperature; however, it is useful to measure absorption spectra at liquid nitrogen temperature because peaks are sharpened, and overlapping components become more evident. Components resulting from the analysis of spectra at low temperature may then be used to estimate at least the number, and possibly the widths and shapes, of chlorophyll components in room temperature absorption spectra.

As emphasized in *Year Book 66*, p. 171, we are continuing to study mainly the spectra of finely disintegrated chloroplasts or of very pale mutants to avoid distortion effects that would make the data unsuitable for precise curve analysis. This year the improved chloroplast fractionation method of J.-M. Michel and M.-R. Michel-Wolwertz has made it possible to get spectral measurements of more significant material. The use of a digital computer has greatly extended our ability to analyze the shapes of the spectra. In addition to our own measurements we have obtained some original records of particularly significant spectra from colleagues in other laboratories.

Comparisons of spectra at 23° and \(-196\)°C. In order to use the sharper low-temperature spectra as a guide in the analysis of room temperature absorption curves we would like to know how the spectra of the individual forms of chlorophyll \( a \) change with temperature.

Fig. 46 gives the absorption spectra of a very pale *Chlorella* mutant showing only very slight narrowing of the absorption at low temperature. The middle curves of this figure, for a pale mutant *Aesculus* leaf (California buckeye), also show a small narrowing combined with a shift to shorter wavelength by low temperature. The upper curves of Fig. 46, however, illustrate the more usual effect of low temperature. Here we see a strong narrowing of \( Ca \, 677 \) and \(Cb \, 648 \) while \( Ca \, 668 \) appears to be only slightly sharpened. From this figure it is evident that a simple general quantitative relation between all bands at room temperature and at low temperature is not likely to exist. This effect of temperature on the shape of the absorption bands is one of the questions susceptible to investigation by curve analysis.

The RESOL program. Dr. Don D. Tunnicliff of the Shell Development Laboratory, Emeryville, California, gave us a very versatile computer program for the analysis of spectra. This was written for the IBM 7090 and 7094 computers and modified by Mrs. Gilman and Mr. Beebe of the Stanford University Computation Center, User Services Group for the 360/67 computer to which we have access. The plotting routine has been adapted to our needs and some further program modifications are still being made by one of us (Mark Lawrence).

To analyze spectra the absorbance is entered in digital form usually at 1-nm wavelength intervals. Trial component bands are estimated by inspection of the curve or from previous analyses of the same or of other curves. Their wavelength peaks, heights, halfwidths, and the proportions of Gaussian to Lorentzian shape are roughly estimated and entered. The computer will then optimize these four parameters of each band to give the best fit to the absorbance curve. Because all parameters of each component are adjusted for each iteration the convergence is rapid. A dozen or fewer
Fig. 46. The absorption spectra of three plants are shown at room temperature and at liquid nitrogen temperature. The amount of band sharpening is not the same for all plants nor for all the bands in a single plant.

iterations will usually suffice to bring the standard deviation to a value that changes less than the specified amount (usually 0.2%) per iteration.

Gaussian curves are wider near the top and narrower at the bottom than are Lorentzian curves of the same width at half height. Since the program optimizes the proportions of the two functions for each component band, this adjustment of the band shape can improve the fit near the tails of the bands over that given by Gaussian curves alone.

By appropriate selection of the trial input bands, some “guidance” toward one or another possible combination may be
made when the number of bands entered is adequate for more than one solution. The corollary of this flexibility is that if too many bands are used, a mathematically correct solution can be achieved that may have no physical meaning in terms of pigment components. The input bands are modified by the program until the change of standard error of fit for each iteration is within the limit specified. The procedure is thus essentially a means of improving the parameters of the input bands selected by eye. By using a preliminary solution as a guide in the choice of input band parameters for a second solution, improved fits can be had and occasional absurdities may thus be avoided.

The output is given as plotted curves for the derived components, for their sum, for the original data, and for the difference between the sum and the data. The parameters of the derived components are also tabulated. This versatile program allows for choices of types of input data, for variations in the operations performed, and for various presentations of output. Assorted options of fitting procedure, weighting factors, and tolerances may be selected. Conversions of input data to frequency or wavelength scales may be made. If desired, transmission can be entered for conversion to absorbance before the curve analysis.

The provisions for handling a background correction may also be used as a means of entering known bands and using the program to evaluate the residual components. The program itself may be used to determine the number and position of bands from the peaks and inflections. Above all, results can be obtained rapidly and at reasonable cost.

The DSPEC program. It is often useful to compare spectra by subtracting one curve from another. Before doing this it is necessary to have one of the curves plotted to a scale that would make the heights attributable to one or another of the components equal in the two curves. Generally the factor needed to accomplish this objective is unknown. Therefore, a whole family of difference curves have to be plotted with a series of factors applied to one of the curves before the subtraction. Inspection of such families of curves can often lead to an approximate estimate of the wavelength peak, the halfwidth, and shape of components that differ in their quantities in the two spectra. Furthermore, these difference spectra can show, by sharp discontinuities, if the two curves compared differ in the types of components as well as in their relative amounts.

A computer program, DSPEC, was written for this purpose with help from Mr. Beebe of the Stanford Computation Center. This program uses curve input data in the same form as does RESOL and produces a family of difference curves each with the desired adjustment factor.

The GRAPHIC program. So far, surprisingly little use has been made of log absorbance curves in analyzing the spectra of chlorophyll complexes. Absorption spectra plotted as log of absorbance against wavelength give, for a pure substance, curves of identical size and shape that differ with concentration only in their position on the height axis. A program to plot spectral data in a standardized logarithmic form has been prepared for use with the new on-line plotter at the Stanford Computation Center. Comparisons of data so plotted are used to select curves significant enough for detailed analysis by RESOL.

Comments on curve analyses. This year J.-M. Michel and M.-R. Michel-Wolwertz found that the width and shapes of components having nearly the same peak wavelength in two fractions of the same chloroplasts can be very different from each other. Thus there seems to be a distinction of width and shape as well as of peak wavelength between several natural forms of chlorophyll. This fact greatly changes the previous simple assumption that all "Ca 670" and "Ca 680" components used for curve analysis should be identical.

Each chlorophyll form must have its own complete spectrum although we can
only study the shapes of the red part of the spectrum because of carotenoids and other colored substances that interfere below about 500 nm. The major red peak has a far more distinctive shape than do the lower bands near 625 and 585 nm. We need to know not only the characteristics of the major red band but also the relation of the shorter bands to the main peak for each form of chlorophyll. At present the emphasis is on the major red peak. To match it with simple curves of known shape it is, however, necessary also to use some sort of approximation to the bands near 585 and 625 nm because the tail of the 625-nm band overlaps the main component. Attempts to resolve the 625-nm band into several components have not yet succeeded.

Some illustrations of work in progress. During the year two reports have been prepared that include curve analyses of chlorophyll in vivo (French et al. and French and Prager, 1968). We will give here a few examples of the potentialities and limitations of the curve fitting procedure as an aid in the understanding of complex absorption spectra.

To evaluate the comparative merits of different curve fitting procedures, the same curves were analyzed in various ways. One of these was the absorption spectrum of pure chlorophyll a in 80% acetone prepared from Euglena by M.-R. Michel-Wolwertz. Even this simple curve seems to have a "hidden" band at about 650 nm. It is unlikely that this component represents a trace amount of residual chlorophyll b because the starting material contained very little chlorophyll b even before purification and the peak of this hidden component is nearer 650 nm than 645 nm, where it would be for chlorophyll b in 80% acetone. Furthermore, a similar hidden component was also necessary to match the data of Smith and Benitez for pure chlorophyll a in ether.

The following comparisons of different curve fitting procedures were made: all Gaussian, all Lorentzian, mixed Gaussian and Lorentzian; wavelength, wave-number; equal weights for all points, weight proportional to square of absorbance. For each case the standard error of the computed fit is given in Table 12.

Components of pure Gaussian shape gave much better fits than those of pure Lorentzian shape but mixed Gaussian and Lorentzian shapes of the same width at half height and the same wavelength center were better than pure Gaussian curves. This improvement was particularly evident on the long wavelength tail. If mixed Gaussian and Lorentzian shapes are used, the need for another small, long-wavelength band may be avoided in some cases. The fit obtained by weighting the upper part of the bands was better than with equal weighting. Matching the curve with symmetrical components on a wavenumber plot, while perhaps theoretically preferable to a wavelength plot, has so far not given appreciably better fits. The very satisfactory fit for mixed Gaussian and Lorentzian components on a wavelength plot is given in Fig. 47(A).

The standard errors refer to the error of fit between the measured heights of the spectral curves and the sum of the hypothetical components at each wavelength, as illustrated in the error curve below the analyzed spectra. The height units are the counter readings of the curve digitizer; 1 unit = 0.00842 in.

<table>
<thead>
<tr>
<th>Shapes of Component Curves</th>
<th>Wavelength Scale</th>
<th>Wavenumber Scale</th>
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</thead>
<tbody>
<tr>
<td>Points Weighted Proportional to (Absorbance)^2</td>
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<td></td>
</tr>
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<tr>
<td>Mixed</td>
<td>2.79</td>
<td>2.73</td>
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</table>
Fig. 47. (A) The absorption spectrum for pure chlorophyll a in 80% acetone from M.-R. Michel-Wolwertz, shown by points, was matched by the sum of four components by RESOL. The standard error of fit was 2.26. (B) The absorption spectrum at -196°C of fraction 2 from *Stichococcus* was matched by hypothetical components. The standard error of fit was 5.35.
Fig. 48. Absorbance spectra of two fractions from disintegrated *Chlorella* chloroplasts separated by centrifugation in a sucrose gradient matched with hypothetical component curves. Data from M.-R. Michel-Wolwertz.
A much more complicated curve analysis is presented in Fig. 47(B) for the absorption of a fraction of chloroplast particles prepared from Stichococcus. This is a particularly interesting sample because of its high chlorophyll b content and its sharp 677.5-nm peak. This represents a second attempt but nevertheless still shows deviation from our concept of the most believable components. The 630.5-nm band is probably too broad; the chlorophyll b at 649.5 nm is probably nearly reasonable; the 667.7-nm band should probably be narrower and more Gaussian; and the 686.5-nm component should be lower and at a longer wavelength. Both the accuracy of fit and the plausibility of the component curves representing bands of the pigments present can certainly be improved.

In Fig. 48 are comparable analyses of two Chlorella chloroplast fractions of M.-R. Michel-Wolwertz. Again this is a second approximation and subject to further modification. Another component near 695 nm combined with the Lorentzian shape of the 677- and 679-nm bands would be preferable. The standard errors of fit 3.46 and 3.45 have been reduced from the previous values of 5.75 and 6.05 (French et al., in press, Fig. 6). This pair of analyses is based on the idea that the main difference in the two curves is in the width of the 679-nm component rather than in the possible presence of another long wavelength pigment in fraction 1 at 687 nm.

A very rough attempt was made to characterize the difference between the two spectra for the Oenothera mutants in Fig. 18 of Fork, Heber, and Michel-Wolwertz (this report, p. 504). One of these mutants lacks photosystem 1 activity while the other lacks system 2 activity. The curves have their greatest difference at wavelengths longer than 680 nm. This difference may be due to an extra chlorophyll a component specifically active in photosystem 1. However the distinction could equally be due simply to differences in the bandwidth and wavelength position of some chlorophyll components. On that assumption, the “Ca 680” component in the curve for the mutant with system 2 activity would have its peak at 677 nm, and a narrower bandwidth, while the “Ca 680” analogue in the curve for the system 1 pigment would have a peak near 682 nm and a larger bandwidth. A situation of that sort is believed to account for the difference in the two Chlorella fractions illustrated in Fig. 48.

A possible test for the plausibility of the extra component theory versus the above explanation is to examine the calculated difference spectra between the two curves, using a series of fractions applied to one curve before the subtraction. These difference curves are uninterpretable if made from spectra that differ otherwise than in the relative amounts of common components. Thus difference spectra having sharp discontinuities indicate that comparisons have been made of curves composed of dissimilar components.

The results of this application of the DSPEC program to the curves of Fig. 18 is shown in Fig. 49. The small differences between the curves are magnified about 3.5 times so most of the small irregularities have no significance in this plot. A smoothed line was drawn more or less between the two top curves, corresponding to a factor of 0.895. At 641 nm there is a dip perhaps due to a form of chlorophyll b in the system 2 preparation. This dip was omitted in drawing the hypothetical spectrum for the extra component of photosystem 1. The hypothetical component so derived is shown in the lower part of Fig. 49. Its peak at 687 nm has a halfwidth of 13.5 nm and the side band, H = 0.26, has a broad maximum somewhere near 650 nm. There is a wide tail possibly due to a component with a 708-nm peak. The accuracy of the records is hardly sufficient to do more than to suggest the
Fig. 49. Enlarged difference spectra between the curves of Fig. 18 with a series of fractions applied to the photosystem 2 curve before subtraction. If an extra component in the photosystem 1 curve that is lacking in the other is the cause of the difference between the two, the spectrum of that component will resemble the lower curve. The small component at 641 nm shown below has been omitted from the estimated difference component attributed to system 1.
possible presence of this long wavelength component.

In general the experience with curve analyses so far has been that a complete solution from a single attempt is likely only if the bands are clearly evident in the input data. For more complex curves, susceptible to being fitted by a variety of mathematically adequate solutions, some judgment in choice of input bands can be made to guide the fitting toward a solution having a reasonable physical meaning in terms of actual pigment components.

References

STAFF ACTIVITIES

The Botanical Society of America awarded one of its two annual Certificates of Merit to Dr. William M. Hiesey "In recognition of distinguished achievement in and contributions to the advancement of botanical science, ecological physiology and imaginative experiments. A pioneer in elucidating the geneecological nature of species; he has done much to encourage and help students in all areas of plant science."

Dr. Olle Björkman was awarded the degree of Doctor of Philosophy "with great distinction" at the University of Uppsala in December 1967. This degree is awarded in Sweden only to outstanding scholars and confers the title of Member of the Faculty at the University of Uppsala. Dr. Björkman's paper "Comparative Studies on Photosynthetic Properties of Species and Races of Higher Plants from Ecologically Diverse Habitats" reviews some of his research that prompted the awarding of the degree.

The Experimental Taxonomy group served as host to visiting classes of biology students from Stanford University, the University of California at Berkeley, and the University of California at Santa Cruz. Classes led by Dr. Herbert Baker of Berkeley, Dr. Jean Langenheim of Santa Cruz, and Dr. John Thomas of Stanford included field trips to the Stanford, Mather, and Timberline field stations.

Members of the Institution staff have lectured to advanced students on special topics in experimental ecology and on photosynthesis. Drs. Björkman and Fork were appointed "Associate Professor of Biology by Courtesy" at Stanford University for the year 1968-1969.

In addition to the work reported for this year, the Department facilities were used by some Stanford faculty and the following individuals:

Dr. Dan McMahon of the Department of Biophysics at the University of Chicago spent the month of September 1967 at our laboratory in an effort to isolate the enzyme ribulose diphosphate carboxylase in clones of Mimulus cardinalis, originally from different climates, known to differ in their capacity for CO₂ uptake at normal air concentrations. Crude extractions were made at this Department for final purification at the University of Chicago. Mrs. Mary Mantuani, a graduate student at Duke University, together with her husband, utilized the facilities at the Mather station during the summer of 1967. She obtained climatological and ecological field data as background information for experimental laboratory work being continued at Duke. Her study embraces a comparison of tolerance to water stress in ecological races of Solidago. Since the spring of 1967, Mr. Lafayette C. Eaton, a graduate student in the Department of Biological Sciences at Stanford, has been conducting a comparative study on the pattern of varia-
tion in morphology, seasonal responses, and survival in the Ranunculus californicus complex as expressed at the Stanford and Mather transplant stations.

Dr. Hiesey participated in an International Biological Planning Conference and a field trip that was held at Caracas, Venezuela, in November 1967.

Dr. Brown served as Secretary-Treasurer for the Stanford Chapter of Sigma Xi and attended the Annual Meeting in New Hampshire in October.

This year a small lot was purchased from the fund given the Institution by Dr. Bush for staff recreation. Plans for a cabin are being considered. This heavily forested land is nearly adjacent to the Tomales Bay State Park and within a short distance of the Point Reyes National Seashore.

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Björkman, Olle, Comparative studies on photosynthetic properties of species and races of higher plants from ecologically diverse habitats. Printed privately, 1967.


Björkman, Olle, Further studies on differentiation of photosynthetic properties of species and races of higher plants in a cabin are being considered. This heavily forested land is nearly adjacent to the Tomales Bay State Park and within a short distance of the Point Reyes National Seashore.


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


French, C. S., see also Heber, U.


Heber, U., see also Fork, David C.

Kouchkovsky, Y. de, see Fork, David C.

Michel-Wolwertz, M.-R., see Brown, Jeanette S.

Nooteboom, W., see Amesz, Jan.

SPEECHES

Amesz, J., D. C. Fork, and W. Nooteboom, Function of the pigments of the reaction center of system 1, Symposium, Plastidenpigmente und ihre rolle im photosynthese process, Gatersleben, East Germany, October 2, 1967.

Björkman, Olle, Photosynthetic differentiation and adaption in plants from ecologically diverse habitats, Seminar, Department of Biological Sciences, Stanford University, Stanford, California, May 1, 1968.

Björkman, Olle, Differentiation of photosynthetic properties among plants native to habitats with contrasting levels of irradiance, Seminar, Department of Botanical Sciences, University of California at Los Angeles, Los Angeles, California, June 4, 1968.

Clausen, Jens, The genetic structure of natural ecological entities, Lecture, Department of Biology, University of California at Santa Cruz, Santa Cruz, California, October 10, 1967.

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Heber, Ulrich W., Photo-induced shrinkage in leaves, Seminar, Department of Biochemistry, University of California at Davis, Davis, California, April 26, 1968.

Heber, Ulrich W., Control of cyclic electron flow in vivo by photosystem II and electron acceptors, Seminar, Laboratory of Chemical Biodynamics, Lawrence Radiation Laboratory, University of California at Berkeley, Berkeley, California, April 30, 1968.


Michel, Jean-Marie, Fractionation of the photosynthetic apparatus in chloroplasts, Seminar, Department of Physiology, University of California at Berkeley, Berkeley, California, March 20, 1968.


PERSONNEL

Biochemical Investigations

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

Carnegie Corporation Fellow: Ulrich W. Heber

Institution Research Fellows: Marie-Rose Michel-Wolwertz, Jean-Marie Michel, Eckhard E. Loos, James M. Pickett

Technical Assistants: Mark C. Lawrence, Lillian K. Prager

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Staff: Olle Björkman, William M. Hiesey, Malcolm A. Nobs; Jens C. Clausen, Emeritus

Institution Research Fellows: Eckard Gauhl, James H. Silsbury

Technical Assistant: Frank Nicholson

Visiting Investigator: Daniel McMahon

Gardener: Archibald H. Lawrence

Summer Research Assistant: Steven G. Wood
Part-time Garden Helpers: James M. Barnes,11 Andrew J. Libertone12
Illustrator: Carolyn R. Clark13
Clerical Assistant: Marylee Eldredge
Accountant-Administrative Secretary: Clara K. Baker
General Department Secretaries: Lena R. Barton,14 Wilta M. Stewart15
Mechanical Engineer: Richard W. Hart
Custodian: Jan Kowalik
Custodian-Helper: Wayne E. Miller16

3 From January 6, 1967. From Institut für Angewandte Botanik, Technische Hochschule, Munich.
4 From October 1, 1965, through July 28, 1967. From Department of Zoology, University of Texas, Austin, Texas.
5 From July 1, 1967.
6 From April 11, 1967. From Botanisches Institut der Johann Wolfgang Goethe-Universität, Frankfurt.
7 From July 21, 1967, through May 1, 1968. From University of Adelaide, Waite Agricultural Research Institute, Glen Osmond, Australia.
8 From August 24, 1967, through October 2, 1967. From the Department of Biophysics, University of Chicago, Chicago, Illinois.
9 From July 1, 1967.
12 From October 3, 1967.
13 From October 4, 1967.
15 From April 12, 1966, through October 31, 1967.
16 From November 3, 1967.
Plate 1. Growth of *Mimulus cardinalis*, Jacksonville 7211-4, under 21% and 4% oxygen and two different levels of CO₂.
Plate 2. Growth of *Zea mays*, Ferry-Morse hybrid 901, under 21% and 4% oxygen and a low CO₂ concentration.