Carnegie Institution

Annual Report of the Director
Department of Plant Biology

290 Panama Street, Stanford, California 94305

1975–1976

Reprinted from
Carnegie Institution Year Book 75
for the year July 1, 1975–June 30, 1976
Issued December 1976
THE CARNEGIE INSTITUTION OF WASHINGTON is engaged in education and basic research in the physical and biological sciences. It was founded on January 28, 1902, by Andrew Carnegie and uses its resources "to encourage, in the broadest and most liberal manner, investigation, research, and discovery, and the application of knowledge to the improvement of mankind."

The Institution's program and activities are reviewed annually in May by a Board of twenty-four Trustees. During the year the Board of Trustees is represented by an Executive Committee which meets quarterly. The chief administrative officer of the Institution is the President.

Administrative Offices of the Institution are at 1530 P Street, Northwest, Washington, D.C. 20005.

The Hale Observatories, 813 Santa Barbara Street, Pasadena, California 91101, are operated jointly by the Institution and the California Institute of Technology, in a program of astronomical research on the structure and dimensions of the universe and the physical nature, chemical composition, and evolution of celestial bodies.

The Geophysical Laboratory, 2801 Upton Street, Northwest, Washington, D.C. 20008, conducts physicochemical studies of geological problems, with particular emphasis on the processes involved in the formation and evolution of the earth's crust.

The Department of Terrestrial Magnetism, 5241 Broad Branch Road, Northwest, Washington, D.C. 20015, treats a wide range of studies in physics and related sciences, including astrophysics, geophysics, nuclear and atomic physics, and biophysics.

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

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

Introduction (Briggs) .................................................. 351
Sequence organization in pea DNA (Thompson) ...................... 356
DNA sequence comparisons in Atriplex (Belford and Thompson) .......... 362
Plasmid modification associated with the loss and acquisition of virulence in Agrobacterium tumefaciens (Rogler and Thompson) ....................... 367
Blue light–induced absorbance changes in membrane fractions from Neurospora crassa (Brain and Briggs) ......................... 372
Blue light–induced absorbance changes in membrane fractions from Zea mays (Briggs, Freeberg, and Weiss) ........................................ 377
An evaluation of markers for plasma membranes in membrane fractions from Zea mays (Cross and Briggs) ....................... 379
A multi-sample automatic monitoring device for the circadian rhythm of transmittance change in Ulva (Britz) ...................... 383
Two applications of a multi-sample automatic monitor for rhythmic transmittance changes in Ulva (Britz) ......................... 392
Measurements of auxin binding to solubilized receptor sites of corn seedling tissue (Dohrmann and Ray) ......................... 395
Comparison of the heat stability of photosynthesis, chloroplast membrane reactions, photosynthetic enzymes, and soluble protein in leaves of heat-adapted and cold-adapted C₄ species (Björkman, Boynton, and Berry) ........................................ 400
Genetic variation affecting metabolic phenotypes: an approach to analyzing photosynthetic carbon reduction in a C₃ plant (Enama) ......................... 407
Molecular weight variation of phosphoenolpyruvate carboxylases from C₄ plants (Enama) ........................................ 409
Photosynthetic capacity of in situ Death Valley plants (Mooney, Björkman, Ehleringer, and Berry) ........................................ 410
Leaf absorbance and photosynthesis as affected by pubescence in the genus Encelia (Ehleringer) ........................................ 413
Carbon dioxide and temperature dependence of the quantum yield for CO₂ uptake in C₃ and C₄ plants (Ehleringer and Björkman) ......................... 418
Hybridizations in Atriplex (Nobs) ........................................ 421
Growth and photosynthesis of Chlamydomonas reinhardtii as a function of CO₂ concentration (Berry, Boynton, Kaplan, and Badger) ........................................ 423
Effects of temperature on respiration and uptake of Rb⁺ ion by roots of barley and corn (Carey and Berry) ......................... 433
Possible use of hollow fiber hemodialysers in soil-plant water relations research (Mahall) ........................................ 438
Enzyme polymorphism in the butterfly Colias: selection on metabolic phenotypes (Johnson) ........................................ 440
INTRODUCTION

On Friday, September 12, 1975, the Department of Plant Biology welcomed over 100 plant scientists to a brief symposium on the scientific programs of the department, followed by a reception and dinner. The purpose of the occasion was to celebrate the completion of construction and the beginning of normal scientific activity in the Department for the first time since the autumn of 1973. At that time, of course, we had no idea what constituted normal scientific activity, although we had made some guesses. One of these guesses is found in the introduction to last year's Report, in which I stated that we anticipated a total of about 15 scientific visitors at steady state. A brief glance at the section entitled "Personnel" at the end of this report will reveal how wide of the mark this estimate was. In addition to 11 postdoctoral visitors whom we were able to support with our fellowship funds, we had an additional 8 who came with their own support, and the steady-state level was 17 most of the time. There were in addition 15 students, both undergraduate and graduate, yielding an overall steady state of 32, over twice that predicted a year ago!

The principal reason for the large increase in the population of visitors doing science is the increased emphasis of the Department of Plant Biology on education below the postdoctoral level. Thus, three Stanford University undergraduates have been doing senior honors research with us, and five Stanford graduate students spent some or all of their research time at the Department. Four graduate students are finishing thesis work, having started it with a present faculty member while he was elsewhere, and two of our senior guests, with us on sabbatical leave, brought an additional three graduate students with them. These dry statistics should not hide the kinds of interactions that have made the Department's contribution to predoctoral education remarkable: A single example will suffice. Dr. George Johnson, a senior visitor, brought two graduate students with him from Washington University of St. Louis, Mary Enama and William Curtis, both in the beginning stages. Curtis learned gas exchange measurement techniques from Professor Mooney of Stanford, another senior visitor; he learned about the genus Viola from Professor Solbrig of Harvard, a third senior visitor. Curtis, who has developed a sound thesis problem on this genus, will spend the rest of the summer of 1976 working with Professor Solbrig at Harvard. Enama began learning the kinds of powerful electrophoretic techniques that Johnson has used to uncover hidden genetic variability in insects, with the object of applying them to higher plant proteins. That they can be used effectively is indicated by what she has contributed to this Year Book. Both Distichlis spicata and Atriplex lentiformis, species that fix CO₂ by the C₄ pathway of photosynthesis, are elegantly adapted for such analysis. Enama also has found a sound thesis project. We expect to see both of these students again.

The Department made a number of formal teaching contributions to Stanford University. Dr. Briggs joined Professor Philip Hanawalt of the Department of Biological Sciences to offer a course in photobiology. Drs. Berry, Brown, Fork, and Stemler gave lectures in the series on photosynthesis in the course. In addition, Drs. Briggs and Berry participated in a series of lectures on photosynthesis for the Biochemistry Department at Stanford, and Drs. Fork, Briggs, Berry, and Thompson gave lectures in Professor Ray's plant physiology course. We fully expect this sort of teaching contribution to continue in the future.
Before considering some of the scientific accomplishments of the past year, a brief word about instrumentation is in order. The most important major addition is a Hewlett-Packard minicomputer the Department purchased last fall. It has analog-to-digital capability, and two spectrophotometers are already on line with it. Thus absorption or fluorescence spectra or kinetic data can be stored and manipulated. We anticipate putting more instruments on line in the near future. A fine library of programs has already been developed and is continually being augmented. For most applications at present, two people can work simultaneously at different terminals without interference. With ample core memory and double disc storage capacity (one disk is interchangeable in addition) we are far from reaching the full potential of the system.

I am gratified to report that the past year has been extremely productive scientifically. The large increase in the number of active workers makes the sorting out and summarizing of the year's accomplishments a very difficult task, however, and one that I undertake with some trepidation. Thompson's group, the newest in the Department, has made substantial progress in several directions. Rogler and Thompson have made a notable contribution to our knowledge of what is required for virulence in the crown gall bacterium Agrobacterium tumefaciens. They have been able to show, using restriction endonuclease, that only a relatively small portion of plasmid DNA from the bacterium is correlated with infectivity. Less than the entire plasmid is required. Thompson has continued and extended his careful analysis of the sequence organization of pea DNA. The pea genome shows a pattern much like that of the amphibian Xenopus, with single-copy DNA sequences less than 3000 nucleotides in length extensively interspersed with repetitive sequences. Belford and Thompson have successfully applied DNA-DNA hybridization techniques to an analysis of the relationships between several species of the genus Atriplex, a genus already the subject of an enormous amount of physiological and ecological work in the Department. Preliminary results from hybridization of single-copy DNA from each species did not completely support the current evolutionary scheme for relationships between these species. Though further experimentation is obviously required, it is already clear that the technique is going to be useful in assessing evolutionary relationships within the genus, particularly with regard to the origin of C4 photosynthesis within the genus and the DNA sequence homology distance between C3 and C4 species. These experiments are an important counterpart to the continuing interspecific hybridization studies in the genus Atriplex being carried out by Nobs.

Briggs' group also has progress to report on several fronts. Efforts have continued to isolate and identify the pigment system that serves as the photoreceptor for a large number of blue-ultraviolet sensitive processes both in higher plants and fungi. Brain, working with the mold Neurospora, and Freeberg and Weiss, working with corn, have isolated membrane fractions that respond to light by showing absorbance changes. In darkness the absorbance changes decay. The responses are caused by the reduction of a b-type cytochrome, and the actual photoreceptor is almost certainly a flavoprotein. The behavior of the system in vitro is strikingly similar to in vivo systems previously studied by others.

Cross has shown that the corn fraction contains a monovalent cation-dependent ATPase, and Brain has verified this result with Neurospora. Most recently Cross has used a fluorogenic reagent with an extremely short lifetime in water to attempt surface labeling of corn cells. The object was to determine which of the membrane fractions found in homogenates was the outermost one, the plasma membrane in intact cells. Preliminary experiments strongly suggest that the ac-
cells and their flux pump activity to convert the substrate for anhydrase.

It may be that papers in the Department of Plant Biology, studies of which have been collected, have analyzed the area of membrane regulation of ion movement.

In keeping with long Department tradition, Britz has developed some elegant instrumentation to use in his studies on the circadian movement of chloroplasts in the alga Ulva lactuca, sea lettuce. He has developed an automatic monitor that can handle 30 samples simultaneously, measuring transmittance of light through the samples once an hour. The measuring beam is dim enough to have no influence on the rhythm itself. Using the monitor he has been able to demonstrate antagonistic effects of the two inhibitors colchicine and cytochalasin B on chloroplast movement. Since the first is known to interact with microtubules and the second with microfilaments, the results suggest that these two contractile protein systems regulate chloroplast position by operating in opposing directions in a push-pull fashion. The data are sufficiently precise that extremely small perturbations of chloroplast movement are readily detected. For example, in less than two hours of a phase-shifting pulse of light there is a dramatic change in the course of chloroplast movement. Some progress has also been made in putting the data into the computer for normalization, averaging, and other manipulation, but the system is not yet on line.

In another kind of study with corn membrane fractions, Dohrmann and Ray of Stanford University have made some exciting progress in isolating and solubilizing a membrane-bound binding site for the plant growth hormone 2-indoleacetic acid. Though hormone receptors are by now well known in several animal systems, plant receptors have remained refractory despite strenuous efforts in a number of laboratories. The Dohrmann and Ray results are extremely promising and could be a prelude to a major step forward in our knowledge of the action of this important plant hormone.

The Physiological Ecology group have continued and extended their series of studies on the ways in which plants adapted to extreme environments withstand stress, and on which systems are normally limiting. Thus Björkman, Boynton, and Berry have investigated a number of systems in two species, an extremely thermophilic species, Tidestromia oblongifolia, and a cool temperate species, Atriplex sabulosa, both C₄ species. They compared the effect of temperature on the quantum yield for photosynthesis by intact leaves with its effect on photosystem I and II activities; ribulose 1,5-diphosphate carboxylase activity (the C₃ enzyme for CO₂ fixation); phosphoenolpyruvate carboxylase activity (the C₄ enzyme for CO₂ fixation); and heat coagulability of soluble proteins as a measure of irreversible denaturation. It is clear that the best fit with thermal inhibition of photosynthesis in both cases is with system II activity. In addition, however, there were interesting differences in heat stability of the C₃ enzyme from the two species, suggesting that not just lipids but proteins may differ between high- and low-temperature-adapted plants. An additional finding, obtained in collaboration with Enama, was that the molecular weight of the C₄ enzyme from Tidestromia was almost twice that of the various Atriplex species, another oddity of this remarkable plant.

Mooney, Björkman, Ehleringer, and Berry have continued their seasonal studies of photosynthetic capacity of Death Valley plants. The creosote bush Larrea divaricata maintained an almost constant photosynthetic capacity under natural conditions all through the year. This homeostatic adjustment could be partially explained by changes in the temperature optimum for photosynthesis with the season, a response...
above which irreversible thermal damage occurred. The maximum for the chlorophyll-protein reaction center complex was very high, at 63°C, consistent with evidence from elsewhere that photosystem I is more resistant to high temperature than photosystem II. Parallel measurement of photochemical activity versus temperature with some of the organisms showed good correlation between change in fluorescence and change in photosynthetic activity.

Whereas Fork's studies involved relatively slow and steady temperature changes, Schreiber studied the effects both of slow heating and of a very rapid temperature jump (largely completed within 2 sec) on fluorescence kinetics with the alga *Scenedesmus obliquus*. He used cells grown at two different temperatures, and monitored fluorescence under two extreme conditions: with photosystem II reaction centers closed (high light intensity, electron transport blocked by an inhibitor) or with photosystem II reaction centers open (low light intensity, system kept aerobic). The fluorescence under the second condition, \( F_0 \), shows a sharp rise between 46° and 52°C, while the fluorescence under the first, \( F_{max} \), showed a continuous decline with increasing temperatures, with a suggestion of a steeper decline above 46°C. The slope changes began a degree higher for cells grown at higher temperature. The kinetics for changes in \( F_0 \) and \( F_{max} \) with a sharp temperature jump revealed an inverse relationship with an increase in \( F_0 \), paralleling a decrease in \( F_{max} \). Although the kinetics are complex, the relationship holds for jumps to temperatures below those causing irreversible damage. A working hypothesis is that temperature affects the distribution of energy between photosystems I and II, with higher temperatures favoring photosystem I, as monitored by the \( F_0 \) fluorescence. Schreiber also documented a role of preillumination in the expression of heat damage in fluorescence measurements. His studies, together with Brown's and Fork's, further underline the value of fluorescence studies in attempts to unravel the extreme complexities of photosynthesis.

Finally, Stemler has made some detailed studies of the binding of \( \text{HCO}_3^- \) to washed thylakoid membranes. He had previously demonstrated the importance of bicarbonate in activation of the \( \text{O}_2 \)-evolving system (entirely independent of its role as carbon donor). By careful binding studies, he has now shown that thylakoids have at least two classes of bicarbonate binding sites: a high affinity class, saturated by about 1 mM \( \text{HCO}_3^- \), and a lower affinity class, saturated above 10 mM. The second class is more strongly implicated in the \( \text{O}_2 \) evolution role, since at least 10 mM bicarbonate is required to restore normal oxygen-evolving capacity to washed thylakoids.

The above summary can hardly do justice to this past productive year. It should, however, provide evidence for a viable and vigorous group of investigators working on fundamental problems of plant biology and making exciting progress on a number of fronts. As we inevitably must depend more and more on solar energy for food, fuel, and fiber, basic knowledge of solar energy conversion as practiced in photosynthesis, of the nature of adaptation to conditions permitting extremely rapid conversion in nature, and of the fundamental mechanisms of plant growth and development must assume high priority.

**SEQUENCE ORGANIZATION IN PEA DNA**

_W. F. Thompson_

In a majority of animal genomes so far studied, single-copy DNA sequences are extensively interspersed with repetitive sequences. A variable but usually prominent fraction of the repetitive DNA in these genomes ap-
pears to be composed of elements some 200–400 nucleotides in length. Additional repetitive elements appear to be much longer (greater than about 1500 nucleotides). This pattern of sequence organization is often called the *Xenopus* pattern, in recognition of the extensive studies carried out on the DNA of this organism. Although different patterns have been found in *Drosophila* and honey bee DNA (Manning *et al.*, 1975; Davidson *et al.*, 1975a), the main features of the *Xenopus* pattern have been found to hold for a surprisingly wide variety of animal genomes (Goldberg *et al.*, 1975; Davidson *et al.*, 1975a, b). Extensive interspersion of repetitive and single-copy DNA sequences has provided the basis for an attractive model of eukaryotic gene regulation (Davidson and Britten, 1973) which is consistent with a rather large body of experimental evidence from animal systems. Until recently, however, no information was available concerning interspersion of sequences in DNA of higher plants. Higher plants typically have much larger genomes, with much higher percentages of total repetitive sequence DNA, than most animals. Information on sequence organization in plant DNA will be of use in studies of molecular evolution and in experiments on gene regulation during development.

A recent study of the cotton genome (Walbot and Dure, 1976) shows that repetitive and single-copy sequences are interspersed in a fashion consistent with the *Xenopus* pattern, although both types of sequences are longer in cotton DNA, and the size distribution of repetitive elements is considerably more heterogeneous. The cotton genome (0.795 pg/cell, haploid) is considerably smaller and contains a smaller proportion of repetitive sequences (40%) than the genomes of many other higher plants. For example, Flavell *et al.* (1974) studied 23 species with DNA contents (1C) ranging from 0.75 to 49 pg and found repetitive sequences accounted for between 46% and 92% of the total DNA. Excluding polyploid species, the range of DNA content was from 0.75 to 31 pg, with the same range of repetitive sequence content. Thus, while the cotton genome data represent a significant advance in our understanding of plant DNA sequence organization, studies of plant species with more typical genome sizes and repetitive sequence contents are clearly required. We report here the results of some preliminary experiments on the garden pea (*Pisum sativum* L.), which has a haploid nuclear DNA content of 5 pg and about 75% repetitive DNA.

**Interspersion**

Figure 1 depicts the reassociation of short fragments of pea DNA (350–400 nucleotides) in 4 M NaClO₄ (Hoyer and van de Velde, 1974). Almost two-thirds of the DNA reassociates by Cot 5 (corresponding to approximately Cot 18 in 0.12 M Na phosphate). The remaining

![Fig. 1. Reassociation of pea DNA in 4 M NaClO₄-0.18 M Na phosphate buffer at T_m-25. Open circles represent the reaction of short (350–400 nucleotide) fragments as measured by hydroxylapatite binding, while the solid circle shows the binding (standard deviation of 5 determinations) of 3200 nucleotide fragments reassociated to Cot 5 under the same conditions. Samples were diluted into 0.12 M Na phosphate buffer and fractionated on columns equilibrated at 60°C; bound DNA was eluted with the same buffer by raising the temperature of the column to 97°–98°C. Cot values are not corrected for the accelerating effect of NaClO₄ (about 3.5-fold, relative to 0.18 M Na+).](image-url)
approximately one-third is composed of a small fraction of more slowly reassociating repetitive sequences and single-copy DNA. When DNA fragments 3200 nucleotides long are reassociated to $C_{ot} = 5$, about 93% bind to hydroxylapatite, indicating that the majority of slow repetitive and single-copy sequences are distributed in such a way that most fragments of 3200 nucleotides containing such sequences also contain repetitive DNA capable of forming duplexes by $C_{ot} = 5$. Or, put another way, the slowly reacting sequences are mostly interspersed with rapidly reacting sequences, and there appear to be relatively few regions of slowly reacting DNA in excess of about 3000 nucleotides in length. Experiments in progress are designed to provide a more quantitative analysis of single-copy sequence lengths in pea DNA.

**LENGTH OF REPETITIVE SEQUENCES**

In most plant genomes, repetitive sequences account for 2-3 times as much DNA as do single-copy sequences. If all the repetitive DNA were present as 200-400 nucleotide elements separated by single-copy elements, the average length of single-copy sequences could only be of the order of 100-150 nucleotides. This length is less than that observed in other organisms—and smaller than a typical structural gene—by about an order of magnitude. Thus we would expect a priori that regions of repetitive DNA substantially longer than 200-400 nucleotides must exist in plant genomes, including pea genomes. Aggregation (or hyperpolymerization) during repetitive sequence reassociation with short DNA fragments from peas and other plants is probably related to the presence of such long repetitive sequence regions (Thompson, 1975, 1976).

However, several possibilities exist for the organization within a long repetitive region. On one extreme, an entire region may be composed of a single long sequence (or a series of identical short sequences), repeated in its entirety at different sites in the genome. The other extreme would involve clustering of several smaller repetitive units. Clusters at different locations in the genome could involve different orders and/or combinations of short sequences, so that any given sequence would have different neighbors each time it occurred.

In beginning experiments on organization of repetitive sequences in pea DNA, we have tried to determine how much of this DNA is contained in long repetitive regions or tandem repeats. The experiments involve reassociating long fragments so that only repetitive sequences can react ($C_{ot} = 5$ in 4 M NaClO$_4$ buffer). Single strands are then removed by digestion with the single strand-specific nuclease S1 (Ando, 1966; Britten et al., 1974), and the size distribution of the remaining duplexes is determined. Long repetitive sequences or tandem repeats are expected to produce long S1-resistant duplexes, while short repeats occurring in different combinations or interspersed with single-copy sequences will yield short duplexes after S1 digestion.

Figure 2 shows the specificity of S1 nuclease for single strands under our digestion conditions. Native or denatured $^3$H-E. coli DNA was mixed with reassociated 3200-nucleotide fragments of pea DNA and subjected to S1 digestion. Digestion products passed through hydroxylapatite in 0.03 M Na phosphate at room temperature; essentially all the resistant DNA remained bound to the column at 60°C in 0.15 M Na phosphate and was eluted by raising the temperature to 97°C. Completely single-stranded DNA is digested rapidly, and there is little or no digestion of native DNA. Reassociated pea DNA is digested rapidly early in the reaction, presumably reflecting the removal of completely single-stranded regions. Subsequently the reaction is slow but significant, which may reflect
slow digestion of DNA in regions of mismatched base pairs within otherwise duplex structures. To minimize this second process while ensuring complete digestion of single-stranded DNA, experimental treatments were carried out for 30 min. After correcting for the approximately 5% of $A_{260}$ which failed to bind in 0.03 M NaPB without S1 treatment, about 58% of the pea DNA is resistant to digestion. This value is in reasonable agreement with the binding of short fragments to hydroxylapatite after reassociation to the same $C_d$, and it therefore appears that these S1 conditions set approximately the same criterion for recognition of duplex structure as fractionation on hydroxylapatite at 60°C.

Nuclease-resistant duplexes were analyzed by chromatography on agarose (A50 m) gel filtration columns in 0.12 M Na phosphate (Davidson et al., 1974; Goldberg et al., 1975). These columns are thought to exclude double-stranded DNA longer than about 1000-15000 nucleotide pairs. Fragments of pea DNA 3200 nucleotides long were reassociated and treated with S1 nuclease as above. The resistant duplexes were isolated by binding to hydroxylapatite and eluting with 0.4 M Na phosphate. This material was then mixed with marker $^{3}H$-E. coli DNA (400 nucleotide pairs) and applied to the column. The results are shown in Fig. 3A. About one-third of the sample was in the form of long duplexes in the exclusion peak. The remainder eluted as a broad band, indicating a heterogeneous distribution of lengths, although there is some suggestion of a peak in the region of 300-400 nucleotide pairs. These re-
Fig. 3A. Agarose gel filtration of S1 nuclease-resistant duplexes. Pea DNA fragments (3200 nucleotides) were reassociated and treated with S1 nuclease for 30 min under the conditions of Fig. 2. Resistant duplexes were eluted from hydroxylapatite with 0.4 M Na phosphate buffer and mixed with trace amounts of sheared, native $^3$H-E. coli DNA (400 nucleotide pairs) and $^{14}$C-thymidine. The mixture was applied to a 1.2 x 45 cm column of Bio-Gel A50m equilibrated with 0.12 M Na phosphate buffer. Filled circles, pea DNA $\Lambda_{260}$; open circles, $^3$H-E. coli DNA; filled triangles, $^{14}$C-thymidine inclusion marker.

Results may underestimate the proportion of long duplexes which form by $C_{ot}$, since some 10%–15% of the S1-treated DNA could not be recovered from hydroxylapatite by salt elution, and we have observed in other experiments that hydroxylapatite fractionation reduces the size of long DNA fragments. In a preliminary experiment in which an S1 digest was analyzed without prior fractionation, about 60% of the DNA eluting prior to the void volume was in the excluded peak.

In alkaline sucrose gradients, most of the DNA from the excluded fraction sedimented as a relatively symmetrical peak around approximately 1500 nucleotides. Thus reassociation, S1 treatment, and HAP fractionation caused about a twofold reduction in the single-stranded length. Rice (1974) has found a similar reduction after S1 treatment of reassociated T-4 DNA, which is presumably caused by the failure of randomly sheared fragments to reassociate over their entire length. Smith et al. (1975) have recently determined the average first collision overlap for randomly sheared simple DNA to be 0.55 of the single-stranded length. Thus, the data do not differ markedly from results predicted on the assumption that most of the DNA giving rise to the excluded fraction was composed of repetitive sequences as long as or longer than the 3200-nucleotide fragments used. Further analysis of the size distribution of long repetitive sequences will therefore require DNA fragments much longer than 3200 nucleotides. However, it is clear that individual repetitive sequences of substantial length are a prominent feature of the pea genome.

In several animal DNAs, including Xenopus, sea urchin, clam, and cow, it has been found that the longer repetitive sequences melt with a higher $T_m$ than the short, interspersed repetitive sequence elements, even after correcting for the effect of duplex length on thermal stability (Davidson et al., 1974; Davidson et al., 1975a, 1975b; Goldberg et al., 1975). This observation appears to hold also for pea DNA, as
shown in Fig. 3B. Duplexes eluting in the exclusion peak of agarose columns exhibit high thermal stability, with a $T_m$ only about 1.5° lower than that of native DNA, but the $T_m$ is rapidly reduced in later fractions. Length effects per se would be expected to result in only about a 2°-3° reduction in $T_m$ for fragments in the range of 300 nucleotides in length (Britten et al., 1974). Thus, most of the $T_m$ reduction seen in short duplexes from the agarose column profile must be attributed to mismatched bases, and the short duplexes must therefore contain a larger fraction of mismatched bases than longer duplexes. While the significance of this observation is not understood, one may speculate that the shorter sequences are, on the average, evolutionarily older and more diverged than are longer repetitive sequences.

CONCLUSION

Although many details remain to be worked out, it is clear from the data so far that sequence organization in the pea genome resembles the pattern for Xenopus and is distinctly different from that for Drosophila. Pea single-copy sequences are mostly less than 3000 nucleotides in length and are extensively interspersed with repetitive sequences, which is consistent with the Xenopus pattern but contrasts sharply with the very long (10,000 nucleotides or more) single-copy elements in the Drosophila genome (Manning et al., 1975). Short (200-400 nucleotide) repetitive elements are less prominent—and longer repetitive sequences more prominent—in pea DNA than in the DNA of most animals (Goldberg et al., 1975). However, the main features of the Xenopus pattern—extensive interspersion of single-copy DNA sequences and the presence of at least some short repetitive elements—are to be found in pea DNA as well. A more quantitative comparison must await more data.

We do not yet know whether all the short repetitive sequences in pea DNA are adjacent to single-copy sequences, as they appear to be in several animal

![Fig. 3B](image-url)
At present it is equally possible that some, or even most, of these short sequences might be arranged in variously ordered clusters, as in the "permuted tandem repetition" model proposed by Cech and Hearst (1976) for highly repetitive mouse main-band DNA. We hope that more information on this question can be obtained in future experiments.

References


DNA SEQUENCE COMPARISONS IN Atriplex

Heather Strong Belford and W. F. Thompson

Last year (Year Book 74, pp. 780-791) we reported on the early stages of a project involving evolutionary studies of Atriplex DNA. Our objectives are to provide information on the phylogenetic relationships among the species in this genus which we hope will be of value in interpreting the evolution of C3 photosynthesis and other adaptations, and (more generally) to characterize DNA changes associated with speciation and evolution in higher plants. This year, we report our first results from comparisons of single-copy DNA sequences in four species. While still preliminary, these results clearly demonstrate the applicability of the technique to Atriplex species and raise some questions concerning previous phylogenetic interpretations. To our knowledge, this communication represents the first report of evolutionary comparisons carried out with single-copy DNA sequences from higher plants.

The use of single-copy DNA for evolutionary comparisons has several advantages, in spite of the fact that the experiments are technically more difficult than those using total DNA. In contrast to total DNA, in which rapid reassociation between related but nonidentical repetitive sequences occurs, single-copy sequences reassociate slowly and form precisely paired duplexes. Thus interspecific DNA hy-
bridizations may be carried out with purified single-copy tracer under conditions in which the reaction of tracer sequences with themselves is insignificant and in which virtually all the mispairing in interspecific duplexes can be attributed to evolutionary divergence between the species being compared (Kohne, 1970). In addition, the use of single-copy DNA avoids problems associated with possible addition or differential amplification of repetitive sequences in different species, with the result that measurements of single-copy DNA homology can be more simply related to evolutionary divergence time than measurements using total DNA.

Because these considerations simplify interpretation of data obtained from experiments with single-copy DNA (and because we are able to isolate a reasonable amount of single-copy DNA from *Atriplex*), we have chosen to focus our initial experiments on this fraction. We hope that experiments with single-copy DNA will yield useful phylogenetic information and provide a more solid foundation on which to base future studies of repetitive sequence evolution.

Table 1 shows the evolutionary relationships thought to exist among a variety of *Atriplex* species and indicates the four species chosen for initial investigation. We wished to determine the degree of DNA homology between representatives of closely related species groups as well as between species presumed to be much more distantly related. *Atriplex hortensis* and *A. triangularis* were therefore selected as representing groups closely related by morphological criteria. *A. serenana* should be distinctly different, since it is considered to represent a relatively high degree of advancement in a different subgenus. *A. sabulosa* was expected to be intermediate, although closer to *A. hortensis* than *A. serenana*.

Single-copy sequences were purified from *A. hortensis* DNA which had been sheared to about 350–400 base pairs in a Vir-Tis “60” homogenizer (Britten et al., 1974). It was heat denatured and allowed to reassociate at 25°C below the *T_m* of native DNA (*T_m* = 25°C) in 4 M NaClO₄ containing 0.18 M Na phosphate buffer (Hoyer and van de Velde, 1974) to a Cot of 100. (Under these conditions reassociation proceeds at about 3.5 times the rate in 0.12 M NaPB at 60°C [Hoyer and van de Velde, 1974; our unpublished results] and thus the “equivalent Cot” for this reaction is about 350.) After reassociation, the samples were diluted 30-fold in 0.12 M Na phosphate and fractionated on hydroxylapatite columns at 60°C by standard techniques. About 70% of the total DNA bound to the column and was considered to be reassociated repetitive sequences. The 30% which failed to bind (containing unreassociated single-copy DNA) was passed over a column of AG-50W × 8 cation exchange resin (Bio Rad) to remove Ca++ and any other residual basic impurities. It was then chromatographed on a column of agarose gel (A1.5 m, Bio Rad) to remove any degraded fragments, and the material eluting in the void volume (about 75% of the A260) was used to prepare highly radioactive single-copy tracer by iodination.

Iodination was carried out for 10 min at 60° with Na¹²⁵I (Amersham, pH 8–11) following the technique of Orosz and Wetmur (Orosz and Wetmur, 1974), except that the buffer was 10 mM Na acetate, pH 5.2, and most of the unbound iodine was removed by chromatography on Sephadex G50 prior to the dialysis step.* The product had a specific activity of 5 × 10⁶–6 × 10⁶ cpm/μg (approximately 5% of cytosine residues iodinated) and a mean fragment length of 250–300 bases. It was highly enriched in single-copy sequences, as shown in Fig. 4.

*These conditions were designed for iodination of single-stranded total DNA under conditions minimizing reassociation of repetitive sequences during the reaction.
TABLE 1. Phylogenetic Scheme for the Genus Atriplex Based on Morphological Criteria

To measure sequence homology between this tracer and DNA from other species, aliquots of the tracer at low concentration (1.7 μg/ml) were mixed with a 3000-fold excess of total sheared DNA (5 mg/ml) from each of the four species. The mixtures were heat denatured and reassociated at $T_m - 25°C$ in NaClO$_4$ buffer. Under these conditions, the tracer is too dilute to reassociate significantly with itself (this was experimentally verified in control reactions containing only the tracer DNA), and the observed duplexes therefore result from reassociation between tracer and unlabeled DNA sequences. Since the high concentration of unlabeled DNA is required to drive the reaction, this DNA is often called "driver" DNA in the following discussion.

After dilution in 0.12 M NaPB, the reassociated mixtures were passed over hydroxylapatite at 60°C. The bound fraction, containing duplexes between
125I-A. hortensis single-copy sequences and driver DNA from various species, was then subjected to thermal elution with 0.12 M Na phosphate at increasing temperatures. The apparatus used consisted of multiple columns maintained in the same (rapidly stirred) water bath in order to assure temperature uniformity among the various samples. Results may be expressed both in terms of the extent of tracer reaction (cpm bound to HAP = total interspecific duplex) and the thermal stability of the hybrids.

Figure 5 shows the results of a thermal elution experiment using duplexes formed by incubation to a driver DNA Cot of 1200 at Tm -25°C. The data are plotted in a differential form which allows simultaneous visualization of the amount of interspecific duplex and the distribution of this material in various thermal stability classes. In the control reaction, most of the tracer forms high-stability duplexes as expected for single-copy DNA. The shoulder at 70°C is consistent with the small contamination by repetitive sequences noted in the reassociation kinetics (Fig. 5), while most of the duplexes elute as a symmetrical peak around the Tm for native DNA (indicated by the arrow at 85°C).

In contrast, most products of the interspecific reactions show much reduced thermal stability, melting as broad peaks between 65° and 85°C. Data from this experiment are summarized in the left column of Table 2. Relative to the control, mean thermal stability of all three interspecific products is depressed to nearly the same extent (7.3°-8°C), indicating that the duplexes formed involve sequences having nearly the same degree of evolutionary divergence from the A. hortensis single-copy sequences. Although estimates of the correlation between mismatching and thermal stability vary between 0.7° and 1.5°C per 1% mismatched base pairs, it is often assumed, as a convenient approximation, that a 1° Tm depression corresponds to 1% mismatch (Bonner et al., 1973). Thus, these duplexes would be mismatched in 7%-8% of the bases.

TABLE 2. Reaction of 125I Single-Copy DNA from Atriplex hortensis with Total Unlabeled DNA from Atriplex Species

<table>
<thead>
<tr>
<th>Unlabeled DNA</th>
<th>Tm -25, Cot = 1200</th>
<th>Tm -25, Cot = 5000</th>
<th>Tm -35, Cot = 1200</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Bound</td>
<td>ΔTm (°C)</td>
<td>% Bound</td>
</tr>
<tr>
<td>A. hortensis</td>
<td>71</td>
<td>...</td>
<td>89</td>
</tr>
<tr>
<td>A. triangularis</td>
<td>51</td>
<td>7.3</td>
<td>71</td>
</tr>
<tr>
<td>A. sabulosa</td>
<td>43</td>
<td>8.0</td>
<td>50</td>
</tr>
<tr>
<td>A. serenana</td>
<td>33</td>
<td>8.0</td>
<td>48</td>
</tr>
</tbody>
</table>
Correlation of Altered Serological Properties with the Deletion of Plasmid Sequences

One approach to identifying the specific sequences on the Agrobacterium plasmid which code for pathogenicity is to obtain plasmid deletion mutants that have lost virulence. In these studies, we have used two non-pathogenic mutants of Agrobacterium tumefaciens obtained from Dr. James DeVay of the University of California at Davis. These mutants were of interest because DeVay and co-workers had observed that one (strain 210) had serological properties different from those of its parent strain (209), while the other (strain 226) remained serologically similar to the parent. In view of the association of virulence with the presence of a large plasmid, we wished to determine whether or not the mutant strains still contained a plasmid and, if so, whether or not the nucleotide sequences had been altered. Dye buoyant density centrifugation in cesium chloride-ethidium bromide gradients revealed the presence of a plasmid in both mutant strains (210 and 226) as well as in the parent strain (209). We then determined the molecular weight of these plasmids by analysis of their contour length in the electron microscope (Fig. 6 and Table 3). It appears that the plasmid of strain 210 (abbreviated pAT 210) resulted from the deletion of sequences totaling $4.8 \times 10^7$ daltons from pAT 209 while no measurable loss of DNA occurred during the derivation of pAT 226. However, definitive proof that the lesions affecting virulence are located on these plasmids will require that they be tested in a strain of Agrobacterium with a known permissive chromosomal background.

In strain 210 the loss of plasmid sequences can be correlated with changes in the serological properties of the strain. It seems likely that the antigenic determinant causing the precipitin bands close to the antigen well in strain 209, which are absent in the reaction with 210 antigen (Fig. 7), are coded by some of the plasmid sequences deleted from pAT 210. This conclusion is still speculative because we cannot eliminate the possibility of independent chromosomal mutations. However, transformation experiments with pAT 209 may answer this question. If chromosomal mutations have not occurred, strain 210 should regain both virulence and its original serological properties as a result of transformation with pAT 209 DNA. If so, these experiments will establish a new biochemical marker on the plasmid.

The Acquisition of Plasmid DNA Sequences Associated with the Transformation to Virulence

Many attempts have been made in the past to demonstrate virulence transfer between strains of Agrobacterium using standard methods of mating. These attempts have generally been unsuccessful. Recently, however, Alan Kerr reported successful transfer of virulence in Agrobacterium (Kerr, 1969, 1971). His experiments involved a novel system in which recipient strains are inoculated into a growing crown gall tumor that still contains the original inciting bacteria (donor strain). The donor and recipient strains carry appropriate chromosomal genetic markers which permit subsequent reisolation of recipients on selective media. After a period in which both strains grow together in the tumor, virulence transfer occurs and a high percentage (in many cases 50%) of the recipient bacteria reisolated from the tumor have acquired virulence. Details

<table>
<thead>
<tr>
<th>Strain</th>
<th>Pathogenicity</th>
<th>Plasmid Molecular Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>209</td>
<td>+</td>
<td>$110 \pm 4.6 \times 10^6$</td>
</tr>
<tr>
<td>210</td>
<td>-</td>
<td>$62 \pm 1.3 \times 10^6$</td>
</tr>
<tr>
<td>226</td>
<td>-</td>
<td>$110 \pm 3.8 \times 10^6$</td>
</tr>
</tbody>
</table>
Fig. 6. Molecular weight determination by contour length analysis of *Agrobacterium* plasmids. Top: standard plasmid pSC 120, molecular weight = $18.7 \times 10^6$, with *Agrobacterium* plasmid from strain 209 (pAT 209). Bottom: pSC 120 with nonpathogenic plasmid from strain 210 (pAT 210).
Fig. 8. Diagram representing profiles of DNA fragments obtained by EcoRI digestion of plasmid DNA from strains K18, K18A, and K27. Molecular weight of fragments greater than $3.4 \times 10^8$ d was determined from their mobility in 1.0% agarose gels and the molecular weight of fragments less than $3.4 \times 10^8$ d was determined from their mobility in 1.5% agarose gels. EcoRI fragments of R6-5 plasmid DNA run simultaneously in each gel were used as molecular weight standards. Solid circles: fragments common to pAT K18A and pAT K27 vir. Triangles: fragments common to pAT K18A, pAT K18, and pAT K27 vir. Open circles: fragments present in pAT K27 and not represented in pAT K18A.

4. Plasmid pAT K27 has an additional set of fragments which are not present in pAT K18A (Fig. 8). These results are consistent with the report of Watson et al. (1975) in which strain K27 was shown to contain two plasmids. The additional fragments found in pAT K27 and not in pAT K18A can be inferred to represent the second plasmid in strain K27. The common fragments between pAT K27 and pAT K18A must therefore comprise the virulence plasmid which is transferred to strain K18.

These results provide indirect evidence for incompatibility between the virulence plasmid of strain K27 and the cryptic plasmid of strain K18. The stability of plasmid pAT K18 in strain K18 when it is grown in a crown gall tumor has not been directly tested, and it is therefore possible that strain K18 lost its plasmid before acquiring the virulence plasmid from strain K27. However, pAT K18 is stable in strain K18 under all in vitro growth conditions so far tested.

Although it appears that recombination did not occur between plasmids of strains K27 and K18, preliminary experiments indicate that recombinant plasmids may exist in two other, unrelated exconjugant strains. If recombination does occur, further studies of recombinant plasmids may help to identify portions of the plasmid genome required for virulence, since fragments containing virulence genes would be expected to be conserved in successive crosses.

A major question concerning the mechanism of crown gall tumorigenesis is whether or not the tumor induction process involves the transfer of genetic information from Agrobacterium to the plant cell. Specific fragments of DNA associated with the acquisition of virulence in several exconjugant strains would be likely candidates for transfer to the plant cell. The use of these specific fragments as probes in DNA-DNA hybridization experiments would enable one to test this possibility. Although some previous hybridization experiments with plasmid DNA have been unable to detect the presence of plasmid sequences in certain lines of plant tumor cells, a small fraction of the total plasmid genome might easily have been missed (Chilton et al., 1974). On the other hand, evidence for plasmid sequences in a different plant tumor cell line has been obtained by hybridization with whole plasmid DNA (Matthysse, 1976). Experiments using only a selected portion of the plasmid DNA should provide a much more sensitive test and help to resolve the discrepancy between these two sets of results.
BLUE LIGHT-INDUCED ABSORBANCE CHANGES IN MEMBRANE FRACTIONS FROM Neurospora crassa

Robert D. Brain and Winslow R. Briggs

The effects of blue light on phototropism, the biological clock, oxygen uptake, and a number of other biological processes have been known for some time (see Briggs, 1976). Only recently, however, has real progress been made in the possible identification of the blue light photoreceptor associated with these effects. Following the initial studies of Poff and Butler (1974) with Dictyostelium and Phycomonosys, work by Muñoz and Butler (1975) implicated the flavin-mediated photoreduction of a b-type cytochrome as possibly a central element in the photoreception process in Neurospora. Since Muñoz and Butler worked with intact mycelium of Neurospora, it seemed appropriate to attempt isolation of a cell fraction that would show the same response.

A carotenoid-free albino mutant of Neurospora crassa (albino-timex) was used and treated in the following way: A liquid suspension (100 ml) was made from a dark-grown five-day-old agar culture and poured into 1000 ml of a liquid medium (2% [v/v] Vogel's solution (1956), 0.5% [w/v] Difco Casamino acids, and 0.5% [w/v] dextrose). Liquid cultures were grown aerobically in the dark on a rotary shaker at 250 rpm for 24 hr at 30°C.

Approximately 40 g of mycelium were harvested by suction filtration, torn into small strips, and immersed in about 55 ml of extraction buffer (250 mM sucrose, 100 mM N-morpholino propane sulfonic acid, 14 mM 2-mercaptoethanol, 3 mM ethylenediaminetetraacetate, and 0.1 mM MgSO₄, adjusted to pH 7.4 by titration with KOH). The strips of mycelium were broken by vigorous shaking with small glass beads in a mechanical homogenizer, and the homogenate was then centrifuged at 2000, 9000, 20,000, and 50,000 × g for 10, 15, 30, and 75 min, respectively, at 4°C. The pellets
TABLE 5. Distribution of Marker Enzymes and Light-Inducible Cytochrome in the Various Cell Fractions from Neurospora crassa (assays per mg protein)

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein mg; ml⁻¹</th>
<th>Na⁺-Dependent Adenosine Triphosphatase mM PO₄ × min⁻¹ × 10⁻³</th>
<th>Absorbance Change (Relative)</th>
<th>NADH-Dependent Cytochrome c Oxidase mM × sec⁻¹ × 10⁻²</th>
<th>Cytochrome c Reductase mM × sec⁻¹ × 10⁻³</th>
</tr>
</thead>
<tbody>
<tr>
<td>2KS</td>
<td>224</td>
<td>15.11</td>
<td>5.01</td>
<td>11.33</td>
<td>2.42</td>
</tr>
<tr>
<td>9KP</td>
<td>185</td>
<td>48.96 (38)*</td>
<td>11.26 (39)</td>
<td>65.80 (207)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>20KP</td>
<td>169</td>
<td>130.97 (100)</td>
<td>28.98 (100)</td>
<td>31.82 (100)</td>
<td>0.015 (100)</td>
</tr>
<tr>
<td>50KP</td>
<td>228</td>
<td>84.91 (49)</td>
<td>14.41 (50)</td>
<td>21.47 (67)</td>
<td>6.390 (4.3 × 10⁴)</td>
</tr>
<tr>
<td>50KS</td>
<td>188</td>
<td>2.69</td>
<td>0.723</td>
<td>0.842</td>
<td>2.97</td>
</tr>
</tbody>
</table>

*Figures in parentheses represent amounts relative to the 20KP, normalized to 100.

evidence is still lacking. Calculations (not shown) indicate that recovery was close to 100% for all the markers and for the potential for a light-induced signal, based on what was initially present in the 2KS, except for the ATPase, which showed a loss of about 20%.

Preliminary studies have been initiated to determine more precisely which cytochromes might be involved in the process described above. The 20KP fraction from a poky mutant of Neurospora (Fungal Genetics Stock Center #3627-2) appears to give significantly smaller signals than the albino-timex mutant described here. Many studies have examined mitochondrial cytochrome deficiencies in poky mutants (Lambowitz and Bonner, 1974; Lambowitz et al., 1972a,b), but there are as yet no studies on extra-mitochondrial cytochrome abnormalities. Preliminary reduced-minus-oxidized difference spectra show poky to be deficient in b-type cytochromes generally, as expected, but to be noticeably deficient in an NADH-reducible cytochrome in the 20KP and 50KP. Further studies are planned with low temperature and derivative spectra to analyze these deficiencies in detail. Studies are also under way to determine whether the poky mutant shows altered photosensitivity, measured either by suppression of expression of the circadian rhythm of conidia-

tion (Sargent and Briggs, 1967; Sargent, Woodward and Briggs, 1966) or by induction of carotenoid synthesis in mycelia grown in liquid culture (De Fabo, Harding and Shropshire, 1976; Zalokar, 1955).

In conclusion, the photoreduction measurements and light-minus-dark difference spectrum reported here for the 20KP are very similar to those reported by Muñoz and Butler for intact Neurospora mycelium. In both systems, an intermediate redox state is required for the signals to be obtained. It therefore seems reasonable that the same pigment system is involved in both cases. Hence the signals described by Muñoz and Butler appear to have their origin in a fraction that could be plasma membrane (and is clearly not mitochondria, endoplasmic reticulum, or the soluble fraction). However, it is still premature to state that this system is the photoreceptor complex for the various physiological blue light responses mentioned above. Such a conclusion would require evidence linking the behavior of this fraction to that of the physiological responses in question. The poky mutants seem promising objects for obtaining such evidence.

References

BLUE LIGHT-INDUCED ABSORBANCE CHANGES IN MEMBRANE FRACTIONS FROM Zea mays

Winslow R. Briggs, Jack Freeberg, and Charles V. Weiss

There is now a substantial amount of literature concerning light-induced absorbance changes in various fungi, and a detailed action spectrum for such a change in Neurospora mycelium is very similar to the action spectra for a host of blue light-induced physiological responses both in fungi and in higher plants (Muñoz and Butler, 1975). If such absorbance changes reflect a light reaction of the physiologically important blue light photoreceptor, one would expect to find similar changes in higher plants as well. Widell and Björn (1976) have indeed found light-induced absorbance changes in dark-grown wheat coleoptiles, but the light-minus-dark difference spectrum did not resemble that reported for the various fungi (see Briggs, 1976), and the action spectrum for the absorbance changes suggested that the cytochromes themselves were serving as photoreceptors, perhaps as in the Dictyostelium system described by Poff and Butler (1974). After eliminating spectral changes caused by blue-induced phytochrome phototransformation and protochlorophyll photoconversion, Poff (personal communication) also saw complex absorbance changes in corn coleoptiles. These changes did not resemble those described for the several fungi either. Thus, two studies with intact tissue of higher plant tissue failed to reveal a light-sensitive system with properties like those of Neurospora mycelium, and the evidence to date would suggest that the Neurospora system is not ubiquitous.

Brain and Briggs (this Year Book) report on the isolation of a membrane fraction from Neurospora which yields the characteristic light-induced absorbance changes described for Neurospora mycelium by Muñoz and Butler (1975). This report describes similar absorbance changes in a comparable membrane fraction isolated from corn coleoptiles.

Corn seeds (Zea mays, L. hybrid WF9 × Beo 38, Bear Hybrid Corn Co., Decatur, Ill.) were soaked overnight in running tap water and then grown in darkness in a constant temperature room at 25°C, 90% relative humidity for four days. The seedlings received 2 hr red light every night but the last for suppression of mesocotyl growth and
stimulation of coleoptile elongation. Coleoptiles were harvested directly into ice under dim green light as described by Hertel and Flory (1968).

Corn coleoptiles were harvested, homogenized, and the homogenate subjected to differential centrifugation as described elsewhere (Year Book 74, pp. 807–809). The pellet obtained following the 21,000 \( \times g \) centrifugation (21KP) was resuspended and used for the irradiation studies. For each 6 g of tissue homogenized, the final resuspension volume was 1 ml. This preparation is comparable to the Neurospora 20KP described by Brain and Briggs. The instrumentation and techniques for measuring absorbance changes on irradiation with blue light were also those of Brain and Briggs.

Figure 11 shows light-induced absorbance changes detected when absorbance at 410 nm is subtracted from that at 423 nm. The signal is clearly similar to that described by Brain and Briggs for the Neurospora fractions, with a half-life of about 30 sec. Under identical conditions of sample geometry and actinic light intensity, the dose-response curve for the corn signal is also much like that for Neurospora preparations. Between 10 and 15 sec of the actinic blue light (1.1 \( \times 10^4 \) ergs cm\(^{-2} \) sec\(^{-1} \)) yielded half-saturation, with 1 min close to complete saturation.

The corn preparations gave smaller signals than those from Neurospora, and were far less stable. Whereas from the fungal membranes one could obtain consistent signals repeatedly over a period of hours, the corn fractions rapidly lost the capacity to produce signals (sometimes within 15 min). For this reason, the only fraction examined was the 21KP.

Since it was not possible to obtain extensive data from any one preparation, a complete light-minus-dark difference spectrum could not be made. However, partial difference spectra were obtained on three different occasions. On each of these occasions, a maximum near 425 nm was found, with minima near 410, and on two occasions, 450 nm. Thus the light-minus-dark difference spectrum for corn appears similar to that described for intact Neuro-

![Fig. 11. Blue light-induced absorbance changes in a resuspended 21,000 \( \times g \) pellet from corn. Upward arrow indicates light on; downward arrow, light off. A 30-sec and a one-min radiation are shown.](image)
spora mycelium (Muñoz and Butler, 1975) or Neurospora membrane preparations.

The corn preparations resembled those from Neurospora in another way. Signals could not be seen unless the system was poised with the cytochromes at an intermediate redox state. At the beginning of an experiment, the cytochromes were normally almost fully oxidized. Irradiation yielded either no signal or a slight decrease in the absorbancy difference between 423 nm and 410 nm. Following a series of 1-min irradiations, however, the cytochromes became partially reduced, as with Neurospora, and signals such as those shown in Fig. 11 could be observed. More fully reduced preparations no longer yielded signals, or showed light-induced oxidation of the cytochromes.

In conclusion, corn 21KP and Neurospora 20KP membrane preparations show very similar responses to blue light. The dose-response curves for the light-induced absorbance changes, the dark decay kinetics, and the light-minus-dark difference spectra resemble each other; and both preparations require an intermediate redox state to show the light responses. These similarities between the fungal and the higher plant systems support the hypothesis that they may indeed represent the physiologically active blue light photoreceptor. Future work will involve efforts to stabilize the corn preparations and determine the distribution of the potential for light-inducible absorbance change in the various fractions.

References


AN EVALUATION OF MARKERS FOR PLASMA MEMBRANES IN MEMBRANE FRACTIONS FROM Zea mays

John W. Cross and Winslow R. Briggs

The plasma membranes of plant cells are rapidly becoming the subject of increased research attention. This activity is partly because of the generally increasing interest of biologists in cell surfaces per se, and partly because these membranes are now suggested to be sites of biochemical activities that are uniquely vegetable, including blue light photoreception (Jesaitis et al., 1976; Brain and Briggs, this Year Book) phytochrome action (Haupt, 1973; Yu, 1975), and auxin activity (Hertel et al., 1972). In each of these cases the isolated membrane fractions involved have been identified as plasma membrane by means of enzymatic "markers" or from "specifically" stained electron micrographs. Of course this identification is valid only insofar as the "markers" are authentic. Unfortunately, the specificity of each of the various plasma membrane markers is subject to question.

Table 6 lists these "markers," and the basis for using each of them to identify the plasma membrane. They divide roughly into two groups, those expected on theoretical grounds to be associated with the cell surface, and those empirically selected (see Table 6). As can be seen, the "markers" in Class I are verified either by good correlation of their presence with PTA staining or with naphthylphtalamic acid (NPA) binding. NPA binding itself was
### TABLE 6. "Markers" Used for Plasma Membranes of Higher Plants

<table>
<thead>
<tr>
<th>Marker</th>
<th>Grounds for Validity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Class 1 (chosen on theoretical grounds)</strong></td>
<td></td>
</tr>
<tr>
<td>Glucan synthetase, Mg(^{++}) independent</td>
<td>Thought to be involved in cell wall synthesis; correlation with NPA-binding activity in membrane fractions (VanDerWoude et al., 1974).</td>
</tr>
<tr>
<td>ATPase (cation-dependent)</td>
<td>Thought to be involved in monovalent cation uptake by correlation of its kinetics with the kinetics of ion transport in roots (Leonard and Hodges, 1973); correlation with PTA staining (Hodges et al., 1972).</td>
</tr>
<tr>
<td>Naphthylphthalamic acid (NPA) binding</td>
<td>An inhibitor of auxin transport; correlation with PTA staining (Hertel et al., 1972).</td>
</tr>
<tr>
<td>Steroid content elevated</td>
<td>Analogy with animal cells, which have most steroids in the plasma membrane; correlation with NPA binding (Hartmann et al., 1978).</td>
</tr>
<tr>
<td><strong>Class 2 (empirically selected)</strong></td>
<td></td>
</tr>
<tr>
<td>PTA staining for electron microscopy (PTA = chromic-perchloric-phosphotungstic acid)</td>
<td>Thin sections of whole tissues or cells stain mainly at the plasma membrane (Roland, et al., 1972).</td>
</tr>
</tbody>
</table>

Note: Several well-known markers for the plasma membranes of animal cells are absent from plant cells (i.e., 5'-nucleotidase, Na\(^+\)-K\(^+\)-stimulated ATPase, adenyl cyclase, sphingomyelin, characteristic hexagonal protein complexes (Roland et al., 1972)."

verified by correlation with PTA staining (Hertel et al., 1972), so the validity of each of these "markers" depends ultimately on the validity of this stain.

PTA staining was originally developed as a stain for plant plasma membrane by Roland et al. (1972) and subsequently improved (Leonard and VanDerWoude, 1976). It acts by oxidation and binding of complex membrane carbohydrates (Roland et al., 1972). In thin sections of whole tissues, the stain definitely shows preference for the plasma membrane. However, its use as a selective stain with isolated membrane fractions seems more questionable. Since each membrane fraction is sectioned and stained separately, it is difficult to interpret light staining in one fraction and heavy staining in another, especially since the process of membrane fractionation itself might alter the intensity of staining. Is the observed variation in stain intensity caused by differences in staining or photography, or by actual specific differences in the binding of stain? Membrane fractions that are sedimented to about 38% sucrose consistently stain most heavily, so the variation does not appear random (Hodges et al., 1972; Leonard and VanDerWoude, 1976). However, considerable stain can be found in other fractions. Since the validity of all the other markers currently depends upon that of this stain it would seem best for caution to prevail.

Because of this situation we are attempting to verify the reliability of some of these "markers" for the plasma membrane and to develop new ones. Our initial work in this area centered on the monovalent cation-dependent ATPase. This enzyme activity has been correlated with the plasma membrane not only by coincidence with PTA staining of sucrose gradient fractions but by the similarity of Eadie-Hofstee plots of its kinetics with the plots for monovalent cation uptake in whole roots (Leonard and Hodges, 1973). This analysis assumes that only one such ATPase enzyme exists, and that it is present only in the plasma membrane.

We harvested the coleoptiles of dark-grown corn seedlings and
homogenized them at 0° in the light. The homogenates were then fractionated by differential sedimentation and by isopycnic sucrose density gradients. The highest specific activities of K+-stimulated ATPase were found in the 21,000 × g and 48,000 × g pellets (21KP and 48KP; Table 7). This distribution was clearly distinct from basal ATPase (mostly in the 48,000 × g supernatant) and cytochrome c oxidase (mostly in the 9000 × g pellet (9KP). It is also distinct from the microsomal marker, NADH–cytochrome c oxidoreductase, which generally is enriched in the 48KP pellet (Jesaitis et al., 1976; and data not shown). On isopycnic gradients (not shown) the K+-stimulated ATPase formed two distinct peaks of activity. The lighter was at 50% sucrose, distinctly denser than the microsomal marker, NADH–cytochrome c oxidoreductase (26% sucrose). This membrane fraction does not correspond to the density of any of the other plasma membrane "marker" enzymes. This peak was similar in density to the peak of Mg++-dependent glucan synthetase activity (glucan synthetase I, a golgi membrane marker [Ray et al., 1969]), although the glucan synthetase has not as yet been tested on the same gradient with the ATPase.

The second peak occurred at higher density (38% sucrose), just above the peak of cytochrome c oxidase activity (40% sucrose), but not identical to it. It did not correspond to the peak of NPA binding activity, which was closely correlated with the cytochrome c oxidase peak. Thus it appears that NPA binding and K+-stimulated ATPase do not reside exclusively in common membrane fractions and therefore cannot represent markers for the same membrane. In addition, the NPA binding activity becomes equilibrated in close correspondence to cytochrome c oxidase and may in fact be bound to mitochondria rather than plasma membranes, although this possibility remains to be proven.

The two distinct bands for K+-stimulated ATPase could reflect the observation that plant cells contain two separate internal compartments of K+, believed to be protoplasm and vac-

---

**TABLE 7. Basal and K+-Stimulated ATPase Activity from Maize Coleoptiles: Differential Sedimentation***

<table>
<thead>
<tr>
<th>Sample</th>
<th>ATPase Activity</th>
<th>Cytochrome c Oxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal K+-stimulated</td>
<td>ΔA₅₅₀min⁻¹ mg protein⁻¹</td>
</tr>
<tr>
<td>Crude homogenate</td>
<td>206</td>
<td>8</td>
</tr>
<tr>
<td>500 × gₘₚₙ 15 min pellet</td>
<td>32</td>
<td>30</td>
</tr>
<tr>
<td>9 × 10⁵ × gₘₚₙ 15 min pellet</td>
<td>80</td>
<td>44</td>
</tr>
<tr>
<td>21 × 10⁵ × gₘₚₙ 15 min pellet</td>
<td>108</td>
<td>81</td>
</tr>
<tr>
<td>48 × 10⁴ × gₘₚₙ 60 min pellet</td>
<td>136</td>
<td>67</td>
</tr>
<tr>
<td>48 × 10⁴ × gₘₚₙ supernatant</td>
<td>261</td>
<td>9</td>
</tr>
</tbody>
</table>

*Coleoptiles (10 g) were chopped with a razor and ground in a mortar in 0.25 M sucrose, 50 mM tris-acetate (pH 8.0), 1 mM EDTA, 0.1 mM MgCl₂, and 14 mM 2-mercaptoethanol (2 vol buffer of tissue), filtered through 2 layers of cheese cloth and centrifuged as described in the Sorvall SS34 rotor. Operations were at 0–5°C. ATPase was assayed (Hodges et al., 1972) in a 1 ml reaction mix with 30 mM Tris-MES (pH 6.0), 1.5 mM MgCl₂, 3 mM ATP-Tris (pH 6.0), and 0.050 ml of the washed membrane fraction resuspended in the grinding buffer at 0.5–2 mg protein per ml. Tests began by addition of ATP after 10 min preincubation and were continued for 15 min, when 0.4 ml 20% trichloroacetic acid was added. P₁ in the centrifuged supernatant was determined by the method of Hedman (1964). Basal ATPase activity was corrected for ATP hydrolysis in the absence of enzyme, and K+-stimulated activity represents the activity at 50 mM KCl in the reaction minus basal activity. Cytochrome c oxidase was assayed by the method of Jesaitis et al. (1976).
uole, at different K⁺ potentials (Davis and Higinbotham, 1976). The lighter K⁺-stimulated ATPase could reside in the tonoplast membrane (for which we do not as yet have a marker), and the denser activity in the plasma membrane, although other possibilities exist.

In order to resolve some of the problems mentioned above, we are attempting to label the plasma membrane of intact cells prior to homogenization and fractionation. Our initial experiments have made use of Fluorescamine (FLURAM), which reacts rapidly with primary amines to yield a fluorescent product. This reaction occurs with a t₁ of milliseconds (Udenfriend et al., 1972), and excess reagent spontaneously hydrolyzes to nonfluorescent products within a few seconds. Since this rapid hydrolysis should prevent significant labeling within the cells, fluorescamine should be an ideal cell surface label. Indeed, it has recently been reported that fluorescamine can label surface-specific peptides of chick fibroblasts without labeling internal peptides (Hawkes et al., 1976).

To test the utility of this reagent as a surface label, 2-mm segments of corn coleoptiles were briefly exposed to fluorescamine and then homogenized and fractionated by differential sedimentation (Table 8). The distribution of fluorescence in the fractions was quite similar to that reported above for K⁺-stimulated ATPase, with the greatest label bound to the 21KP and 145KP. In another experiment (not shown) it was found that homogenizing the tissue before adding fluorescamine resulted in a tenfold increase in the labeling of soluble amines with only a twofold increase in the labeling of membranes. From these results it appears that fluorescamine does primarily label the surface of intact cells unless the permeability barriers of the cell are first broken. The reasonable correspondence of fluorescamine labeling with the presence of K⁺-stimulated ATPase suggests that at least one of the ATPases may be present on the plasma membrane. Further experiments are now in progress to optimize the specificity of this label and to verify that it reacts with the cell surface.

**TABLE 8.** Fluorescamine Labeling of Maize Coleoptile Segments: Differential Sedimentation*  

<table>
<thead>
<tr>
<th><strong>Sample</strong></th>
<th><strong>Relative Fluorescence (per mg protein)</strong></th>
<th><strong>K⁺-Stimulated ATPase</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude homogenate</td>
<td>1.0 (0.33)</td>
<td>178 (0.46)</td>
</tr>
<tr>
<td>500 x gmax, 15 min pellet</td>
<td>2.2 (0.73)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>9 x 10⁴ x gmax, 15 min pellet</td>
<td>1.5 (0.50)</td>
<td>164 (0.42)</td>
</tr>
<tr>
<td>21 x 10⁵ x gmax, 15 min pellet</td>
<td>3.0 (1.00)</td>
<td>390 (1.00)</td>
</tr>
<tr>
<td>145 x 10⁵ x gmax, 30 min pellet</td>
<td>2.9 (0.97)</td>
<td>364 (0.93)</td>
</tr>
<tr>
<td>145 x 10⁵ x gmax, supernatant</td>
<td>0.7 (0.23)</td>
<td>151 (0.39)</td>
</tr>
</tbody>
</table>

*Dark-grown corn coleoptiles were collected on ice and weighed into two groups of 10 g each. These were chopped into 2-mm segments, and washed three times at room temperature in 10 mM sodium phosphate-pyrophosphate buffer, pH 7. The first two washes were accompanied by vacuum infiltration (5 min each). Washed segments were rinsed and suspended in 30 ml labeling buffer (0.25 M sucrose, 1 mM EDTA, 25 mM Na₃P₂O₇, brought to pH 8.5 with 25 mM NaH₂PO₄). The samples received either 0.30 ml of fluorescamine freshly dissolved in pure acetonitrile (10 mg/ml), or 0.30 ml of acetonitrile alone. After 1 min at 20° the solutions were aspirated and the samples rinsed three times in the same buffer at 0°, then rinsed and resuspended in 30 ml of grinding buffer (see Table 7). Coleoptile segments were then homogenized and fractionated as described in the legend to Table 7. To assay fluorescence, a 0.10 ml aliquot was removed from each fraction, diluted in 2.0 ml SDS (0.25%, w/v), and centrifuged at 1000 x g, 5 min. Fluorescence at 475 nm was excited at 390 nm. Half-band widths were 10 and 8 nm for emission and excitation, respectively. The fluorescence of each sample was corrected for protein content and for the auto-fluorescence of the corresponding unlabeled sample (which was at least tenfold lower than the labeled sample). K⁺-stimulated ATPase activity was assayed as described in the legend to Table 7.

†Figures in brackets represent amounts relative to the 21KP, normalized to 1.00.
A MULTI-SAMPLE AUTOMATIC MONITORING DEVICE FOR THE CIRCADIAN RHYTHM OF TRANSMITTANCE CHANGE IN Ulva

S. J. Britz

As discussed last year (Year Book 74, p. 794), a circadian rhythm of chloroplast movement in the green alga Ulva lactuca L. causes a differential sieve effect and results in a corresponding rhythm of visible light transmittance change (Britz and Briggs, 1976). The vegetative thallus of Ulva forms a flat sheet two cell layers thick, with each cell containing one large, cup-shaped chloroplast. In daily light-dark (LD) cycles the chloroplasts cover the outer cell face (face position) during most of the day, and the transmittance is low. Starting late in the day and continuing through most of the night, the chloroplasts move slowly down the side walls, expose the central portion of the cell (profile position) and greatly increase the transmittance (up to 200%). Movement back to face position begins before the end of the dark period and continues more rapidly with the onset of light. Rhythmic chloroplast movement continues during periods of constant darkness (DD) or constant light (LL).

The relation between chloroplast position and thallus transmittance has been studied in detail by direct techniques and computer modeling (Britz, manuscript in preparation). The results of these studies show that within certain bounds, the correlation between chloroplast position and transmittance is excellent. For a given orientation change, the transmittance change depends mainly on the absorbance; the higher the absorbance, the greater the transmittance change and the better the correlation with chloroplast position. At wavelengths at which absorbance is low, the transmittance changes not only become much smaller but are in-
fluenced more by changes in other factors such as light scattering and reflectance. These features of chloroplast movement can be matched by a computer model (a preliminary version of which has been described; *Year Book* 74, p. 794) indicating the movement-related transmittance changes can be described quantitatively in terms of known and measurable parameters.

For various reasons (ease, objectivity, integration of the result over many cells), transmittance measurements are useful for the study of chloroplast orientation. Long-term, rhythmic transmittance changes in a single *Ulva* thallus have been monitored both by an automatic, continuously recording microphotometer and flow-through cuvette combination and by non-flow-through cuvettes measured manually (Britz et al., 1976). The transmittance changes were large, highly regular, and persistent for many days. In both cases sampling noise was minimized because the thalli were kept in a constant geometry with respect to the measuring beam. Moreover, thalli were measured without disturbing the rhythm, something not possible with conventional techniques.

The following paper describes the design, construction, and operation of a practical system to monitor rhythmic transmittance changes automatically for multiple samples. This system is useful for the study of any type of slow transmittance change, such as that accompanying chloroplast movement.

**DESIGN AND CONSTRUCTION**

The basic unit of the monitor is the sample holder. It must be convenient in terms of size, manipulation, and amount of tissue and medium required (the latter, an important consideration in inhibitor experiments). While it is not necessary that the sample holder reproduce a "natural" environment, it must offer physiological conditions for long-term measurement. Moreover, the design should be simple and flexible.

Thus, it was decided to make the sample holder essentially a small dish in which the *Ulva* thallus would be held horizontally and for which the measuring beam would be vertical. This design is suitable for in situ light microscopy (particularly with an inverted microscope) or spectrophotometry. Experiments parallel to those in the monitor can be conducted on the physiological and biochemical properties of other samples maintained in small Petri dishes, a comparable environment.

The sample holder and dimensions are depicted in Fig. 12. The holder is constructed with a clear Plexiglas cylinder melded to a circular black Plexiglas base. The base has a large central hole over which a cover slip masked with black photographic tape is paraffined.

![Perspective view of the disassembled sample holder](Fig. 12)
fined in place. The Ulva thallus can then be positioned flush against the cover slip by dialysis membrane or nylon mesh. Alternatively, as shown in Fig. 12, the thallus can be supported above the cover slip by a nylon filament and Plexiglas ring combination. The tape mask has a 4-mm diameter window such that when the Ulva thallus is positioned over the window only light passing through the sample is transmitted by the holder. In this configuration the sample should be illuminated by a measuring beam coming from above, thereby exposing the entire thallus and eliminating any possible effects of differential light treatment. An advantage of the cover slip is that the size of the window may be varied. The holder is covered by a clear polystyrene top and there is a small notch cut in the top of the cylinder to allow for gas exchange. There are no problems with evaporation or condensation on the clear cover, at least under the operating conditions described below. Pins extending from the holder anchor it reproducibly in the monitor.

The choice of lighting conditions for the monitor poses the next problem; obviously light must be used for transmittance measurements. Since the transmittance changes depend strongly on the absorbance, the optimal wavelength to monitor the rhythm should be about 436 nm, the absorbance maximum. However, there are several problems which urge caution. The period of circadian rhythms depends on the quality and quantity of ambient lighting. Both blue light and red light shift the phase of the Ulva rhythm (Britz, unpublished data), and continuous exposure to blue light (and presumably red) can decrease the free-running period (Britz et al., 1976). For photosynthetically active organisms, light may supply energy and affect the amplitude of the rhythm. In addition, light may modify chloroplast shape, thus influencing transmittance (Britz, manuscript in preparation). Measurements made in higher intensity light run the risk of error due to bubble formation in the measuring beam. Thus, the monitor should operate at light intensities approaching effective DD with the potential to operate under LL.

The requirement for minimal light (less than 1 erg cm⁻² sec⁻¹ continuous exposure of which from 5% to 20% would reach the photodetector; Britz et al., 1976) eliminates at present the possibility of continuously illuminating an array of samples, each with its own inexpensive solid-state photodetector. A more sensitive light-detecting system is required, such as a single photomultiplier past which samples are moved sequentially. For this purpose a device like a fraction collector seems ideal. However, the transmittance signal is quite sensitive to sample position. When many samples must be measured, precise repositioning becomes a problem. Therefore, it was decided to have the monitor consist of 30 sample holders positioned along the rim of a constantly revolving wheel (one revolution per hour) such that the samples are swept through a dim, vertical measuring beam with the transmitted light measured by a photomultiplier beneath. The output from the photomultiplier, amplified and recorded on a strip chart recorder, constitutes a single channel of data on which the records of 30 samples appear in sequence every hour. This number of samples and measurements is reasonable and sufficient for most purposes. A schematic representation of the monitoring device is shown in Fig. 13.

The wheel consists of an aluminum frame and an outer circle of plywood with holes for the sample holders. Next to these holes are raised supports for the orientation pins. A small electric motor drives the wheel, which is connected to the drive shaft by cork pressure plates that allow optional manual rotation.

The entire apparatus (sample holders, wheel, light source, and photomultiplier) is enclosed in a temperature-controlled chamber, with the associated power supplies,
Fig. 14. Typical strip chart recordings of photomultiplier output at times when chloroplasts occupy profile (DDs to DD) and face (DD to DD) positions. The bar represents approximately 1 nA. The spike height (nA) is proportional to transmittance. Note the change in sample transmittance with respect to the reference.

Also shown are the hourly values for the reference.

The individual samples present a regular and even pattern of transmittance change. Thus, one does not depend on sample averaging to relieve hidden noise. While there are obvious differences in the magnitude of transmittance and the amplitude of change, the patterns are remarkably similar to one another and to their average. Such repeatability within an experiment is typical and, on the basis of 16 monitor runs, the reproducibility between different experiments is almost as good.

Considering many experiments and expressing the transmittances as a percentage of the first transmittance maximum, one can state the value for the first transmittance minimum is usually 50%-60%, the second maximum is 80%-110%, and the second minimum is about 70%-80%. These results compare well with transmittance values obtained in parallel experiments that were correlated to direct microscope observation of chloroplast position (Britz, manuscript in preparation). For the first two maxima the chloroplasts are approximately in full profile position, and the highest transmittance values are measured. For the first transmittance minimum the chloroplasts are in full face position, and the lowest transmittance value is measured. At subsequent minima and maxima the chloroplasts fail to reach the extreme positions, and intermediate transmittance values are measured. The rhythm generally damps out with the chloroplasts in an intermediate-to-face position. With the monitor, rhythmic transmittance changes can be detected for at least six cycles, although the last cycles frequently become distorted by noise, at least part of which may be related to reference instability.

There is also excellent repeatability within one experiment for the times of transmittance maxima. The maxima for the five samples from Fig. 15 have been calculated by the parabola slope technique (Britz et al., 1976) and are presented in Table 9. The phase alignment between different samples also
so close to 24 hr, it is impossible to rule out diurnal synchronizing factors such as small temperature fluctuations. However, a major role for such factors is unlikely because it is possible to alter experimentally the length of the free-running period as well as to reset stably the phase of the rhythm.

While the above-mentioned sample differences in transmittance magnitude and amplitude of change are not important for rhythm studies per se, they present serious problems for quantitative application of the transmittance change measurements to chloroplast movement studies. However, the direct dependence of amplitude on the magnitude of transmittance suggests normalization as an empirical correction. This observation is surprising, since spectrophotometry studies determined the amplitude of transmittance change to be inversely proportional to the minimum (i.e., face position) transmittance (Britz, manuscript in preparation). The spectrophotometry studies were done with special attention to keeping the thalli flattened, but in the monitor sample holders the thalli may be more or less wrinkled. Because the sieve effect due to chloroplast orientation is very sensitive to the angle of incidence of the measuring beam, the wrinkling factor will decrease the maximum transmittance more than the minimum, thereby decreasing the amplitude of the change. In fact, maximum transmittance change is not a constant fraction of minimum transmittance, but rather is a function of both minimum transmittance and the degree of wrinkling of the thallus.

**TABLE 9. Phase Coincidence of *Ulva* Transmittance Change Rhythm Maxima**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Maxima (hr after start of DD)</th>
<th>First</th>
<th>Second</th>
<th>Third</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td></td>
<td>5.37 ± 0.51</td>
<td>30.72 ± 0.14</td>
<td>53.54 ± 0.12</td>
</tr>
<tr>
<td>26</td>
<td></td>
<td>5.51</td>
<td>30.96 ± 0.13</td>
<td>52.55 ± 0.28</td>
</tr>
<tr>
<td>27</td>
<td></td>
<td>4.99 ± 0.29</td>
<td>30.89 ± 0.14</td>
<td>53.33 ± 0.16</td>
</tr>
<tr>
<td>28</td>
<td></td>
<td>4.76 ± 0.36</td>
<td>30.8 ± 0.15</td>
<td>52.89 ± 0.16</td>
</tr>
<tr>
<td>29</td>
<td></td>
<td>5.27 ± 0.28</td>
<td>30.8 ± 0.17</td>
<td>53.28 ± 0.15</td>
</tr>
</tbody>
</table>

*The values are the zero slope times in hours ± 95% confidence limits of the estimate. The larger errors for the first maxima result from taking fewer points (5 vs. 7) over a shorter period of time (5 hr vs. 12 hr) than for the second and third maxima.

†This value is estimated because the fifth point is higher than the fourth (Fig. 15), which causes aberrant behavior of the parabola slope method.
tance values are less than expected. There is also a fair amount of variability, probably caused by the wrinkling.

The effects of normalization are shown in Fig. 16 where the average of the raw data from Fig. 15 plus standard errors of the mean are depicted along with the average of the same data after normalization to 1.0 at the estimated transmittance value for the first maximum. Normalization has reduced the standard errors to 25%-50% that of the raw data when expressed in terms of the transmittance change. Thus, the data within one experimental treatment can be made more compatible, and the data between different treatments can be made more comparable.

With this background, a brief survey was made to ascertain reasonable sample preparation methods for reproducible operation of the monitor. The first concern was the method of fastening *Ulva* in the sample holder. As mentioned above, samples could be held flush against the cover slip or supported slightly above the surface. Contact of the thallus with nutrient medium may be critical in terms of free-running period, amplitude, and damping characteristics (Britz et al., 1976).

In the present study it has been observed for flush-mounted thalli that bottom cells not exposed to medium are about twice as large as top cells after a typical experimental protocol of three days' adaptation in LD cycles and six days in the monitor. The bottom cells are apparently inhibited from dividing but not from enlarging. This effect was not observed in samples mounted directly before the start of monitoring (i.e., without LD adaptation in cuvettes) and may therefore depend on events during adaptation. It is not known whether there is a differential movement rhythm between top and bottom cells, although the transmittance rhythm curves appeared "normal" (i.e., no peak splitting or broadening of the peaks was observed). The flush-mounted samples had rhythms with similar amplitude and damping characteristics but somewhat longer free-running periods than samples in the raised mounts (24 vs. 23 hr based on one trial with three samples for each treatment). No top-bottom cell size differential has been observed in samples adapted in support rings.

The raised support mode offers advantages for the removal of oxygen bubbles that form in bright light and become trapped under the thallus. However, thalli grow under these conditions, and with the raised support mode they can become wrinkled. Thus, it is necessary to rearrange the samples once during adaptation. The increased distance of the sample from the cover slip obviates the use of inverted...
microscopy and requires a larger thallus disc to ensure that none of the measuring beam leaks past. The agreement between monitor studies and parallel spectrophotometric studies indicates this increased distance does not introduce complications caused by light scattering.

The amount of nutrient medium in the sample holder is important in terms of volume-to-surface ratio (gas exchange relies on diffusion) and volume-to-thallus area ratio. The smallest thallus disc that can be used reliably with the support ring is 11.5 mm in diameter, and the cuvette can conveniently hold up to 10 ml. This combination provides a volume-to-thallus area ratio about twice that in the growth flask. The use of either 7.5 or 10 ml of medium made no difference in the results.

Since the length of adaptation in the sample holders can affect the sample, this factor was also tested. Adaptation of one day or less consistently gave irregular rhythms with drastically skewed baselines, nonmonotonic damping patterns, and periods greater than 25 hr. However, no difference appeared between samples adapted 2½ and 3½ days.

In earlier work, *Ulva* from the "slowly growing" phase of culture was used, as it gave the longest-lasting rhythms (Britz and Briggs, 1976). The monitor was therefore used to compare the rhythm patterns of samples from rapidly growing and slowly growing phases of culture. The "younger" thalli were similar through three to four days of monitoring. Then in about 50% of the cases (out of 14 trials) the transmittance change rhythms appeared to become uncoupled from the clock and began to drift around in varying patterns. Control samples continued to give normal patterns. No systematic differences in the rhythms have been detected for *Ulva* from various times during the slowly growing phase of culture. Also, for a three-week-old culture it did not appear to matter from where in the thallus sample discs were cut.

The most perplexing matter has been that of pH control. Because of contamination problems the culture medium is unbuffered. During several weeks of growth under LD the pH can therefore rise to 9.0 or 9.5, conditions under which *Ulva* can still actively photosynthesize. During adaptation the medium in the sample holders becomes stabilized around 8.5 in the daytime and drops a few tenths of a pH unit at night. During constant darkness in the monitor the pH drops to about 8.0. Since certain studies require pH control, the effect on the rhythm of buffered medium was tested.

Five mM Tris buffer did not stabilize the pH except near 8.0 and, in any case, appeared to have no effect. Ten mM Tris had some distinct effects independent of pH (tested were pH 8.1 and 8.8 which bracketed the pH of the unbuffered controls); these included lengthening of the free-running period and some initial inhibition of the transmittance change. Ten mM HEPES had only a slight effect on the period (also lengthening it, apparently independently of the pH at 8.1 and 8.6) and no effect on the initial amplitude. The rhythm under both Tris and HEPES appeared to damp more rapidly and was noisier by the fifth cycle than the unbuffered controls; the Tris-treated samples were worse. The actual transmittance for the pH 8.1 samples under both Tris and HEPES was considerably lower than for the higher-pH buffered samples or the unbuffered controls. It was not clear whether this feature was caused by the chloroplasts being more in face position or, for example, by increased pigment.

On the basis of the above discussed results, a routine protocol was selected. Thallus discs 11.5 mm in diameter are cut from *Ulva* in the slowly growing phase, mounted in holders with raised support rings, filled with 9–10 ml of unbuffered culture medium per sample, and then allowed to adapt for 2½ days in LD cycles before loading in the monitor. With this protocol, samples mounted in the wheel and shielded
from the measuring beam for the first 72 hr of monitoring (DD$_6$ to DD$_{72}$) had rhythms from DD$_{16}$ to DD$_{114}$ identical in terms of amplitude and phase with those of the continuously monitored controls. The effects of the measuring beam are therefore negligible and the monitor may be assumed to offer conditions approaching DD.

**DISCUSSION**

The results of the present study show the *Ulva* transmittance change rhythm to be not only large and regular but also remarkably precise, quantitative, and reproducible, provided that certain methods of sample preparation and data handling are employed. For example, adaptation in sample holders seems to enhance reproducibility and may have been a factor in an earlier study where samples were not adapted and where substantial variability in rhythm patterns between samples was observed (Britz et al., 1976).

It was not intended here to characterize different sample preparation methods exhaustively or to optimize monitoring conditions. Certainly, many details remain unresolved, such as the role of pH control, temperature, and prior culture conditions. Nonetheless, a sufficient basis has been established to realize the advantages of multiple sample monitoring and to employ rhythmic *Ulva* transmittance changes as an experimental system for chloroplast movement and circadian rhythm studies.

**ACKNOWLEDGMENTS**

The author thanks Drs. J. Pfau, W. Nultsch, C. S. French, and W. R. Briggs for helpful discussions leading to the design of the monitoring device, and Mr. R. Hart for technical expertise in transforming these designs into reality.

**References**


**TWO APPLICATIONS OF A MULTI-SAMPLE AUTOMATIC MONITOR FOR RHYTHMIC TRANSMITTANCE CHANGES IN Ulva**

*S. J. Britz*

The completion of an automatic monitoring device for rhythmic changes in transmittance in *Ulva* offers expanded possibilities to study the underlying chloroplast movement, from the standpoints of the motility mechanism and the controlling circadian rhythm. This paper presents the results of two experiments that exemplify the quantitative application of the monitor in such studies.

Inhibitors have been applied to examine the mechanism of chloroplast movement. Experiments with the monitor have repeated and expanded upon preliminary work with conventional transmittance measuring techniques (Britz, 1975). Several features of the monitor have aided in the experiments. The ability to measure the same sample in the same position repeatedly increases the accuracy. The ability to gather data at hourly intervals and to continue measurements for at least three to four days facilitates the determination of small effects and effects that do not become apparent until after the first cycle has passed. Moreover, it is easier to distinguish possible action on the clock from inhibition of movement mechanism.

Table 10 shows the effects of two common inhibitors of cellular movement, colchicine and cytochalasin B, on
TABLE 10. Cytochalasin B Reversal of Colchicine-Inhibited Rhythmic 
Ulva Chloroplast Movement*

<table>
<thead>
<tr>
<th>Treatment and Number of Samples (n)</th>
<th>Movement in the Direction from Profile to Face, Relative Thallus Transmittance Change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>First</td>
</tr>
<tr>
<td>Control</td>
<td>0.437 ± 0.028</td>
</tr>
<tr>
<td>52 µM Cytochalasin B (n = 4)</td>
<td>0.461 ± 0.014</td>
</tr>
<tr>
<td>1 mM colchicine (n = 4)</td>
<td>0.421 ± 0.017</td>
</tr>
<tr>
<td>52 µM cytochalasin B + 1 mM colchicine (n = 3)</td>
<td>0.385 ± 0.054</td>
</tr>
</tbody>
</table>

*Presented are the first three successive transitions in the direction from profile to face. Intervening face to profile movement is not shown. "First" refers to the movement from the first transmittance maximum to the first minimum in DD. Control exhibits normal damping. The extent of movement is measured by reference-corrected and normalized relative thallus transmittance changes ± 2 × S.E. Note that values are amplitudes and not absolute transmittances. All treatments were given starting at DDu, immediately following the first transmittance maximum and point of normalization. Concentrated solutions of the drugs were added to nutrient medium, partially removed from the sample holders (cytochalasin B formed a flocculent upon addition and required a minute or two to dissolve), and then redistributed, to yield the above final concentrations. These treatments do not seem to disrupt greatly either the amplitude or phase characteristics of the rhythm. All treatments are 0.5% (v/v) dimethylsulfoxide (DMSO) which is used as a carrier for the cytochalasin B. DMSO by itself does have an effect upon both the rhythm and movement.

Chloroplast movement in Ulva. Colchicine is a presumptive inhibitor of microtubule-related phenomena (Weisenberg et al., 1968), and cytochalasin B is a presumptive inhibitor of microfilament-related phenomena (Wessells et al., 1971). Microfilaments and microtubules are major candidates for the contractile, movement-generating forces with cells (Hepler and Palevitz, 1974). Both colchicine and cytochalasin B have side effects which limit their application as diagnostic tools for microtubules or microfilaments. However, the attempt here has been to dissect various aspects of the movement through the differential sensitivity of the two phases (face-to-profile and profile-to-face) to these drugs.

The data in Table 10 are amplitudes and therefore do not show the relative thallus transmittance relationships between treatments. Thus, Table 10 does not present a complete view of the action of these inhibitors. Nonetheless, it is clear that colchicine dramatically inhibits the extent of movement after the passage of one cycle, while cytochalasin B alone has little or no effect. However, if both cytochalasin B and colchicine are added at the same time, the colchicine effect fails to develop. Cytochalasin B has in a sense reversed the action of colchicine.

The present experiment and others indicate that chloroplast movement involves the action of two different and opposing mechanisms (possibly microtubules vs. microfilaments) and that actual rhythmic movement may result from changes in the balance between these two systems.

Light-induced phase shifting experiments have also been undertaken with the monitoring device. Preliminary results are shown in Table 11. A 1-min light pulse was given at DDu, the first transmittance maximum and, as previously determined, the time of maximum sensitivity to phase advances. The times of subsequent transmittance maxima were determined with the parabola slope tech-
TABLE 11. Light-Induced Phase Shifting of the *Ulva* Transmittance Change Rhythm$^a$

<table>
<thead>
<tr>
<th>Sample</th>
<th>Second</th>
<th>Third</th>
<th>Fourth</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>26.52 ± 0.24</td>
<td>49.56 ± 0.13</td>
<td>73.47 ± 0.22</td>
</tr>
<tr>
<td>5</td>
<td>26.72 ± 0.16</td>
<td>49.81 ± 0.11</td>
<td>73.59 ± 0.16</td>
</tr>
<tr>
<td>6</td>
<td>30.38 ± 0.15</td>
<td>53.77 ± 0.14</td>
<td>77.49 ± 0.21</td>
</tr>
<tr>
<td>7</td>
<td>30.72 ± 0.10</td>
<td>54.43 ± 0.09</td>
<td>78.76 ± 0.18</td>
</tr>
<tr>
<td>8</td>
<td>30.57 ± 0.16</td>
<td>54.32 ± 0.06</td>
<td>78.04 ± 0.17</td>
</tr>
<tr>
<td>average phase shift (hr)</td>
<td>3.94 ± 0.16</td>
<td>4.49 ± 0.28</td>
<td>4.57 ± 0.47</td>
</tr>
</tbody>
</table>

$^a$Samples 4 and 5 were given 1 min of white light (67 kerg cm$^{-2}$ sec$^{-1}$ from a 500-W tungsten projection lamp filtered through 3 cm of 1.4% CuSO$_4$, IR-opaque as judged by a Corning 7-56 IR-transmitting filter and a thermopile) at time DDt. Samples 6, 7, and 8 received no treatment. Times of relative transmittance maxima and 95% confidence limits are calculated by the parabola slope technique (Britz et al., 1976).

The 1-min light treatment has resulted in a steady-state, 4 1/2-hr phase advance within two cycles. Dose-response characteristics have not yet been determined, but such a treatment is probably many times beyond saturation.

Four aspects of the analysis deserve emphasis. First, the parabola slope technique gives a very accurate estimate for the times of the maxima. Second, the behavior of the samples is very reproducible. Third, based on the first two points, it is possible to estimate the magnitude of the shift with great accuracy. Fourth, the treatment came to equilibrium quickly (i.e., minimal transient behavior). As a result it should be possible to get multiple estimates of the shift within one experiment.

The mechanics of measuring shifts is clearly well established. The ability to produce a shift with as little as one minute of light indicates that *Ulva* is considerably more sensitive to such treatments than other photosynthetic organisms (Cummings and Wagner, 1968). Since a shorter light exposure is less likely to influence processes other than the rhythm, *Ulva* should be an attractive organism for further phase shifting studies.

References


Kinetics and properties of membrane-associated binding sites for auxins have been studied with an in vitro system of homogenized and purified membrane preparations from corn coleoptiles; the physiochemical interaction between ligand and target unit was demonstrated by the pelletable binding of radioactive-labeled auxin (mostly 14C-naphthaleneacetic acid = NAA) to membrane vesicles, which are formed during extraction (Hertel et al., 1972; Ray et al., in preparation, 1976).

The binding can be considered specific, since it is a saturable, reversible process and is in agreement with the physiological characteristics of auxin action: Analogs compete according to their affinity in growth and transport tests; further, binding is mainly seen with hormone-sensitive tissues (Ray et al., in preparation, 1976). Graphical analysis of the data indicates a single class of binding sites with a \( K_d \) of \( 407 \times 10^{-7} \) M as being responsible for the specifically bound radioactivity; total binding is about 10% if a concentration of labeled NAA below the \( K_d \) is mixed with the particles, whereas 6% is bound specifically. The receptor is expected to be a protein, but in its function may be dependent upon lipids: Pronase digestion, for example, only results in a significant loss of binding if it is preceded by an incubation with phospholipase C (Ray et al., in preparation, 1976).

Binding studies on fractionated, subcellular membranes showed significant quantitative and certain qualitative differences of auxin binding properties within the three investigated classes of ER, golgi, and plasmamembrane vesicles (Ray et al., in preparation, 1976; Dohrmann, 1975). In an isopycnically centrifuged, linear sucrose density gradient, a sharp peak of specifically bound 14C-NAA is seen at 25% sucrose, coinciding with the ER-marker enzyme NADH-cytochrome \( c \) reductase, while a minor amount of binding occurs at densities higher than 30% sucrose; these fractions should contain mainly golgi and plasmamembrane vesicles, as suggested by the simultaneously measured activity of the markers \( \beta \)-1,4- and \( \beta \)-1,3-glucansynthetase and \( ^3 \)H-naphthylphthalamic acid binding. By changing the binding assay conditions it has been possible selectively to demonstrate specific binding characteristics for each of these membrane types.

In order to get further information about the nature of the receptor site(s), we are presently attempting to isolate and identify the molecular binding component. The binding pattern of density-separated vesicles raises the question, whether there are chemically and kinetically different receptors present in those membranes or whether the sites may be basically similar but somewhat modified, for instance as a result of membrane flow sequences. Also, we hope to obtain data that will explain the function of the ligand-receptor complex.

Isolation and purification steps after membrane disruption have to be monitored by assaying for capacity to bind auxin, using a method other than the previously employed centrifugation assay, which is not applicable to a soluble receptor. Several possible approaches have been tried:

(a) Equilibrium dialysis did not turn out to be satisfactory; in the sedimentation assay, bound and unbound ligand (about 90%) are separated to a maximal extent, whereas in equilibrium dialysis the concentration of free

---

1 Carnegie Department of Plant Biology Fellow and Stanford University, Stanford, California.
2 Stanford University, Stanford, California.
for equilibration and elution causes a dilution of radioactivity in the suspension. Correcting calculations show that the difference in labeled auxin, which has to be bound to gain just the concentration of \(^{14}\)C-NAA present in the Sephadex bed before additional ligand can be bound, is exactly reflected in the difference between trough and peak. Considering this, the amount of bound \(^{14}\)C-NAA can be calculated as:

\[
\text{% bound} = \frac{(\text{cpm}^1_{\text{peak}}) - (\text{cpm}^1_{\text{basal}}) \times 100}{(\text{cpm}^1_{\text{total}})}
\]

where (cpm\(^1\)) = amount of \(^{14}\)C-NAA in cpm measured in all fractions which contain radioactivity exceeding the basal value, and (cpm\(^2\)) = cpm per volume of column (sample before run).

The amount of bound \(^{14}\)C-NAA is directly proportional to the amount of sample added to the column and is also dependent upon the concentration of unlabeled NAA used in the assay, as seen in Fig. 18. These data were obtained from a series of column assays using increasing concentrations of nonradioactive NAA up to saturation of all specific receptor sites with ligand. The elution figures show gradually decreasing peaks and troughs and finally come close to zero deviation from the base line. For a clear illustration only the data with \(5 \times 10^{-7} M\) NAA (in the range of half-saturation) and \(10^{-4} M\) NAA (virtually all specific sites should be occupied by unlabeled NAA) are plotted. With the first NAA concentration the peak is reduced by about 50% (cpm\(_{\text{peak}} = 2273\) with \(^{14}\)C-NAA alone vs. 1230 when \(5 \times 10^{-7} M\) NAA is added), whereas addition of \(10^{-4} M\) NAA reduces the amount of bound radioactive NAA almost completely (144 cpm).

These data show that the column assay is a quantitative measure for the binding capacity of solubilized receptor sites for auxin. Differences in the binding properties between solubilized and membrane-associated receptor sites on the other hand may be expected, as seen in Fig. 18.

![Graph](image)

**Fig. 18.** Membrane suspensions were solubilized and centrifuged as described in the text. To equal amounts of the supernatant mixed with \(^{14}\)C-NAA none, \(5 \times 10^{-7}\) or \(10^{-4}\) unlabeled NAA was added; the samples were assayed with Sephadex G25 columns which had been equilibrated with the \(^{14}\)C-NAA buffer to which in each case nonradioactive NAA was added to yield the same concentration as the sample.
further experiments indicate. Table 12 is a summary of assays done so far to compare the specific binding features. Addition of benzoic acid, structurally related to auxin but not growth promoting, at a concentration of $10^{-5} M$, which only at this high concentration will affect $^{14}C$-NAA binding to intact vesicles at all, competes to some extent when used in the column test. Differences are also seen with dithioerythritol (DTE); it has a strong inhibitory effect on the auxin binding to membranes, but it is less effective on solubilized preparations. Furthermore, data reflecting the heat sensitivity are included in Table 12. The samples were incubated at different temperatures for 60 min, before the binding tests were done at 4°C; these results (see values for 50°C and 100°C) suggest that solubilized receptor sites have a reduced heat lability. Digestion with pronase or phospholipase C causes only partial inactivation of binding activity, both with vesicle suspensions and with solubilized binding sites. Although one has to account for differences in the binding properties of unsolubilized and detergent-treated particles, there is no doubt that the Sephadex G25 test can be taken as a quantitative auxin binding test. Questions concerned with these discrepancies will be approached in the future, following the planned isolation of the component that is responsible for the binding.

Preliminary data from fractionation experiments using appropriate Sephadex or agarose gels for separation of larger molecular weight substances resulted in a pattern of binding activity not identical with the general protein profile.

References


TABLE 12. $^{14}C$-NAA Binding Assay on Membrane-Associated and Solubilized Receptor Sites

<table>
<thead>
<tr>
<th>Treatment</th>
<th>By Sedimentation</th>
<th>Sephadex G25 Column</th>
</tr>
</thead>
<tbody>
<tr>
<td>25°C (60 min)</td>
<td>83*</td>
<td>71*</td>
</tr>
<tr>
<td>50°C (60 min)</td>
<td>3.5</td>
<td>43</td>
</tr>
<tr>
<td>100°C (60 min)</td>
<td>...</td>
<td>4.6</td>
</tr>
<tr>
<td>3mM DTE (0°C, 120 min)</td>
<td>16</td>
<td>49</td>
</tr>
<tr>
<td>Pronase (18.5 mg/ml; 25°C, 60 min)</td>
<td>45</td>
<td>43</td>
</tr>
<tr>
<td>Phospholipase C (1.5 mg/ml; 25°C, 60 min)</td>
<td>39</td>
<td>79</td>
</tr>
<tr>
<td>$10^{-3}M$ Benzoic Acid</td>
<td>88</td>
<td>43</td>
</tr>
</tbody>
</table>

*The numbers represent the relative specific binding (sedimentation) or the relative radioactivity over basal activity compared to the controls (= 100).

The original suspensions from washed membrane preparations were mixed with $^{14}C$-NAA and divided in half. One portion was treated with 1% Triton X-100 and centrifuged for 60 min at 133,000 $\times g$; the supernatant from this step and the original suspension with unsolubilized vesicles were incubated as indicated in the left column. Aliquots of each incubate were then assayed for binding: 0.1 ml particles/ml $^{14}C$-NAA assay buffer $\pm 10^{-3}M$ NAA were pelleted at 133,000 $\times g$ for 20 min; 0.3 ml supernatant was passed through a 0.5-ml Sephadex G25 column equilibrated with $^{14}C$-NAA buffer.
During the year studies have been continued to determine the thermal stability of key components of the photosynthetic machinery responsible for the marked differences in temperature tolerances between thermophilic higher plant species such as *Tidestromia oblongifolia* and cool-temperate plants such as *Atriplex sabulosa*. As reported last year (Year Book 74, pp. 743–759), the two C4 species differ in their high-temperature stability in several respects, including the maintenance of semipermeability of the cell membranes, respiratory activity, and photosynthetic activity. While onset of high-temperature damage differed by about 10°C in each case, photosynthesis was affected at considerably lower temperatures than either respiration or membrane semipermeability. Complete inhibition of photosynthesis occurred before other symptoms of high-temperature damage could be detected. Thus, the high-temperature inhibition of photosynthesis could not result from a general breakdown of the integrity of the cell.

Light-saturated photosynthesis was inhibited at 37°C and above in *A. sabulosa* (a C4 plant native to cool oceanic habitats), but no signs of inhibition were evident in *T. oblongifolia* (a summer-active C4 plant native to the floor of Death Valley) until the leaf temperature exceeded 47°C. It was unequivocally shown that the high-temperature inhibition of photosynthesis in *A. sabulosa* and *T. oblongifolia* was not even in part due to changes in the resistance to diffusive transport of CO2. This conclusion was valid for CO2 transport through the stomata as well as inside the leaf parenchyma.

Our previous studies indicated that thermal inhibition occurred at very similar temperatures for light-saturated and light-limited photosynthesis. Measurements of fluorescence kinetics on leaf discs and photochemical activities of chloroplasts isolated from heat-treated leaves pointed to the possibility that inactivation of photosystem II was primarily responsible for the observed high-temperature inhibition of the quantum yield of photosynthesis in intact leaves. Studies this year comparing the quantum yield for CO2 uptake by intact leaves and the quantum yield for DCIP reduction by isolated chloroplasts, as a function of the temperature at which the leaves had been pretreated, provide strong evidence that inactivation of a component of photosystem II, or a breakdown of the integrity of the membranes with which this photosystem is associated, constitutes one of the sites most sensitive to high temperature.

As shown in Fig. 19A and 19B, the responses to the quantum yield of photosynthesis measured at a non-limiting temperature for intact leaves exposed to increasing pretreatment temperature closely matches changes in the relative quantum yield for DCIP reduction of chloroplasts isolated from pretreated leaves of each species. In both cases, *T. oblongifolia* is able to sustain a constant quantum yield at much higher temperatures than *A. sabulosa*. In contrast the quantum yield of electron transport driven by photosystem I (reduction of NADP using DCIP ascorbate as electron donor in the presence of DCMU) in chloroplasts isolated from similar heat-treated leaves, remains fully active at temperatures that cause nearly complete inactivation of both photosystem II and CO2 fixation by intact leaves. This result agrees with our previous findings that the light-saturated rate
Fig. 19. Comparison of the heat stability in vivo of complete photosynthesis of *A. sabulosa* and *T. oblongifolia* with that of selected light and dark reaction components. Quantum yields for complete photosynthesis (A) and for photosystem I and II activities (B and C) of isolated chloroplasts together with activities of RuDP and PEP carboxylases (D and E) and extractability of total soluble protein (F) are shown for leaves treated at the temperatures indicated. The quantum yield of complete photosynthesis by attached leaves as influenced by pretreatment was measured at 30°C as described in *Year Book 74*, pp. 760-761. Chloroplasts were isolated as a 2000 x g pellet from leaves after 5 min pretreatment, using media containing 0.33 M sucrose, 0.05 M Tricine, 15 mM KCl, 1 mM MgCl₂, 1 mM MnCl₂, pH 7.4. Photosystem I was assayed as NADP reduction at 340 nm and photosystem II as DCIP reduction at 600 nm in a Perkin-Elmer 356 spectrophotometer equipped with an actinic illumination attachment. The intensity of actinic illumination was varied by use of neutral density filters from a maximum intensity of 3 nE cm⁻² sec⁻¹. The reaction mixtures contained: (1) for NADP reduction, media plus chloroplasts to 25 μg chlorophyll/ml 3 x 10⁻⁵ M DCIP, 10⁻⁷ M ascorbate, 2.5 x 10⁻⁶ M NADP and 50 μg/ml spinach ferridoxin; (2) for DCIP reduction, isolation media plus chloroplasts to 10 μg chlorophyll/ml, 3 x 10⁻⁵ M DCIP and 10⁻⁴ M NH₄Cl₂. The light-dependence curves were linear, and since chlorophyll concentrations were identical, changes in the slopes of these curves with pretreatment were taken to indicate changes in the relative quantum yield of the reactions assayed. The chloroplasts used were derived predominantly from mesophyll cells. Leaves heat-treated for 10 min were extracted by grinding exhaustively with glass beads in a pre-chilled mortar followed by homogenization in a glass tissue grinder. Microscopic examination indicated nearly complete breakage of the bundle sheath cells. The supernatant after 40,000 x g centrifugation was assayed for soluble protein by the Lowry Method; RuDP carboxylase was activated and assayed according to Lorimer et al. (1976), and PEP carboxylase was assayed according to Björkman and Gauhl (1969).

These studies substantiate the hypothesis that thermal damage to specific sites of the photochemical apparatus is a major factor limiting photosynthetic performance after exposure of intact tissue to damaging temperatures. Furthermore, substantial differences exist among plants in their resistance to such damage. While photosystem II is the most heat-sensitive photosynthetic component assayed, neither the mechanism responsible for the damage nor how specific this damage may be is clear. Several reports (Krause and Santarius, 1975; Mukohata et al., 1973; Emmett and Walker, 1969) suggest that ATP formation by photophosphorylation is also sensitive to heat in isolated chloroplasts. Our studies indicate that chloroplasts damaged by pretreatment of intact leaves are also uncoupled. We feel that thermal damage is probably not specific to only one site or component in photosystem II but rather may be a secondary result of either ionic or pH changes in the chloroplast envir-
environment or a modification of the organization of the lipid–protein membrane structure necessary for photosynthetic activity.

One factor known to play an important role in membrane function is the degree of unsaturation of the fatty acids of the membrane lipids. The fatty acids of the major chloroplast lipids—monogalactosyl diglycerides (MGDG), digalactosyl diglycerides (DGDG), and phosphatidyl glycerol (PG)—are somewhat more saturated in *Tidestromia* than in *A. sabulosa* or in *A. glabriuscula*, a C₃ species also from cool marine habitats (Table 13). However, this difference in extent of saturation of the glycolipids is small. In contrast, the relative difference in unsaturation of the fatty acids of phosphatidyl glycerol is much greater than that of the other chloroplast lipids. Anderson (1975) suggests that this lipid, because of its charge, may play a specific role in lipid–protein associations within the membrane. We are currently examining the hypothesis that changes in the unsaturation of fatty acids of this lipid might have specific effects upon the heat stability of chloroplast membranes.

While differences observed in the susceptibility of photosystem II activity to heat inactivation in the two species are sufficient to explain the observed high-temperature inhibition of the quantum yield for complete photosynthesis in intact leaves, one cannot rule out the possibility that other photosynthetic components, such as key enzymes of the carbon fixation and reduction process, have the same temperature sensitivity and are being inactivated together with photosystem II. Inactivation of nonphotochemical components would not affect the quantum yield, but might have substantial effects upon the light-saturated photosynthetic capacity, which as previously noted (Year Book 74, pp. 748–751) is affected at lower temperatures than those which cause inhibition of the quantum yield.

This year systematic comparative studies of the heat susceptibility in vivo of a number of key photosynthetic enzymes have been initiated. To date we have examined the heat stability of the activity of the two carboxylation enzymes, ribulose-diphosphate carboxylase (RuDPCase) and phosphoenolpyruvate carboxylase (PEPCase). In addition, we have followed the effect of heat treatment of intact leaves of denaturation of the bulk soluble protein.

As shown in Fig. 19D, RuDPCase is a remarkably heat-stable enzyme. In *T. oblongifolia* leaves, no inactivation was observed until the temperature exceeded 55°C and even after exposure of the leaves to 60°C for 10 min, 80% of the activity remained. In *A. sabulosa* leaves inactivation of this enzyme could be detected at about 10°C lower temperature. Yet this temperature is much higher than the temperature at which inhibition of light-saturated photosynthesis sets in. After heat treatment of the leaves at 47°C, which causes complete inhibition of photosynthesis in this species, at least 90% of the RuDPCase activity remained. Thus, even though a difference be-

### Table 13. Comparison of the Extent of Unsaturation, Expressed as Double Bonds per Carbon Atom, of the Fatty Acids Separated by Gas Chromatography from the Chloroplast Lipids of *Tidestromia oblongifolia*, *Atriplex sabulosa*, and *A. glabriuscula* *

<table>
<thead>
<tr>
<th>Lipid</th>
<th><em>T. oblongifolia</em></th>
<th><em>A. sabulosa</em></th>
<th><em>A. glabriuscula</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Monogalactosyl diglyceride</td>
<td>0.150</td>
<td>0.151</td>
<td>0.161</td>
</tr>
<tr>
<td>Digalactosyl diglyceride</td>
<td>0.120</td>
<td>0.140</td>
<td>0.151</td>
</tr>
<tr>
<td>Phosphatidyl glycerol</td>
<td>0.052</td>
<td>0.086</td>
<td>0.092</td>
</tr>
</tbody>
</table>

*Data is from an undergraduate honors research thesis by Thomas H. Payne (1976).
between the species in apparent heat stability of this enzyme in vivo was detected, the onset of heat denaturation was at a temperature 10°C higher than that required to inactivate complete photosynthesis. These results indicate that RuDPCase from both a cool- and a heat-adapted C₄ species is highly heat stable and that heat inactivation of this enzyme is not involved in high temperature inhibition of photosynthesis.

PEPCase (Fig. 19E) exhibits a considerably lower heat stability in vivo than RuDPCase, although marked differences again exist between the two species. However, inhibition of PEPCase activity in each species also occurs at considerably higher temperatures than inhibition of photosynthesis. In contrast, Phillips and McWilliam (1971) report that RuDPCase and PEPCase from *Atriplex nummularia*, a C₄ species, begin to show a temperature-dependent decline in activity at considerably lower temperatures when detached leaves are heat treated for several hours. These studies do not necessarily contradict our findings, since such long-term changes in activity may be due to factors other than heat-induced protein denaturation per se. The studies reported here are for short-term (10 min) heat exposure during which changes other than protein denaturation should be minimized.

As shown in Fig. 19F, one or several unidentified components of the soluble protein in the leaves evidently undergo heat denaturation and aggregation so that they are no longer soluble in the aqueous extraction buffers. The temperature at which this denaturation commences in each species is very close to the temperature at which heat inhibition of leaf photosynthesis can first be observed and is considerably lower than the corresponding temperatures for inactivation of the two carboxylases. Hence, high-temperature inhibition of photosynthesis may involve both inactivation of photosystem II, associated with the chloroplast membranes, and denaturation of one or more soluble, non-membrane-bound enzymes which constitute a significant fraction of the bulk soluble protein. Studies are now being initiated to determine whether this denaturation involves other heat-labile photosynthetic enzymes in *A. sabulosa* and *T. oblongifolia*.

The heat stabilities of the various components of the photosynthetic apparatus observed in vivo for the two species might not necessarily reflect the intrinsic heat stabilities of the components. For example, heat inactivation of a certain component may be an indirect effect caused by a high-temperature-induced change in its immediate cellular environment, such as a change in pH or ionic composition, both of which are known to influence strongly the stability of proteins.

Therefore, differences in heat stability between the species could in part be due to factors determining the heat stability of the cells' partitioning membranes. Experiments reported last year (*Year Book 74*, pp. 751–759) showed that differences in the heat resistance to loss of semipermeability of the membrane which prevent leakage of solutes from immersed leaves into the surrounding aqueous medium cannot account for the differences in heat stability of the photosynthetic apparatus. However, these results do not rule out the possibility that the heat stability of other partitioning membranes within the cell are involved.

Krause and Santarius (1975) have shown that chloroplasts isolated with their outer membranes intact are more resistant to thermal damage than are chloroplasts lacking this membrane. Presumably the only effect of the outer membrane on thermal stability of the photosynthetic membrane is to maintain a more favorable internal environment than that provided in the external medium. Within an intact leaf cell the possibilities of such interactions are manifold.

Therefore, we chose to compare the heat stabilities of the various photo-
synthetic components in vitro as well as in vivo. To date, our in vitro studies have been limited to comparisons of isolated and purified RuDP carboxylase from leaves of *A. glabriuscula* and of RuDP carboxylase and PEP carboxylase from leaves of *T. oblongifolia*. For this purpose, extracts containing all of the soluble leaf protein were applied to 38 ml 0.29–0.88 M linear sucrose density gradients (Boynton *et al.*, 1972). After centrifugation for approximately 40 hr at 25,000 rpm in an SW-27 rotor at 2°C, the gradients were fractionated and the distribution of protein, RuDPCase, and PEPCase activities determined. With most C₃ and C₄ species, in which Fraction I protein (RuDPCase) constitutes a large fraction of the total soluble protein, this technique provides an excellent yield of highly purified enzyme in a single-step procedure (Fig. 20A and B). With *T. oblongifolia*, in which RuDPCase constitutes only a small fraction of the total protein (Fig. 20C), prior enrichment using (NH₄)₂SO₄ fractionation is necessary to obtain adequate amounts and purity of the enzyme.

Preparations of RuDPCase from *A. glabriuscula* and *T. oblongifolia* appeared homogeneous as determined by sedimentation velocity centrifugation in the analytical ultracentrifuge and by polyacrylamide gel electrophoresis. No differences between RuPCase in the two species in molecular weight (ca. 530,000 daltons), net charge, or specific activities (2.0 μmole CO₂ • min⁻¹ mg protein⁻¹ at 30°C) could be resolved. Similar analyses are in progress on RuDPCase purified from *A. sabulosa*.

Heat inactivation of the proteins in vitro was assayed either as the time-dependent decline in catalytic activity in high-speed supernatants subjected to various temperatures or as the increase in absorbance at 285 nm of the purified enzyme. These two assay techniques gave very similar estimates of denaturation in our experiments. The heat stability of RuDPCase and PEPCase in vitro was strongly influenced by pH during treatment of the proteins in 10 mM Mg²⁺ and 5 mM dithiothreitol (omitted in the absorbance assays) in 0.1 M HEPES or MES buffers over the pH range of 5.5–8.5. Maximum heat stability for PEPCase was observed in pH range 5.5–6.0, and the rate of inactivation increased sharply as the pH was increased from 6 to 7. RuDPCase exhibited maximum heat stability at pH 7.0–7.6, and the rate of inactivation increased steeply below pH 6.8 and above pH 7.8. At their respective pH optima for maximum heat stability, appreciable inactivation of the *T. oblongifolia* PEPCase and RuDPCase was not detected until 48° and 57°C, respectively. This difference in high-temperature inactivation is similar to that found in vivo, suggesting that the two carboxylation enzymes have intrinsically different heat stability characteristics. Comparative data on the heat stability in vivo of RuDPCase and PEPCase from *A. sabulosa* remain to be obtained.

Preliminary results also indicate that the heat stability of RuDPCase in vitro is appreciably lower in *A. glabriuscula* than in *T. oblongifolia*, again resembling the situation in vivo. Further comparative studies of the heat stability of this enzyme isolated from *T. oblongifolia, A. sabulosa*, and other cold-adapted and heat-adapted species of higher plants, together with determinations of the relative hydrophobicities of the proteins should provide additional insight into a problem of considerable evolutionary and ecological interest.

It is also interesting from an evolutionary viewpoint that PEPCase shows a large difference in molecular weight between the two C₄ species *A. sabulosa* and *T. oblongifolia*, whereas RuDPCase does not. Clearly different peak positions for PEPCase activity are seen in Fig. 20B and 20C, with the *T. oblongifolia* enzyme position at higher density (greater molecular weight) than the *A. sabulosa* enzyme. This difference in molecular weight was con-
Fig. 20. Comparison of the distribution of total soluble protein with RuDP and PEP carboxylase activities in *A. glabriuscula*, *A. sabulosa*, and *T. oblongifolia*. 40,000 x g supernatant fractions prepared as in Fig. 19 were loaded on 38 ml 0.29–0.88 M sucrose gradients containing 25 mM Tris, pH 7.5, 25 mM MgCl₂, 25 mM KCl, 5 mM Dithiothreitol (Boynton et al., 1972) and centrifuged in an SW-27 rotor at 25,000 rpm, 2°C for 40 hr. PEPCase and RuDPCase activities were assayed on aliquots of 1 ml fractions as outlined in Fig. 19. Protein content was determined after TCA precipitation by the Lowery Method.

Firmed by polyacrylamide gel electrophoresis with crude leaf extracts, followed by a staining procedure specific for PEPCase activity. The molecular weights estimated by this method were 170,000 and 340,000 for *A. sabulosa* and *T. oblongifolia* enzymes, respectively. We previously reported (Year Book 71, pp. 135–141) two electrophoretic forms of PEPCase in C₄ species of *Atriplex* and a third isozyme in C₃ species of this genus. A survey of the molecular weights of PEP carboxylase in a number of related species, covering a very wide geographical and ecological range, has now been started to determine the extent and possible significance of this variation.

In addition to providing a convenient method for enzyme purification, sucrose density gradient centrifugation also permits an accurate, direct, and reasonably simple means of assessing the fraction of the total soluble leaf...
protein that different plants allocate to a single major protein, RuDPCase, and how this allocation is influenced by environmental factors. As shown in Table 14, this enzyme accounts for 41%-46% of the total protein in A. glabriuscula, a value typical of those reported in the literature for C₃ plants. A. sabulosa (C₄), which like A. glabriuscula (C₃) is native to cool oceanic habitats, allocates one-fifth of its soluble protein to RuDPCase when grown under identical conditions (22°C day/15°C night). However, both species have similar growth and photosynthetic rates (Year Book 73, pp. 748-767; Year Book 74, pp. 743-748). The amount of total soluble protein is similar in the two species. In contrast, the thermophilic C₄ plant T. oblongifolia allocates less than 10% of its soluble protein to RuDPCase when grown under a 45°C day/32°C night regime, which is close to the optimum temperature for growth in this species. This low proportion of RuDPCase is not compensated for by a higher content of total soluble protein, since the soluble protein content is considerably lower than in the other species. The smaller amount of RuDPCase detected in this C₄ species is not a result of an incomplete breakage of the bundle-sheath cells, because special precautions were taken to make certain that complete breakage of all cells was obtained.

One might ask how T. oblongifolia is able to get by with a much smaller amount of RuDPCase than, for example, A. sabulosa, and yet is capable of at least as high photosynthetic rates at the optimum temperature. A simple answer to this question may be that at a temperature of 45°C, the total catalytic activity of this amount of RuDPCase is as high as that of the larger amount of enzyme in A. sabulosa at 22°C.

Preliminary studies suggest that while total RuDPCase activity is not limiting photosynthesis in T. oblongifolia at 45°C, it may well be rate limiting at lower temperature. This suggestion could explain the rapid decline in photosynthetic rate with decreasing temperature (Year Book 70, pp. 511-520), and thus also the high optimum temperature for photosynthesis in high-temperature-grown plants of this species (Year Book 74, pp. 743-759). These early results further suggest that the amount of RuDPCase increases with decreasing growth temperature over a 25°C range, as does also the photosynthetic capacity at rate-limiting temperatures. Studies designed to test the validity of such a proposed temperature-controlled adjustment of RuDP carboxylase synthesis are planned.

This work was supported in part by NSF Grant No. BMS to O. Björkman. We thank Ms. Mary Enama, a CIW graduate student from the Department of Biology, Washington University, St. Louis, for carrying out the polyacrylamide gel electrophoresis experiments.

References

| TABLE 14. Comparison of the Total Leaf Protein, Total Soluble Protein, and Fraction I Protein (RuDPCase) in A. glabriuscula, A. sabulosa, and T. oblongifolia |
|----------------|----------------|----------------|
| Species        | Fraction I Protein, % of total soluble protein | Total Soluble Protein, mg/g dry wt | Total Leaf Protein, mg/g dry wt |
| A. glabriuscula, C₃ | 41-46            | 130             | 260             |
| A. sabulosa, C₄  | 19-21            | 117             | 250             |
| T. oblongifolia, C₄ | 4-8              | 64              | 185             |
GENETIC VARIATION AFFECTING METABOLIC PHENOTYPES: AN APPROACH TO ANALYZING PHOTOSYNTHETIC CARBON REDUCTION IN A C₄ PLANT

Mary Enama

The physiological processes of an organism depend on the action of many enzymes. Because the various pathways are coordinated, it has been argued that the associated allelic variation should show similar coordination, comprising a "metabolic phenotype." To date, there has been no reported study of genetic variation among the enzyme loci of a single physiologically important metabolic pathway in plants. This paper describes a series of investigations to provide this information for a C₄ plant.

A multi-locus study of evolutionary adaptation of a metabolic process should have the following characteristics in addition to those described for a single locus study:
(1) The metabolic process should be of primary physiological importance.
(2) The process should be well characterized biochemically.
(3) The organism studied should be found in a variety of habitats in which individuals of one habitat are known to differ physiologically and genetically from those in other natural locations.
(4) The organism should be easily grown and should produce a sufficient quantity of tissue to allow analysis of several enzymes from each individual.

In plants, such a metabolic process is photosynthetic carbon reduction. Gas-exchange analysis has shown it to be influenced by and adapted to environmental parameters such as light, temperature, and water stress (Björkman et al., 1975). The biochemical pathway is well known, and its various components are under intensive investigation in many laboratories. In addition, for a variety of plants, transplant studies have indicated the existence of ecotypes in which primary productivity varies in individuals of a given species transplanted between two natural habitats of the species (Björkman et al., 1974). That is, the physiological plasticity of individual plants is genetically controlled and differs with site of origin. Thus, it would be very informative to do a multi-locus analysis of such ecotypes with regard to photosynthetic carbon fixation.

Two species that are likely candidates for such a study are Distichlis spicata and Atriplex lentiformis, both NAD–malic enzyme C₄ species. Both species grow naturally in desert and coastal habitats. Existing data indicate that a biochemical analysis might show interesting differences between ecotypes (Björkman et al., 1974; Pearcy and Harrison, 1974).

The set of enzymes chosen for the analysis should include those thought to be of major regulatory importance for the metabolic process under consideration. For photosynthetic carbon re-
duction in a C₃ plant one might consider the following ten enzymes: phosphoenolpyruvate carboxylase (E.C. 4.1.1.38) [PEP carboxylase]; ribulose-1,5-diphosphate carboxylase (E.C. 4.1.1.39) [RuDP carboxylase]; glutamate-oxaloacetate transaminase (E.C. 2.6.1.1) [GOT]; NAD–malic enzyme (E.C. 1.1.1.39); phosphoglyceric acid kinase (E.C. 2.7.2.3); NADP glyceraldehyde-3-phosphate dehydrogenase (E.C. 3.1.3.11) [NADP G-3-PDH]; fructose-1,6-diphosphatase (E.C. 3.1.3.11) [FDPase]; phosphoribulokinase (E.C. 2.7.1.19); pyruvate, P, dikinase (E.C. 2.7.9.1); and adenylate kinase (E.C. 2.7.4.3) [AK].

Electrophoretic assays for some of these presently exist (Shaw and Prasad, 1970; Hatch et al., 1972) and are being developed for the others. Individuals of Distichlis spicata have been used as a trial system to test and develop assays. To date, electrophoretic assays for PEP carboxylase, RuDP carboxylase, GOT, NADP G-3-PDH, FDPase, and AK have been successful in a Tris-glycine electrophoretic system (Davis, 1964) using crude leaf extract. Of these six enzymes, three show multiple bands: four bands for GOT, two for FDPase, and two for AK. As the isozyme literature shows, these multiple bands may represent isozymes from different organelles, participating in separate metabolic functions (Scandalios et al., 1975; Viswanathan and Krishnam, 1962). Since the interest of the proposed study is to characterize allelic variation in photosynthetic carbon reduction, it will be necessary to show by subcellular fractionation or other means which isozyme(s) are involved in the process under consideration.

The band which appears for NADP G-3-PDH seems to be NAD-specific, so the same basic assay can be used for both by adding NAD when assaying MDH or NADP for a malic enzyme assay. For some species NAD–malic enzyme requires Mn⁺⁺ for activity (Hatch, Mau, and Kagawa, 1974). This information offers a possibility for distinguishing the various malate-utilizing enzymes since different bands do appear, depending on what combination of Mn⁺⁺, Mg⁺⁺, NAD, and NADP are used in the electrophoretic stain. Whether this approach is valid will have to be confirmed by an independent method.

Thus, the outline of the proposed study is to collect plants of the same species from two dissimilar habitats and to survey the suggested ten enzymes from each individual, using standardized Ferguson plots as described by Johnson elsewhere in this Report. It will be interesting to see if distinct arrays of structural alleles characterize the different ecotypes and if in vitro characteristics of the enzymes can reasonably be correlated with in vivo differences in photosynthesis.

References
MOLECULAR WEIGHT VARIATION OF PHOSPHOENOLPYRUVATE CARBOXYLASES FROM C₄ PLANTS

Mary Enama

The phosphoenolpyruvate carboxylase reaction is the first step in photosynthetic carbon fixation in C₄ plants. As such, it plays an important role in metabolic regulation and is present and highly active in C₄ plants. In the course of purifying this enzyme by means of sucrose density gradient centrifugation it was noted that the enzyme from Tidestromia oblongifolia had a considerably larger molecular weight than that from Atriplex sabulosa (this Year Book). An electrophoretic Ferguson plot analysis is a quick means of measuring size and relative charge of proteins even from crude extracts when a specific stain is available.

A Ferguson plot analysis has been done for the phosphoenolpyruvate carboxylases from T. oblongifolia, A. sabulosa, Atriplex hymenelytra, and Distichlis spicata. Leaf tissue was prepared by grinding in 2-4 volumes of 0.1 M Tris, pH 7.8, 0.01 M MgCl₂, 2.5 × 10⁻⁴ M EDTA, 2% Na ascorbate, 5 mM DDT, and 4.5 mM diethyldithiocarbamic acid at 4°C. The mixture was then centrifuged at 27,000 g for 30 minutes at 4°C. The supernatant was run on 4%, 5%, 6%, 7%, 8%, and 9% polyacrylamide gels in a Tris-glycine electrophoretic system (Johnson, 1975). The gels were stained with 2 mM phosphoenolpyruvate, 1 mM NaHCO₃, 10 mM MgCl₂, 6.5 mM Fast Violet B Salt, and 2.5 × 10⁻⁴ M EDTA in a 0.1 M Tris buffer, pH 7.8. A red band appears within 15 minutes at the location of the phosphoenolpyruvate carboxylase. Figure 21 shows the results of a sample run for each species. Replicate runs for each species yield similar results within experimental error.

The equation which describes the lines depicted in Fig. 21 is discussed elsewhere in this Report. The y-intercept is the free electrophoretic mobility (μₑ) and the slope is the retardation coefficient (Kᵣ), which can be correlated with molecular weight (Johnson, 1975). Table 15 shows the values of Kᵣ and the estimated molecular weights for the phosphoenolpyruvate carboxylases of the four species tested and average values for the two internal standards used in every gel, hemoglobin and ferritin. These estimates indicate that the Tidestromia

<table>
<thead>
<tr>
<th></th>
<th>Kᵣ</th>
<th>Est. Mol. Wt.*</th>
<th>Actual MW</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. sabulosa</td>
<td>-0.094 ± 0.006</td>
<td>170,000</td>
<td></td>
</tr>
<tr>
<td>A. hymenelytra</td>
<td>-0.105 ± 0.006</td>
<td>190,000</td>
<td></td>
</tr>
<tr>
<td>D. spicata</td>
<td>-0.099 ± 0.005</td>
<td>180,000</td>
<td></td>
</tr>
<tr>
<td>T. oblongifolia</td>
<td>-0.142 ± 0.005</td>
<td>340,000</td>
<td></td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>-0.049 ± 0.002</td>
<td>50,000</td>
<td>64,500</td>
</tr>
<tr>
<td>Ferritin</td>
<td>-0.164 ± 0.009</td>
<td>440,000</td>
<td>450,000</td>
</tr>
</tbody>
</table>

*The molecular weight value shown was calculated from a standard curve (Johnson, 1975). As can be seen from the value for hemoglobin there can be up to 20% error in the values obtained. Thus, there is no significant difference between the estimated molecular weights from the first three species, but these values are significantly different from that for T. oblongifolia.
enzyme is about twice the molecular weight of the phosphoenolpyruvate carboxylases from the other three species. Whether or not the apparent size difference is an evolutionarily and structurally significant increase of the enzyme size is not yet known. The available information in the plant literature indicates a molecular weight of 350,000 for the phosphoenolpyruvate carboxylase from *Atriplex spongiosa* (Ting and Osmond, 1973).

Other C₄ plants, including *A. spongiosa* and plants in the Amaranthaceae related to *Tidestromia*, will be tested to see if *Tidestromia* has a unique phosphoenolpyruvate carboxylase or if there are apparently two size classes of phosphoenolpyruvate carboxylase among C₄ plants.

References

PHOTOSYNTHETIC CAPACITY OF *in situ* DEATH VALLEY PLANTS

H. A. Mooney, O. Björkman, J. Ehleringer, and J. Berry

As part of our long-term studies on the physiological ecology of plants we have been concentrating our efforts on the characteristics of components of the native flora of Death Valley, California. While Death Valley is often identified as one of the hottest and driest environments on earth, these extremely high air temperatures (exceeding 50°C) occur only during the summer months (Fig. 22). In the winter months relatively cool temperatures (20°C) predominate, and in the spring and fall months air temperatures are
often around 30°C. Consequently, Death Valley provides us with a broad variety of thermal environments and a unique opportunity to study the mechanisms plants possess to adapt to these seasonal thermal regimes.

We report here studies of the intrinsic photosynthetic capacities of several species in response to seasonal changes in thermal regimes of their natural habitat. The measurements were made on plants growing under natural conditions on the floor of Death Valley (Year Book 73, pp. 748–757).

Using the mobile laboratory, we measured the photosynthetic capacity of plants during the winter (January), spring (March), summer (late May), and fall (October) seasons. Plants studied included a winter annual (*Camissonia claviformis*), a summer-active herbaceous perennial (*Tidestromia oblongifolia*), and two evergreen shrubs (*Larrea divaricata* and *Atriplex hymenelytra*).

Under natural conditions the two evergreen shrub species were photosynthetically active throughout the year, although their maximum photosynthetic rates were considerably below those of the two that hold their leaves for a short period (Fig. 23). The two herbaceous species *Tidestromia* (*C₃*) and *Camissonia* (*C₄*) possess extremely high maximum photosynthetic rates (exceeding 5 nmol CO₂ cm⁻² sec⁻¹), yet their physiological activities are restricted for the most part to a single season during the year. There appears to be an inverse relationship between maximum photosynthetic rate and leaf longevity among the species in this
study. Of the species with the highest rates observed, the winter-active annual *Camissonia* is a C₃ plant, whereas the summer-active herbaceous species *Tidestromia* is a C₄ plant.

At the 30°C measurement temperature, *Larrea*, the C₄ evergreen species, maintained a relatively constant photosynthetic capacity under natural conditions at all times of the year. This homeostasis persisted in spite of widely disparate midday seasonal air temperatures (20° to 45°C) and leaf water potentials (−25 to −50 bars). The unusual homeostatic adjustment comes about, in part, because of changes in the photosynthetic thermal optimum (Fig. 24). These data were obtained with plants receiving irrigation to eliminate the effect of water stress. Although the photosynthetic rate was relatively constant at the various seasonal temperature optima (2.4–2.7 nmol CO₂ cm⁻² sec⁻¹), the temperature optimum shifted from 20°C during the cool season to over 30°C in the fall.

*Atriplex*, the C₄ evergreen species, exhibited its highest photosynthetic rate under natural conditions in the spring with newly produced leaves. The photosynthetic rates were reduced during the other season coincident with changes in leaf reflectivity (Year Book 73, pp. 846–852). Associated also with the lower photosynthetic rates were decreases in leaf water potential, leaf conductance, and leaf nitrogen. In contrast to *Larrea*, *Atriplex* showed little change in its temperature optimum for photosynthesis between seasons (data not shown). As shown in Fig. 25 the optimum temperature for photosynthesis was 30°C.

The summer-active C₃ plant *Tidestromia* is characterized by a very high photosynthetic rate during its principal growth period. In the summer months when *Tidestromia* is active, leaf temperatures may be in excess of 45°C. The optimum temperature for photosynthesis was similar (Year Book 74, pp. 743–751). Under spring and winter temperatures *Tidestromia* would have very low photosynthetic rates. The measured photosynthetic rates were lower in the fall, however, because of the onset of leaf senescence.

The C₃ winter-active annual *Camissonia* germinates following heavy winter rains and can, under certain conditions, complete its entire life cycle in six weeks. This short-lived species has a remarkable capacity to capture sunlight and fix carbon. *Camissonia* converted incident photosynthetically active radiation (400–700 nm) into

---

**Fig. 24.** Seasonal photosynthetic temperature responses of *Larrea* in Death Valley. These values were measured on irrigated plants under conditions of 170 nE cm⁻² s⁻¹, an ambient CO₂ concentration of 325 μbar, and a water vapor pressure deficit of less than 15 mbar.
chemical energy with an efficiency of 8.5%. This is a consequence of the lack of light saturation at midday irradiances, a feature that is also characteristic of Tidestromia (Year Book 70, pp. 540–550). The in situ midday photosynthetic rate of Camissonia was nearly 6 nmol CO₂ cm⁻² sec⁻¹, a rate that is higher than has been measured on such productive crop species as corn, sorghum, and sugar cane.

The thermal optimum of photosynthesis of the various species coincides with the prevailing temperatures during their principal growing period (Fig. 25). The winter-active C₃ Camissonia has a thermal optimum near 20°C and the summer-active C₄ Tidestromia, of over 45°C. The C₄ plant Lorrea can potentially grow throughout the year, except during midsummer. This plant shifts its thermal optimum in concert with the prevailing air temperatures. The C₄ plant Atriplex grows only during the winter and spring months and maintains a thermal optimum at 30°C.

The major results from these studies will be published elsewhere in greater detail along with a full analysis of the physiological components responsible for the changing seasonal photosynthetic responses of the plants.

This work was supported in part by the National Science Foundation. We thank the U.S. Park Service in Death Valley for their considerate assistance.

LEAF ABSORPTANCE AND PHOTOSYNTHESIS AS AFFECTED BY PUBESCENCE IN THE GENUS Encelia

James Ehleringer

Among higher plants there is a trend toward increasing leaf pubescence (presence of leaf hairs) along environmental gradients of decreasing precipitation (Schimper, 1903; Warming, 1909; Clausen, Keck, and Hiesey, 1940). These hairs, covering the surface of the leaf, are generally considered to be an adaptive feature of plants occupying arid habitats. The reason is that pubescence can reduce the heat load of leaves by increasing the reflectance from the leaf surface, reducing the amount of radiation absorbed. The adaptive value
californica. Field observations of solar absorption coefficients for both species were made in December (1974), March (1975), and July (1975). *E. californica* was sampled at Point Mugu (362 mm mean annual precipitation) and San Diego (240 mm) in southern California. *Encelia farinosa* was sampled at Superior (433 mm), Tucson (270 mm), Tonopah (155 mm), and Ehrenberg (90 mm) in southwestern Arizona. Five representative samples were collected from each site. The means of all samples of a species at a given sampling time were reduced to a single value.

As shown in Fig. 28 the mean solar absorption coefficients for *E. californica* were 83.9%, 83.8%, and 82.4% and for *E. farinosa* 71.7%, 63.4%, and 52.5%, respectively, for sampling dates of December, March, and July. The range of values between sites averaged 2% for *E. californica* and 10% for *E. farinosa*, while the range within a site averaged 2% for both species. These field data clearly show that the solar absorption coefficient for *E. californica* remains higher than that for *E. farinosa* at all times through the growing season. Additionally, whereas the solar absorption coefficients for *E. californica* remained constant through the season, the absorption coefficients for *E. farinosa* steadily declined as the season progressed. By July *E. farinosa* averaged 30% less visible light absorbed than *E. californica*. This decrease in leaf absorptance by *E. farinosa* through the season occurred in conjunction with an increase in mean maximum air temperatures. This correlation suggests a role for pubescence in modifying the leaf energy balance as the environment becomes harsher and reduces leaf temperatures. Cunningham and Strain (1969) have also shown that leaf size decreased through the season, further providing a more favorable energy balance.

If pubescence is adaptive for *E. farinosa*, then a positive correlation should exist between leaf absorptance and precipitation for sites occupied by this species. Solar absorption coefficients from each of the *E. farinosa* during March, the period of peak productivity, were plotted against the precipitation received up to the sampling date at the site during the current growing season (Fig. 29). These data reveal a strong correlation ($r^2 = 0.995, P < 0.01$) between the solar absorption coefficient and precipitation, suggesting, first, that the reduction in energy absorbed by pubescent leaves is directly dependent on the aridity of the environment and, second, that the degree of pubescence is a plastic response by the leaves to the amount of precipitation received. Since transmittance through pubescent leaves is quite small, there is also a strong correlation between the solar reflection coefficient and precipitation. It should be noted that it is the plastic response by pubescent leaves which is responsible for most of the variation in solar absorption coefficients between *E. farinosa* sample sites. The possible correlation between pubescence and aridity on a community basis rather than on a single
species basis was discussed by Billings and Morris (1951), but solar absorption coefficients were not reported.

The decrease in light absorbed by pubescent *E. farinosa* leaves over the 400–700 nm waveband is sufficient to cause significant reduction in the heat load of leaves. This decreased radiation load should indeed be of selective advantage to *E. farinosa* in arid desert sites since nearly 50% of the solar radiation load on the leaf and approximately 80% of the radiation absorbed by typical green leaves is in the 400–700 nm range. Yet a reduction in the amount of light absorbed carries with it a great disadvantage because reduction in light absorption means less light is available for photosynthesis. To minimize these effects of pubescence on light-limited photosynthesis it is likely that there is an inverse relationship between the degree of pubescence and the water available to the plant, such that as plants are less stressed the degree of pubescence is lowered. The strong correlation between solar absorption coefficients and precipitation (Fig. 29) serves as indirect evidence for such a relationship.

To determine to what degree the advantages of pubescence—in terms of reduced heat load on the leaf—may be offset by lower rates of carbon gain, photosynthetic rates were measured on individuals of *E. farinosa* differing in degree of pubescence. All plants were grown under conditions of sufficient water and nutrients and full sunlight in phytocells (*Year Book* 72, pp. 393–403). Simultaneous measurements of CO₂ and water vapor exchange were made on single attached leaves using a leaf chamber and gas-exchange system (*Mooney et al.*, 1971; Osmond and Björkman, 1975). All measurements were made in normal air, 325 μbars CO₂, and 21% O₂.

Photosynthesis-light response curves for leaves of *E. farinosa* with solar absorption coefficients of 53%, 65%, and 82% are shown in Fig. 30. Three significant features evident in these curves are: (1) The incident quantum yield (slope of linear part of curve between 0 and 30 nE cm⁻² sec⁻¹) decreases as the pubescence increases; (2) the maximum rates decrease as pubescence increases; and (3) unlike most plants, net photosynthesis under all three pubescence conditions is not light saturated even at 200 nE cm⁻² sec⁻¹, which is equivalent to full noon sunlight during the summer. Quantum yields on an incident light basis were 0.025, 0.033, and 0.041 for absorptances of 53%, 65%, and 82%, respectively. When calculated on an absorbed quanta basis, the quantum yields were 0.048, 0.050, and 0.050. These quantum yields, typical for higher plants (*Year Book* 74, pp. 760–761), indicate that although pubescence in *E. farinosa* increases light reflectance and reduces net photosynthesis, it does not affect the basic photosynthetic process (CO₂ fixed per
Fig. 30. Light dependence of net CO₂ uptake by single attached leaves of Encelia farinosa differing in their degree of pubescence. Rates were determined at a leaf temperature of 30°C, a CO₂ partial pressure of 325 μbar, an O₂ concentration of 21% by volume, and a water vapor pressure deficit of less than 10 μbar. α = absorption coefficient.

quantum absorbed). Net photosynthesis is so dramatically affected by pubescence that at a leaf absorption of 53% the net photosynthetic rate is nearly linear with light intensity up to full sunlight. Stomatal conductances to water and CO₂ exchange were similar for the leaves at any given light intensity, suggesting that CO₂ diffusion limitations were not responsible for differences among the curves. When photosynthetic data from these three curves are plotted against absorbed rather than incident quanta, all data lie on a single curve, indicating that the principal differences among the curves were due primarily to decreases in light absorption due to pubescence and not to physiological differences.

Studies on the ecophysiology of the genus Encelia will continue in the oncoming year. Having documented the extraordinary capability of the pubescence layer to reflect light and also to affect physiological processes, this coming year's work will focus on three main questions that have arisen during the past year: (1) What are the causes of the extremely high photosynthetic rates observed in leaves of Encelia species? (2) What other heat transfer or energy balance functions does the pubescence layer have? In particular, does the pubescence serve as an insulating layer between the metabolically active tissues and the hot, arid external environment? (3) What are the ecological relationships in the tradeoff between carbon gain and reduced heat load on the leaf in pubescent leaves along aridity gradients?

References


Schimper, A. F. W., Plant Geography upon a Physiological Basis, Clarendon, Oxford, 1903.


CARBON DIOXIDE AND TEMPERATURE DEPENDENCE OF THE QUANTUM YIELD FOR CO₂ UPTAKE IN C₃ AND C₄ PLANTS

James Ehleringer and Olle Björkman

Last year we reported that at a leaf temperature of about 30°C and in normal air the quantum yield for CO₂ uptake in plants possessing the C₃ path-
way was equivalent to that of plants possessing the C$_i$ pathway (Year Book 74, pp. 760–761). This seemed remarkable since it means that the decrease in the quantum yield of C$_i$ plants by its inherent higher energy requirement (2 additional ATP molecules per CO$_2$ fixed) offsets the decrease in the quantum yield of C$_i$ plants caused by oxygen inhibition under normal atmospheric conditions. This year we have extended this study to include the effects of changing CO$_2$ partial pressure and leaf temperature on the quantum yield of leaves to determine how these factors interact with the inhibitory effect of O$_2$, and if the quantum yields of C$_i$ and C$_i$ plants are indeed equivalent over a range of temperatures. All measurements were made at strictly rate-limiting light intensities on intact leaves attached to the plants. All plants were potted and grown under conditions of sufficient water and nutrients in growth cabinets.

The quantum yield for CO$_2$ uptake at normal atmospheric CO$_2$ concentration is markedly inhibited by 21% O$_2$ in C$_i$ plants, but it is unaffected by oxygen in C$_i$ plants (Björkman, 1966; Year Book 68, pp. 629–631; Year Book 70, pp. 522–524; Year Book 71, pp. 141–148; Year Book 74, pp. 760–761). Similarly, at normal atmospheric O$_2$ concentration the quantum yield is markedly CO$_2$ dependent in C$_i$ but not in C$_i$ plants (Year Book 70, pp. 522–524; Year Book 71, pp. 141–148). Figure 31 shows the quantum yield of the C$_i$ plant Encelia californica as a function of intercellular pressure in 21% and in 2% oxygen. Leaf temperature was 30°C.

![Figure 31. Quantum yield for CO$_2$ uptake in Encelia californica (C$_i$) determined as a function of intercellular pressure in 21% and in 2% oxygen. Leaf temperature was 30°C.](image)

The steep dependence of the quantum yield in low oxygen suggests that the carboxylase activity of RuDP carboxylase-oxygenase in vivo is saturated by 300 μbar CO$_2$ at rate-limiting light intensities. The presence of a strong CO$_2$ dependence of the quantum yield of C$_i$ plants at higher O$_2$ concentrations is consistent with the view that the O$_2$ inhibition of net CO$_2$ uptake is primarily caused by the oxygenase activity of RuDP carboxylase.

The steep dependence of the quantum yield in C$_i$ plants such as E. californica (and other C$_i$ species) on CO$_2$ concentration at normal atmospheric O$_2$ concentration and the independence of the quantum yield on CO$_2$ in C$_i$ plants such as Atriplex rosea point out one of the selective pressures favoring the evolution of the C$_i$ pathway. The ability of the C$_i$ pathway to concentrate CO$_2$ at the Calvin cycle
carboxylation sites effectively makes light-limited photosynthesis of C₄ plants independent of intercellular CO₂ pressure over a very wide range. However, in a primitive atmosphere of high CO₂, or low O₂ concentrations, or both, selective pressures would strongly favor the C₃ pathway because of its lower intrinsic quantum requirement for CO₂ fixation.

Figure 32 compares the temperature dependence of the quantum yield for CO₂ uptake in the C₃ species E. californica with that of the C₄ species Atriplex rosea in normal air of 325 μbar CO₂ and 21% O₂ (Fig. 32). These results show clearly that in normal air the quantum yield of the C₃ plant is superior to that of the C₄ plant at leaf temperatures below approximately 30°C, but at higher temperatures the quantum yield of the C₃ plant is superior to that of the C₄ plant. This change in the quantum yield in the C₃ species with temperature cannot be accounted for by changes in the liquid phase solubilities of CO₂ and O₂ over the temperature span, since corrections for changes in the solubilities of these gases fail to alter the observed quantum yields significantly. The change in the quantum yield in C₃ plants such as E. californica with leaf temperature must therefore be due to a change in the degree of oxygen inhibition with temperature. Oxygen inhibition of the quantum yield increases exponentially as the temperature is increased from 10° to 40°C. In normal air the oxygen inhibition is only 14% at 14°C but increases to 47% at 38°C. Over this same temperature span there is no change in the absolute value of the quantum yield of E. californica when measured in low oxygen. The change in quantum yield due to inhibition by 21% O₂ in E. californica follows the Arrhenius equation, giving an activation energy approximately equivalent to -8 Kcal mol⁻¹. If the view is correct that the O₂ inhibition of the quantum yield is caused by the oxygenase activity of RuDP carboxylase, then the present results imply that oxygenase activity increases more steeply with temperature than does carboxylase activity. Experiments designed to determine if this is the case are being conducted with the purified enzyme by Dr. Murray Badger in this laboratory.

The distribution of C₃ and C₄ species in nature correlates generally with daylight temperature, i.e., C₄ species are more common in hot climates than in cool or cold climates. Since the rate of photosynthesis and primary production in many plant canopies is strongly light limited, the observed difference in quantum yield between C₃ and C₄ species as a function of leaf temperature may be an important factor in determining their distribution. Under conditions of sufficient soil moisture, a C₃ plant will have greater potential for carbon gain at low temperatures. Conversely, a C₄ plant will have greater potential for carbon gain at high temperatures with a crossover point at approximately 25°-30°C. This greater potential for carbon gain in C₃ plants at low temperatures would imply that C₄ photosynthesis would be at a disadvantage in cool, low-light habitats such as the floor of cool temperate forests and the arctic tundra. On the other hand,
the C₄ pathway would be selectively more advantageous in shaded habitats of high temperature and in dense stands in high-light, high-temperature habitats such as tropical grasslands. C₄ photosynthesis would of course be particularly advantageous in hot, sun-baked desert habitats where little mutual shading of the leaves occurs within the plant stands. However, under these conditions the advantage of C₄ photosynthesis is largely due to the increased capacity for photosynthesis at high light intensities. Nevertheless, the higher quantum yield at high temperatures would also be expected to confer a significant advantage. It is apparent that both the increased capacity for photosynthesis at high light intensities and the higher quantum yield at high temperatures are the results of the same mechanism, namely the ability of the C₄ pathway to increase the concentration of CO₂ at the site of fixation by RuDP carboxylase.

References

HYBRIDIZATIONS IN Atriplex

Malcolm A. Nobs

In 1968 the first hybrid between a species having the C₄ photosynthetic pathway and one with the C₃ pathway was successfully obtained. This F₁ hybrid, *Atriplex rosea* L. × *A. triangularis* Wildenoo (*A. patula* ssp. *hostata*, Hall and Clements), was a diploid with 2n = 18. During the reduction division in this hybrid, only four pairs of chromosomes formed, resulting in less than 10% fertility. The progeny obtained in the second generation formed a series of polyploids ranging from triploid to above pentaploid, making a critical genetic analysis of the inheritance of the C₄ vs. the C₃ pathways impossible.

During the ensuing years, 22 additional intraspecific hybridizations have been attempted. These attempts were made for three basic reasons. The first was to obtain a hybrid combination between C₃ and C₄ species which would remain diploid and be sufficiently fertile for genetic analysis. The second was to obtain hybrid combinations between different C₄ species to determine whether in later generations there would be segregation within the C₄ characteristics. The third was to obtain data on the genetic relationships within the genus *Atriplex* which could supplement the studies by Thompson on DNA hybridization.

The crossing diagram (Fig. 33) summarizes these accumulated data. It is apparent that during the evolution of *Atriplex* the Obione subgenus diverged profoundly from the subgenus Euatriplex. Only one hybrid combination out of eight attempts, *A. rosea* × *argentea* ssp. *expansa*, has yielded hybrids; within Euatriplex, by contrast, six of the eight attempts have yielded hybrid progeny, and within Obione two out of five have been successful.

Within Euatriplex the species studied have become highly differentiated genetically. Table 16 summarizes the cytological data. In three of the combinations the hybrids were so weak that they never reached maturity. The remaining three form a progressive series from nearly sterile to partially fertile. The low fertility in the *rosea* × *sabulosa* combination, both in the *rosea* group and believed by Hall and Clements (1923) to be very closely related, is in part due to chromosomal rearrangements. Bridges and fragments are commonly seen during the first anaphase of reduction division.

The one fertile hybrid, as judged by regular reduction division resulting in 98% normal pollen, is *A. fruticulosa* × *A. serenana*. Both species are diploids in the pentandra group of Obione. It is
a vigorous hybrid and is intermediate morphologically between the parents. It has regular Kranz-type leaf anatomy as do both of its parents. The second generation of this combination should yield valuable material for the study of the \( C_3 \) photosynthetic system.

The other successful hybrid combination within \textit{Obione} is \( A. \) \textit{serenana} \( \times \) \( A. \) \textit{argentea} ssp. \textit{expansa}. The former is diploid while the later is tetraploid. The hybrid is triploid with 27 chromosomes. At first metaphase in reduction division frequently nine pairs of chromosomes are formed with nine univalents. As the \( A. \) \textit{argentea} parent frequently has 1–3 multivalents at this stage, the observed pairing is pre-
sumed to be autopairing between the chromosomes of the argentea parent.

A similar situation exists in the one hybrid between Euatriplex and Obione, A. rosea (diploid) × A. argentea ssp. expansa (tetraploid). This hybrid is also triploid, and has 27 chromosomes, with nine chromosome pairs at metaphase and nine univalents. If autopairing does occur, it would form a physiologically sound genomic foundation which could accommodate foreign chromosomes. The lack of success in the attempted cross between A. phyllostegia and A. dioeca carne as a surprise, since they are morphologically very similar. Both are diploid, possess the rudimentary perianth in the female flowers, have succulent leaves with classic C\textsubscript{4} anatomy, and have very similar inflorescences. In fact Hall and Clements (1923) consider them closely enough related that they might be united into one species. Apparently, however, they have diverged considerably genetically, for although aborted embryos were formed, no hybrid seed matured.

At the present time not all of our basic objectives have been fulfilled, for we have not achieved a diploid hybrid between a C\textsubscript{4} and a C\textsubscript{4} Atriplex which is sufficiently fertile to produce a second generation for genetic and physiological study. However, considerable progress has been made on our understanding of the genetic structure of the genus.

References


GROWTH AND PHOTOSYNTHESIS OF *Chlamydomonas reinhardtii* AS A FUNCTION OF CO\textsubscript{2} CONCENTRATION

Joseph Berry, John Boynton, Aaron Kaplan, and Murray Badger

Earlier studies by Bowes and Berry (Year Book 71, pp. 148–157) showed that photosynthesis by cells of the green alga *Chlamydomonas reinhardtii* was competitively inhibited by O\textsubscript{2} and that a portion of the carbon fixed (depending upon the CO\textsubscript{2} and O\textsubscript{2} concentration) was excreted to the medium as glycolic acid. These effects were correlated with the effects of O\textsubscript{2} and CO\textsubscript{2} on ribulose 1-5 diphosphate (RuDP) carboxylase/oxygenase, the enzyme that catalyzes CO\textsubscript{2} fixation and appears to be the site of O\textsubscript{2} inhibition in species of higher plants having C\textsubscript{4} photosynthesis.

Experiments were initiated this year to investigate the effect of O\textsubscript{2} and CO\textsubscript{2} concentration upon the growth of *Chlamydomonas*. The initial objective was to find an appropriate combination of high O\textsubscript{2} and low CO\textsubscript{2} concentration which would inhibit growth of the wild type strain and serve as a positive selective method for mutant genotypes with altered CO\textsubscript{2} requirements. We hypothesized that such mutant genotypes might result from changes in the properties of the RuDP carboxylase/oxygenase molecule which would affect its $K_{\text{c}}$ (O\textsubscript{2}) or $K_{\text{c}}$ (CO\textsubscript{2}). Selective screening of mutagenized haploid and diploid genotypes under this condition might permit the identification of nuclear or chloroplast gene loci, respectively (Lee *et al.*, 1973), coding for the peptides comprising the small and large subunits of the enzyme RuDP carboxylase/oxygenase (Wildman *et al.*, 1975).

In similar experiments with higher plants (Year Book 70, pp. 507–511), a controlled atmosphere of 60 ppm CO\textsubscript{2} and 21% O\textsubscript{2} was found to permit very little growth of the C\textsubscript{4} plant *Atriplex patula*, while the C\textsubscript{4} species *A. rosea* grew well under this condition. This growth regime was used to screen F\textsubscript{2} hybrid genotypes derived from a cross between *A. rosea* and *A. patula* for the
more efficient C₄ mechanism of CO₂ fixation.

In the present experiments, the wild type C. reinhardtii (haploid Strain 137C, Stock GB-126) was grown phototrophically in 300 ml shake cultures of HS culture media (Sueoka, 1960) aerated with different mixtures of O₂ and CO₂. Light intensity was 50 nE cm⁻²sec⁻¹ from VHO fluorescent lamps in the physiology and biochemistry experiments and 41 nE cm⁻²sec⁻¹ from cool white fluorescent lamps in the growth experiments. The results of the growth experiments are presented in Table 17. Maximum growth rate (minimum doubling time) was obtained in air enriched with 3 × 10⁴ ppm CO₂. At this CO₂ concentration, growth rate was substantially inhibited by increasing the O₂ partial pressure to 97%. However, decreasing the CO₂ concentration from 3 × 10⁴ to 350 ppm had little effect upon growth, suggesting that CO₂ uptake is nearly saturated at air levels of CO₂ under the light levels and cell densities used. Substantial but slow photosynthetic growth was obtained at less than 100 ppm. At both 65 and 33 ppm, growth rate was not significantly affected by changing the O₂ concentration dramatically. Since growth rate under the low CO₂ regimes appears to be linearly dependent upon CO₂ concentration, the CO₂ compensation point for photosynthetic growth must be quite close to zero (or very low). These results are remarkably different from those obtained with C₃ species of higher plants, and indicate both a greater resistance of O₂ inhibition and a higher efficiency of CO₂ fixation at low CO₂ concentrations. Superficially, these algae would appear to use a mechanism of CO₂ assimilation which differs from that of normal C₃ species of higher plants and resembles C₄ photosynthesis in the sense that the organism can utilize very low exogenous CO₂ concentrations for photosynthetic growth.

**PHOTOSYNTHETIC CO₂ FIXATION**

The earlier studies by Bowes and Berry (Year Book 71, pp. 148-157) indicated that photosynthesis by cells of C. reinhardtii grown on air enriched with 2–3 × 10⁴ ppm CO₂ was quite similar to C₃ photosynthesis of higher plants. They reported a Kᵣ(CO₂) of approximately 30 µM at pH 7.8 and low O₂, incorporating ¹⁴CO₂ into acid stable products as the assay method. CO₂ fixation was competitively inhibited by increased O₂ concentrations. Very efficient CO₂ uptake has been reported for certain algal species. Whittingham

<table>
<thead>
<tr>
<th>Gas Mixture</th>
<th>CO₂ ppm</th>
<th>O₂ %</th>
<th>Doubling Time (hr)</th>
<th>Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 × 10⁴</td>
<td>21</td>
<td>9.1</td>
<td>14.4</td>
<td></td>
</tr>
<tr>
<td>3 × 10⁴</td>
<td>97</td>
<td>25.3</td>
<td>11.9</td>
<td></td>
</tr>
<tr>
<td>350</td>
<td>21</td>
<td>9.9</td>
<td>15.2</td>
<td></td>
</tr>
<tr>
<td>350</td>
<td>97</td>
<td>14.6</td>
<td>...</td>
<td></td>
</tr>
<tr>
<td>65</td>
<td>4</td>
<td>21.9</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>65</td>
<td>84</td>
<td>19.2</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>2</td>
<td>41</td>
<td>0.89</td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>92</td>
<td>37</td>
<td>0.90</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>...</td>
<td>0.29</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>...</td>
<td>0.25</td>
<td></td>
</tr>
</tbody>
</table>

*Flow rates varied from 9 to 18 l/hr per flask. Cultures were inoculated at a density of 1 × 10⁶ cells/ml from log phase phototrophic cultures pre-grown at 350 ppm CO₂–21% O₂. Cell density was followed daily by hemocytometer counts. Experiments were terminated when each culture reached stationary phase, and the doubling time was calculated for the linear portion of the growth curve.
DEPARTMENT OF PLANT BIOLOGY

(1952), Steemann-Neilsen (1955), and Brown and Tregunna (1967) have demonstrated that Chlorella species become CO₂-saturated at CO₂ concentrations less than atmospheric. Brown and Tregunna (1967) and Steemann-Nielsen and Jensen (1958) have shown that this alga has a very low CO₂ compensation point, and Björkman (Year Book 65, pp. 446–454) reports that, in contrast to the findings of Tamiya and Huzisige (1949), photosynthesis of Chlorella is not inhibited by 21% O₂. The growth experiments reported in Table 17 suggest that Chlamydomonas has photosynthetic characteristics similar to those discussed above for Chlorella.

The apparent contradiction of the growth studies with the photosynthetic properties reported by Bowes and Berry (Year Book 71, pp. 148–157) can be explained on the basis of different physiological states of the algae. An abundance of literature, starting with J. S. Turner (unpublished) and R. Howels (1940), reviewed by Briggs and Whittingham (1952), indicates that Chlorella can exist in two distinctly different states depending upon whether the cells were exposed to air (0.03% CO₂) or air enriched with 1%–5% CO₂ during growth. The alga may be induced to change from one state to the other by exposure to the appropriate CO₂ supply. Induction may take from ½ hr to several hours depending upon the strain and direction of the transformation (Briggs and Whittingham, 1952; Graham and Whittingham, 1968; Steemann-Neilsen and Willemoes, 1966). Clearly, the physiological efficiency for use of CO₂ in photosynthesis differs dramatically for Chlorella induced to one state or the other. Such cells also differ in their metabolism of glycolic acid (Nelson and Tolbert, 1969) and their content of carbonic anhydrase (Graham et al., 1971). A similar phenomenon has been documented in the blue-green alga Coccochloris (Ingle and Coleman, 1976), and Chlamydomonas reinhardtii (Nelson et al., 1969; Graham et al., 1971). Thus, Chlamydomonas grown at low CO₂ might be expected to have quite different kinetics of CO₂ assimilation and interaction with O₂ than those reported by Bowes and Berry (Year Book 71, pp. 148–157), for cells adapted to higher CO₂.

While the general features of this adaptive developmental differentiation by algae are known, the mechanism(s) responsible are not clear. Some workers assume the photosynthetic capabilities of high-CO₂ grown cells to be impaired (Osterlund, 1950; Steemann-Neilsen, 1955; Steemann-Neilsen and Willemoes, 1966). Our interest in this topic was whetted by the indication (discussed previously) that high-CO₂ adapted cells have kinetics of CO₂ assimilation which resemble higher plant species with C₃ photosynthesis, and low-CO₂ adapted algae appear to be exceptional and are considerably more efficient in assimilating CO₂. No comparable data of the kinetics of CO₂ exchange of high- and low-CO₂ adapted algae were available to evaluate this hypothesis, nor had the factors governing the kinetics of CO₂ assimilation in the respective states been investigated. For these reasons, a comparative study of C. reinhardtii adapted to low- and high-CO₂ concentration was initiated.

Photosynthesis was measured as O₂ production in a closed electrode chamber (Rank Brothers, Bottisham, Cambridge, England) modified for increased sensitivity as described previously (Year Book 72, pp. 405–407). After depletion of endogenous CO₂ (contained in the media, or as a pool within the cells) O₂ evolution was completely dependent upon addition of CO₂ or NaHCO₃ to the electrode chamber.

The dependence of photosynthesis (measured as O₂ evolution) upon added HCO₃⁻ concentration in 50 mM HEPES, pH 7.0, is shown in Fig. 34 for both low- and high-CO₂ grown cells. Similar maximum rates are achieved on a per-cell basis for cells cultured under both regimes, but the concentration dependence is dramatically different. The apparent Kₐ's on a HCO₃⁻
basis are $3.6 \pm 0.2 \mu M$ and $64.4 \pm 10.8 \mu M$ for the low-CO$_2$ and high-CO$_2$ grown cells, respectively. The $K_{d}(\text{CO}_2)$ calculated for high-CO$_2$ grown cells at this pH is 15 $\mu M$, which is quite similar to the apparent $K_{d}(\text{CO}_2)$ of C$_3$ species of higher plants and that reported earlier (Year Book 71, pp. 148-157) for high-CO$_2$ adapted C. reinhardtii. In contrast, cells grown at low CO$_2$ concentrations have nearly 20-fold greater affinity for CO$_2$ at this pH ($K_{d}(\text{CO}_2) = 0.83 \mu M$) than the high-CO$_2$ grown cells. The CO$_2$ requirement of low-CO$_2$ adapted C. reinhardtii is similar to that reported by Whittingham (1952) and Steemann-Neilsen and Jensen (1958) for low-CO$_2$ adapted Chlorella. These CO$_2$ requirement studies were made under low (<15%) O$_2$ concentration, with the lowest HCO$_3^-$ concentrations always at the lowest O$_2$ concentration, and the ratio of O$_2$ evolved/CO$_2$ fixed was nearly one. As will be discussed later, this ratio falls below 1.0 at limiting CO$_2$ concentrations under high O$_2$.

Chromatographic analysis of the acid stable products formed by low-CO$_2$ and high-CO$_2$ grown cells fed $^{14}$CO$_2$ at half-saturating concentrations for short periods did not indicate any substantial difference in the pathway of CO$_2$ assimilation (data not shown). Sugar phosphate and 3-PGA were major products, and only small quantities of label appeared in malate and aspartate.

Supernatant fractions from extracts of high-CO$_2$ and low-CO$_2$ grown cells were assayed for activities of RuDP carboxylase/oxygenase and PEP carboxylase. As shown in Table 18, there was little difference between cells grown under either condition. RuDP carboxylase is the predominant carboxylase in both cell types.

Kinetic properties of RuDP carboxylase isolated from Chlamydomonas grown at high-CO$_2$ and low-CO$_2$ concentrations were also studied using methods described by Lorimer et al. (1976). We found apparent $K_{d}(\text{CO}_2)$ of the activated form of this enzyme to be $57 \pm 5 \mu M$ for both high- and low-CO$_2$ grown cells. Purification of the cell extract by Sephadex G25 did not affect the apparent $K_{d}(\text{CO}_2)$. Our assays of RuDP carboxylase from the C$_3$ plant Encelia californica by the same method yielded a fourfold lower $K_{d}(\text{CO}_2)$ of 15 $\mu M$. Since the Chlamydomonas RuDPCase was found...
### TABLE 18. Comparison of RuDP Carboxylase and PEP Carboxylase Activities in Cells of *C. reinhardtii* Grown at Low (Air) and High (5%) CO₂ Concentrations*

<table>
<thead>
<tr>
<th>nmol CO₂ (O₂) fixed per min per 10⁷ cells</th>
<th>RuDP Carboxylase</th>
<th>RuDP Oxygenase</th>
<th>PEP Carboxylase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air grown</td>
<td>17</td>
<td>1.5</td>
<td>1.6</td>
</tr>
<tr>
<td>5% CO₂ grown</td>
<td>26</td>
<td>1.8</td>
<td>2.0</td>
</tr>
</tbody>
</table>

*Cells were harvested by centrifugation and broken in the French press at 6000 psi in 0.1 M Tris, pH 8.0, 5 mM Dithiothreitol buffer at a density of 2 × 10⁷/ml. Enzyme assays were carried out on S₁₀₀₀₀ fractions for 2 min at 25°C in 0.1 M Tris buffer, pH 8.2. The RuDP carboxylase/oxygenase enzyme was activated, RuDP carboxylase was assayed at 25°C; 10 mM HCO₃⁻, and RuDP oxygenase assayed at 20°C and 0.251 mM O₂ at saturating RuDP concentrations according to the methods of Lorimer et al. (1976). PEP carboxylase was assayed as described by Björkman and Gauld (1969). Chlorophyll content was 5.2 μg and 6.7 μg per 10⁷ air-grown and 5% CO₂-grown cells, respectively.

Glycolate was measured by the technique of Calkins (1943) on cell-free filtrate of the medium after 20 minutes of steady-state photosynthesis. The ratio of glycolate excreted to HCO₃⁻ assimilated increases when the HCO₃⁻ concentration is limiting. Similar data were obtained with high-CO₂ adapted algae (cf. Fig. 39, *Year Book 71*, p. 153). The data reported here illustrate that low-CO₂ adapted *C. reinhardtii* also excretes glycolate. This had not been detected before because much lower HCO₃⁻ concentrations are required in low-CO₂ adapted cells to induce glycolate excretion (1 μM HCO₃⁻ for the low CO₂ cells vs. 50 μM HCO₃⁻ for the higher CO₂ cells at pH 7.0). Nelson et al. (1969) reported that low-CO₂ adapted *C. reinhardtii* has an increased capacity to metabolize glycolate. While this could contribute to the difference in glycolate excretion, we believe that the difference in the affinity of the low-CO₂ and high-CO₂ grown cells for CO₂ is the reason that glycolate excretion is not normally observed in low-CO₂ adapted algae. This is supported by the finding (not shown) that 10 mM isonicotinic acid hydrazide did not affect glycolate excretion in low-CO₂ adapted cells of *C. reinhardtii* and the blue-green alga *Coccomchloris* (Ingle and Coleman, 1976).

Since glycolate is more oxidized than carbohydrate, the normal product of photosynthesis, less O₂ should be evolved per CO₂ fixed when glycolate is a major product of photosynthesis. This
Fig. 35. Comparison of glycolate excretion, the O₂/HCO₃⁻ ratio and O₂ evolution as influenced by the steady state HCO₃⁻ concentration for high-CO₂ adapted (A) and low-CO₂ adapted (B) cells of C. reinhardtii. Conditions were as described in Fig. 34 except that 10 mM phosphate buffer, pH 7.0, was used and O₂ was 20%–25%. Note that the scale of the bicarbonate axis differs by a factor of 10.

result is in fact obtained with both high-CO₂ and low-CO₂ grown cells. In experiments with several combinations of O₂ and HCO₃⁻ concentrations, we were able to account quantitatively for the changes in the measured O₂/HCO₃⁻ ratio of high-CO₂ adapted cells by the amounts of glycolate formed (Fig. 36). In low-CO₂ adapted cells glycolate excretion followed the same trend but could not quantitatively explain the drop in the O₂/HCO₃⁻ ratio. The significance of this difference is not clear. Under conditions of low O₂ (~ 1%–5%), neither cell types excreted detectable amounts of glycolate, and the O₂/HCO₃⁻ ratio was very close to unity. Because glycolate formation alters the O₂/CO₂ exchange ratio, the effect of O₂ upon the kinetics of CO₂ assimilation cannot be assessed by measuring O₂ evolution with respirometers (cf. Tamiya and Huzisige, 1949), or by using the oxygen electrode system of these studies without also monitoring the exchange ratio. The reports that low-CO₂ adapted algae are resistant to O₂ inhibition of photosynthesis should be reexamined in view of the observation reported here that effects of O₂ upon photosynthesis of low-CO₂ adapted C. reinhardtii are only evident at very low bicarbonate concentrations.
Several lines of evidence reported here including products of CO₂ fixation, studies of carboxylase enzyme levels and effects of O₂ upon photosynthesis suggest that RuDP carboxylase is the mechanism of initial CO₂ fixation utilized by both high- and low-CO₂ adapted cells. Studies of the kinetics of CO₂ assimilation by these cells indicate a very large difference in the quantitative requirement for CO₂. The very low CO₂ requirement of low-CO₂ adapted cells verifies the hypothesis that C. reinhardtii can adapt to become substantially more photosynthetically efficient than C₃ species of higher plants. This also appears to be true of the green algae Chlorella and Scenedesmus, the blue-green alga Coccolithus (Ingle and Coleman, 1976), and perhaps many other algal species. Evidence presented indicates that, as in plants with C₃ photosynthesis, RuDP carboxylase is not more efficient in low-CO₂ adapted C. reinhardtii compared to C₃ plants. In contrast to C₃ plants, no evidence for an alternate initial carboxylation mechanism in C. reinhardtii was obtained either from studies of PEP carboxylase levels or studies of initial products of CO₂ fixation.

Reed and Graham (1968) have suggested that changes in the level of the enzyme carbonic anhydrase might explain the improved efficiency of low-CO₂ adapted Chlorella. Subsequently, carbonic anhydrase activity in low-CO₂ grown cells was found to be manyfold higher than in high-CO₂ grown cells (Graham and Reed, 1971; Graham et al., 1971; Nelson et al., 1969). Furthermore, Graham et al. (1971) showed that when high-CO₂ grown cells of either Chlorella or C. reinhardtii were transferred to low CO₂, both carbonic anhydrase activity and O₂ evolution increased dramatically during the first 90 minutes without detectable changes in activity of the Calvin cycle or β-carboxylation enzymes. We question whether this increase in carbonic anhydrase activity is in itself sufficient to explain the more efficient photosynthetic behavior of low-CO₂ adapted cells. Carbonic anhydrase catalyzes the equilibration of unhydrated CO₂ with HCO₃⁻, but should not be able to alter the position of this equilibrium. Thus, in the presence of carbonic anhydrase alone, the affinity of the system for CO₂ should not exceed that of the carboxylating enzyme. Furthermore, C₃ species of higher plants have more carbonic anhydrase activity than either low-CO₂ adapted C. reinhardtii or C₃ plants (Graham et al., 1971).

In order to explain the behavior of low-CO₂ adapted cells, we postulate that either (1) an unknown and undetected carboxylation enzyme with a greater affinity for CO₂ is present, or (2) a mechanism capable of enhancing the concentration of CO₂ at the site of fixation by RuDP carboxylase is operating. The requirement of such a concentrating mechanism for CO₂, combined with glycolate excretion, may explain the O₂/HCO₃⁻ ratio in low-CO₂ adapted cells.

Raven (1968, 1970, 1974) has provided experimental evidence and reviewed the work of others indicating that some algae are capable of assimilating HCO₃⁻. In contrast to CO₂ assimilation, which appears to be via passive diffusion, HCO₃⁻ assimilation by the alga Hydrodictyon africanum appears to occur via a metabolic influx pump that operates across a cellular membrane. Raven (1970) has invoked this mechanism to explain the ability of certain algae to photosynthesize at alkaline pH. Since metabolic energy may be coupled to ion transport to effect large concentration gradients (Mitchell, 1966), there is no reason in principle why such a mechanism might not also be invoked to serve as a means of concentrating HCO₃⁻ or CO₂ within the cell under conditions of limiting CO₂ supply.

Findenegg (1974) has shown that low-CO₂ adapted cells of Scenedesmus obliquus possess a Cl⁻ influx pump that is absent in high-CO₂ adapted cells. The Cl⁻ uptake is inhibited by
HCO₃⁻, and he suggests that the normal physiological substrate is HCO₃⁻. Whether or not Chlamydomonas has the capacity for HCO₃⁻ uptake is not clear from the literature (see Raven, 1970). Therefore, experiments were conducted to determine if CO₂ or HCO₃⁻ is the form of carbon assimilated. Since the ratio of HCO₃⁻ to H₂CO₃ present in solution can be manipulated by changing the pH, one should be able to assess the effect of pH on the total inorganic carbon requirement for photosynthesis in whole cells and infer which form (CO₂ or HCO₃⁻) is actually utilized (see Raven, 1970).

We have determined Michaelis-Menten constants for photosynthesis in high-CO₂ and low-CO₂ grown cells calculated on the basis of CO₂ or HCO₃⁻ as a function of pH (Table 19). Over the pH range 7.0–8.0, the K_m(CO₂) of the high-CO₂ grown cells is fairly constant (15–23 μM) while the K_m(HCO₃⁻) varies by a factor of more than 10. This result indicates that CO₂ is probably the form utilized in photosynthesis by the high-CO₂ grown cells. In the low-CO₂ grown cells, the K_m(CO₂) falls and the K_m(HCO₃⁻) increases with increasing pH over the range from pH 7.0 to pH 8.0. These data do not permit a clear decision as to which form of carbon is assimilated by the low-CO₂ adapted cells, but rather suggest that the cells are capable of utilizing variable proportions of CO₂ or HCO₃⁻, depending upon the inorganic carbon concentration and pH. Regardless of the form of carbon present, the K_m(CO₂) of low-CO₂ adapted cells of C. reinhardtii is much lower over the pH range examined than for species of higher plants with C₃ photosynthesis. This contrasts with Hydrodictyon africanum (Raven, 1968) and Scenedesmus (Brown and Tregunna, 1967), which can utilize HCO₃⁻ at high pH but do not appear to have enhanced CO₂ uptake at low pH. Either C. reinhardtii has a higher affinity for HCO₃⁻ and thus can utilize HCO₃⁻ at a lower pH, or its accumulating mechanism can utilize CO₂ as well as HCO₃⁻, depending upon the form available.

Another insight into the question of how carbon is assimilated in C. reinhardtii is provided by studies with Diamox, an inhibitor of carbonic anhydrase in Chlorella (Graham and Reed, 1971; Graham et al., 1971) and Cocccochloris (Ingle and Coleman, 1976). We have found that Diamox alters the efficient response of low-CO₂ adapted cells to added HCO₃⁻ (Fig. 37) and shifts the concentration dependence toward that of high-CO₂ adapted cells. The effect of Diamox suggests

### Table 19. The Influence of pH upon the Apparent K_m (Total CO₂ + HCO₃⁻) and K_m (HCO₃⁻) or K_m (CO₂) of Photosynthetic O₂ Evolution by Cells of C. reinhardtii Adapted to High- or Low-CO₂ Concentrations

<table>
<thead>
<tr>
<th>pH</th>
<th>K_m (total)</th>
<th>K_m (CO₂) μM</th>
<th>K_m (HCO₃⁻)</th>
<th>V_max mmol O₂ min⁻¹/10⁶ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low-CO₂ Grown Cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.5</td>
<td>6.3 ± 0.5</td>
<td>3.6</td>
<td>2.7</td>
<td>12.3 ± 0.2</td>
</tr>
<tr>
<td>7.0</td>
<td>2.9 ± 0.1</td>
<td>2.4</td>
<td>0.5</td>
<td>20.0 ± 1.0</td>
</tr>
<tr>
<td>7.5</td>
<td>6.8 ± 0.5</td>
<td>6.3</td>
<td>0.5</td>
<td>13.7 ± 0.2</td>
</tr>
<tr>
<td>8.0</td>
<td>9.0 ± 1.0</td>
<td>8.8</td>
<td>0.2</td>
<td>12.6 ± 0.3</td>
</tr>
<tr>
<td>High CO₂ Grown Cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.5</td>
<td>61 ± 4</td>
<td>35</td>
<td>25.7</td>
<td>28.2 ± 0.6</td>
</tr>
<tr>
<td>7.0</td>
<td>149 ± 9</td>
<td>121</td>
<td>27.9</td>
<td>23.2 ± 0.4</td>
</tr>
<tr>
<td>7.5</td>
<td>504 ± 34</td>
<td>470</td>
<td>34.2</td>
<td>14.5 ± 0.4</td>
</tr>
<tr>
<td>8.0</td>
<td>1163 ± 64</td>
<td>1137</td>
<td>26.2</td>
<td>11.7 ± 0.2</td>
</tr>
</tbody>
</table>

*Conditions as in Fig. 34 except for pH. Kinetic constants were calculated according to Wilkinson (1961).*
that carbonic anhydrase is essential for the mechanism of carbon assimilation by low-CO$_2$ adapted cells. High-CO$_2$ adapted cells of *C. reinhardtii* have only very low levels of carbonic anhydrase (Graham *et al.*, 1971; Nelson *et al.*, 1969).

We found that addition of carbonic anhydrase to the media of high-CO$_2$ adapted cells has no influence on the kinetics of photosynthesis. Thus the difference between low- and high-CO$_2$ adapted cells cannot be explained by the effect of carbonic anhydrase on the rate of CO$_2$ and HCO$_3^-$ equilibration in the medium.

More than a single isozyme of carbonic anhydrase has been observed in 14 species of higher plants (Graham *et al.*, 1971), and Everson (1970) has shown that carbonic anhydrase is present both at the surface of and within spinach chloroplasts. Graham and Reed (1971) discuss several possible intracellular roles for carbonic anhydrase, none of which appears to explain the specific role of this enzyme in low-CO$_2$ adapted algal cells. Findenegg (1974) showed that adaptation of *Scenedesmus* to low-CO$_2$ concentration correlates with induction of carbonic anhydrase activity and a Cl$^-$ (presumed HCO$_3^-$) influx pump. If HCO$_3^-$ is the form of carbon entering the cell, it must be converted to CO$_2$ prior to fixation by RuDP carboxylase (Cooper *et al.*, 1969). Thus, both a bicarbonate influx pump and carbonic anhydrase should be required for the uptake mechanism of low-CO$_2$ adapted cells to enhance photosynthesis.

In summary, key components theoretically required for a CO$_2$-accumulating mechanism based on an HCO$_3^-$ influx pump have been individually characterized in various algae by a number of workers. However, in no single experimental system have these observations been integrated with one another or with studies of the kinetics of CO$_2$ assimilation. If this can be achieved, it may document yet another mechanism, in addition to the C$_4$ and CAM pathways evolved by green plants, to increase photosynthetic competence without altering the molecular properties of RuDP carboxylase.

Studies reported in this paper indicate that the CO$_2$-exchange kinetics of *Chlamydomonas reinhardtii* are dramatically changed by the previous growth conditions of the algae. Cells adapted to 5% CO$_2$ during growth have a CO$_2$ requirement similar to C$_4$ species of higher plants, while cells adapted to air levels of CO$_2$ (0.03%) are substantially more efficient at low-CO$_2$ concentrations. We have shown that the improved efficiency of the CO$_2$ uptake cannot be attributed to a new pathway of CO$_2$ fixation or to induction of a more
efficient form of RuDP carboxylase. The increased efficiency of low-CO$_2$ adapted algae was abolished by the inhibitor Diamox. This suggests that carbonic anhydrase plays an essential role in the mechanism which improves the efficiency of CO$_2$ uptake. We suggest that HCO$_3^-$ may be actively accumulated by a metabolic influx pump in low-CO$_2$ adapted cells. Carbonic anhydrase would be required to convert HCO$_3^-$ to CO$_2$, the substrate for carboxylation by RuDP carboxylase. A concentration gradient between the inside of the cells and their surroundings, established by the influx pump, could account for the increased efficiency of CO$_2$ uptake observed.

**References**


The root has received little emphasis in physiological interpretations of a plant's adaptation to different temperatures. Previous studies have measured the influence of the environmental temperature on various metabolic processes in the aerial portions of several plant species (Billings et al., 1971; Björkman and Holmgren, 1961; Mooney and Billings, 1961). Grobelaar (1963) observed the virtual cessation of root growth in corn grown at 5°C. MacLean and Donovan (1973) and Porter and Moraghan (1975) have shown a differential response to low root temperatures by different cultivars of corn as well as a lower temperature limit for root growth that is above freezing. However, these studies have not examined the physiological mechanisms that determine the influence of temperature on root function. Chapin (1974) found that species of plants from cool soil regimes maintain a greater capacity for phosphate absorption than species native to warm regimes, regardless of whether the growth temperature was 5° or 20°C, but too few temperatures have been analyzed to determine the nature of the effect of temperature on ion uptake.

This paper will investigate how ion accumulation and respiration are influenced by short-term temperature variation and whether the rate of either process is modified by environmental growth temperatures. Uptake of rubidium was used because of its similarity to K⁺ uptake and because of the desirable features of the tracer ³⁷Rb (Epstein, 1972).

**Materials and Methods**

Barley seeds (*Hordeum vulgare* cv. Mariout Ferry Morse) and corn seeds (*Zea mays*, cv. WF9 × Bear 38) were weighed (10 g) and rinsed three times with fresh 0.5 mM CaSO₄ solution. The seeds were spread onto acrylic-framed polyethylene-coated fiberglass mesh screens floating on 3 liters of aerated 0.5 mM CaSO₄ solution in polyethylene boxes. These boxes were placed in the dark either for 5 days at 28° ±0.5°C (warm barley and corn) or for 14 days at 10° ± 0.5°C (cool barley). The seedlings were rinsed daily with deionized water and transferred to clean boxes with fresh CaSO₄ solution. At the end of the growth period the seedlings from the 10° or 28°C regimes were of comparable size. These were rinsed with CaSO₄ solution, and the terminal 10 cm of roots extending below the screen were excised for the experiments described below.

Oxygen consumption at various temperatures was measured on approximately 100 mg excised roots in the 5 ml vessel of a Rank Brothers' oxygen electrode. Oxygen consumption by the electrode was subtracted from the total oxygen consumed by the sample whenever the amount consumed by the electrode exceeded 2% of the total. After conditioning the roots for 15–20 min at each temperature in an aerated solution, respiration measurements were obtained. Data reported is on a fresh weight basis.

Rubidium uptake was measured from an 0.05 mM RbNO₃ solution (0.5 mM CaSO₄ added) labeled with ⁶⁷RbCl (approximately 1 µCi/300 ml). This concentration is rate-saturating for the low concentration isotherm mechanism (Epstein et al., 1963a). Roots with a fresh weight of 200–300 mg in a “tea bag” (Epstein et al., 1963b) were conditioned for 20 min in a 0.5 mM CaSO₄ solution at the appropriate temperature. The “tea bags” were then transferred to 300 ml of labeled solution at the appropriate temperature for either 20 min below 15°C or 10 min above 15°C. After the uptake period, the roots...
were immediately plunged into a (0°-
4°C) desorption solution (0.05 mM
RbNO₃ and 0.5 mM CaSO₄) for 1 min
and then transferred to a room tem­
perature desorption solution of the same
composition for about 30 min. The roots
were then freeze-dried and dry weights
obtained using an electro balance ac­cu­
rate to 3 x 10⁻⁴ mg. The ⁸⁶Rb was
counted on an LKB liquid scintillation
counter by placing the root samples
into vials and directly adding scintilla­
tion fluid toluene, Triton X-100, om­

RESULTS AND DISCUSSION

In Fig. 38 the rate of rubidium ac­
cumulation is plotted as a function of
root temperature for barley grown at
two temperatures. Warm-grown barley
shows a temperature optimum above
30°C in contrast to the 20°-25°C op­
timum of cool-grown barley. The max­
imal rate of rubidium accumulation for
cool-grown barley is 5.0 μmoles g⁻¹hr⁻¹
and for warm-grown barley is 12.2
μmoles g⁻¹hr⁻¹. Even at 20°C the
warm-grown barley has a rate of 9.7
μmoles g⁻¹hr⁻¹, twice the rate of cool-
grown barley at the same temperature.
This difference may indicate that pro-
longed exposure to low temperature
inactivates portions of the ion accumu­
lation mechanism. The change in rate
from 5° to 30°C in warm-grown barley
is from 1.8 to 12.2 μmoles g⁻¹hr⁻¹, giv­
ing a Q₁₀ of 2.11 (5°-30°C). A similar
Q₁₀ for barley roots was suggested by
Epstein (1972) based on the difference
between the rate at 4.5° and at 30°C.
However, in warm-grown barley Q₁₀
does not remain constant over the en­
tire range of 5°-30°C, indicating a
changing activation energy.

The shape of the curve for warm-
grown barley does not fit the mono­
tonic exponential increase in rate nor­mally
associated with the increase in rate of a
single enzymatic reaction with tem­
perature. The very steep temperature
dependence of ion uptake at low tem­
perature lessens at temperatures be­
tween 15° and 30°C. This is seen more
clearly in Fig. 39 where the natural
logarithm of the rate for rubidium ac­
cumulation by barley roots is plotted as
a function of the inverse of tempera­
ture (an Arrhenius plot). The data for
warm-grown barley fit two different
straight lines for the temperature

![Fig. 38. Rubidium accumulation as a func­tion of temperature by roots of warm (28°C) and
cool (10°C) grown barley. Rubidium concentra­
tion was 0.05 mM in the solution. Each data
point represents the average of at least four
separate experiments. Rates are expressed on a
gram fresh weight basis.]

![Fig. 39. Arrhenius plots for rubidium uptake
by warm- and cool-grown barley roots shown in
Fig. 38. Each data point represents the average
and standard error of the mean (vertical bars) of
at least four separate experiments. The numbers
in parentheses equal the activation energy in
kcal mol⁻¹deg⁻¹ calculated from the slope of the
line.]
ranges 11°–30°C and 5°–9°C. At temperatures from 11° to 30°C, apparent Arrhenius activation energy is 3.6 kcal mole⁻¹deg⁻¹, equivalent to a Q₁₀ of 1.3, and indicates that ion uptake is little affected by temperatures above 10°C. Similarly, Holmern et al. (1974) have shown that sulfate accumulation in barley is little affected by temperatures from 20° to 30°C. Below 10°C (Fig. 39), small changes in temperature have a dramatic effect on the rate of Rb⁺ ion accumulation in the roots, with an apparent activation energy of 21.5 kcal mole⁻¹deg⁻¹ and a Q₁₀ of 5.5. Such dramatic changes in Q₁₀ do not occur in cool-grown barley. The reasons for this difference are not clear, but one possibility is that ion accumulation by the roots grown at high temperatures becomes limited by their respiratory capacity at low temperatures.

Temperature control of respiration was also measured. As shown in Fig. 40, the respiratory rate of cool-grown barley roots is in excess of the rate for warm-grown barley roots at temperatures below 30°C. Stimulation of respiratory capacity by cool growth temperature has also been shown by Billings et al. (1971). They reported that lower environmental growth temperatures yielded higher dark respiration at a given temperature by leaves of Oxyria digyna adapted to arctic regimes than by those from warmer alpine regimes. Many other workers have found similar plant responses (Björkman and Holmgren, 1961; Chapin, 1974; and Mooney and Billings, 1961).

Respiration of barley roots grown at either temperature increases exponentially with temperature (Fig. 41), yielding an activation energy of 8.0 kcal mole⁻¹deg⁻¹ for warm-grown barley, and an activation energy of 10.6 kcal mole⁻¹deg⁻¹ or 8.7 kcal mole⁻¹deg⁻¹ for cool-grown barley, depending on the temperature range. Warm-grown barley does not show the same sharp change in activation energy for respiration as seen for ion uptake.

Cool-grown barley roots have a higher respiratory capacity than warm-grown barley and hence more energy available to the root tissue, yet they have a lower rate of ion uptake. Both the higher rate of ion accumulation and the sharp drop in rate below 10°C (Fig. 38) of warm-grown barley are distinct from the pattern in cool-grown barley. Because the rate of ion accumulation

![Graph showing respiratory rate of warm and cool-grown barley roots over temperature range from 5°C to 45°C.](image)
for roots grown at low temperature is much lower than the rate for roots that develop at normal temperatures, we suggest that development of the ion uptake mechanism may be abnormal at low temperature.

Ion accumulation in warm-grown barley is much more severely reduced (by 60%) than respiration (by 30%) as the temperature is reduced from 10° to 5°C. This suggests that respiratory limitation may not be solely responsible at low temperatures. We suggest that the effect of low temperature on ion uptake may be related to a temperature effect upon a cellular membrane. Such membrane-based temperature effects have been extensively studied in relation to chilling injury in chilling-sensitive plants such as corn, sweet potato, and tomato. Sharp changes at low temperature in the activation energy of some membrane-associated reactions and changes in the permeability of membranes have been related to chilling injury in these plants (Lyons and Raison, 1970; Raison, 1973). The temperature response of many membrane-associated activities, including sugar uptake by bacteria (Linden et al., 1973), also show such chilling-sensitive responses.

We have also examined the effect of temperature on respiration and Rb⁺ ion uptake by roots of corn, which is known to be chilling sensitive. In corn, in contrast to barley, rates of ion accumulation and respiration are closely correlated over the temperature range examined, and both have discontinuous Arrhenius plots with high activation energies at low temperatures (Fig. 42). The temperature response of corn root respiration is similar to that reported for isolated corn mitochondria (Raison, 1974). The sharp break in activation energy is taken to indicate a chilling-sensitive response. This author has shown that this type of discontinuity is caused by a change in state or phase of the membrane lipids. Later studies which considered the physical nature of membrane changes with temperature, using techniques such as electron spin resonance, indicated that the membrane phase change was dependent on the degree of saturation of fatty acids in the membrane lipids.
Power *et al.* (1970) have shown that barley germinated at 25°C and then placed at 9°C does not grow for the first 3 weeks and subsequently has a lower maximum growth rate when compared to plants grown with 15.5° or 22°C. The plants grown at low temperatures, however, eventually equal the yields obtained by those grown at the highest temperatures. The sharp effect of low temperature (below 10°C) on growth of barley may be related to the effect of low temperature on the functioning or development of ion uptake capacity by barley roots described in the present study.

Chapin (1974) examined the degree to which plants native to warm and cool edaphic environments can maintain the capacity for phosphate uptake with severe alteration of their normal root temperatures. Data presented here indicate the need to examine these species over a range of temperatures (5°–30°C) to reveal the mechanisms responsible for limitation of ion uptake. Our studies also indicate a need to differentiate between chilling injury to the membranes functioning in ion uptake and the effect of temperature on the capacity for ion uptake or on the turnover of the ion uptake mechanisms.

In summary, we have shown phenotypic adjustment to low growth temperatures. Root respiration is clearly stimulated when barley is exposed to low root temperatures during growth. Activation energies for rubidium accumulation in corn and warm-grown barley indicate that uptake of this ion is not dramatically influenced by root temperature over the normal edaphic range (10°–30°C). A lower capacity for rubidium accumulation in cool-grown barley and a higher apparent activation energy may be caused by a damaged or incomplete ion accumulation mechanism due to prolonged exposure to low temperature. However, a phenological lag in development cannot be discounted. Unlike barley, corn exhibits a parallel pattern for the effect of low temperature on
both ion accumulation and respiration. The effect of low temperature on these activities indicates that both are involved in chilling sensitivity. A similar sharp effect of low temperature upon ion uptake by barley roots at temperatures below 10°C indicates that the ion uptake mechanism of barley may be subject to membrane-based chilling injury at low root temperatures.

References


POSSIBLE USE OF HOLLOW FIBER HEMODIALYSERS IN SOIL-PLANT WATER RELATIONS RESEARCH

Bruce E. Mahall

A major limitation of research in soil-plant water relations has been the inability to control the water potential in soil rooting media at moisture contents of less than field capacity. As pointed out long ago by Shantz (1925), the addition of small quantities of water to dry soil (less water than that necessary to reach field capacity throughout the soil volume) results in nonuniform water potential distributions with large moisture gradients. With such techniques the water potential around roots is unknown and uncontrollable at any particular time. It is also nearly impossible to control soil water potential accurately through time in the presence of a plant transpiring at variable rates. Recent attempts to control soil water content at levels below field capacity have not been successful (Kramer, 1974).

In studies of soil-plant water relations, hydroponic culture has become an important alternative to soil because it allows precise control of water potential uniformly distributed around roots, and the water potential may be easily altered through time. The artificiality of the hydroponic technique results in some important disadvantages, however. Solutes such as polyethylene

¹Current address: Department of Biological Sciences, University of California, Santa Barbara, California 93106.
glycol (PEG), used to maintain osmotic potentials, may be taken up by plants and may have direct physiological effects unrelated to osmotic effects (Leshem, 1966; Greenway et al., 1968; Janes, 1974). Perhaps even more important is the unnatural (for most terrestrial plants) absence of gas space surrounding roots. It has been shown that this absence may affect root anatomy by a response to soil water content rather than directly to oxygen diffusion rates (Varade et al., 1970, 1971). Moreover, Mexal et al. (1975) have demonstrated that availability of oxygen for root respiration may be severely limited in solutions of PEG of higher molecular weights.

The purpose of this note is to introduce the possibility that the problems of control of known water potentials through space and time, solute poisoning, and aeration may be greatly reduced by the use of hollow fiber hemodialyser units as root chambers. These units are now commonly used in artificial kidney machines and in routine laboratory dialysing operations. There are many models of hemodialysers commercially available, but because of their cost (~ $25 each) and geometry, the Cordis-Dow C-DAK Models 4 and 5 appear to be most appropriate for soil-plant water relations research. These units consist of over 13,000 regenerated cellulose hollow fiber membranes aligned parallel to each other and extending 16–20 cm from one manifold to another. The fibers have an inside diameter of 200 μ, a wall thickness of 30 μ, and a collective surface area in excess of 1.3 m². Between the manifolds the fibers are enclosed by a polycarbonate jacket, closed off from the manifold chambers except by passage through the fiber walls. When in use as an artificial kidney, blood is pumped through the hollow fibers and dialysate is flushed through the jacket.

I have modified the jackets of such devices by adding a hole with a collar so that plants may be grown with their roots enclosed in the jackets among the hollow fibers. A nutrient solution containing an osmotic solute too large to pass easily through the fiber membrane pores at the hydrostatic pressures present is pumped slowly through the fibers. Solutes as small as PEG 600 (average molecular weight) show no tendency to leak through these membranes under the small hydrostatic transmembrane pressure produced by a Buchler Polystaltic Pump. When transmembrane pressures exceeding 0.05 kg cm⁻² are produced by adding a solution to the jacket with an osmotic potential exceeding that inside the fibers, even PEG 6000 will slowly diffuse through the membranes. The rationale here is that the relatively small gas volume inside the jacket surrounding the fibers should quickly reach a water potential in equilibrium with the osmotic potential inside the fibers since water moves freely through the membranes. In addition, the physical contact between the fibers and the plant roots should result in a water film connection between the inside of the fibers and the roots through which inorganic nutrients may diffuse to the roots. Because the hollow fibers are cellulose, the root chamber must be maintained free of microorganisms capable of decomposing the fibers.

With this method, uniform distributions of accurately known water potentials around roots may be maintained and controlled precisely through short-term or long-term changes. Since the contact of the osmotic solute with roots will be restricted, the solute poisoning problem may be reduced to a large degree. Because the hollow fiber rooting medium is a composite of gas, liquid, and solid, it much more closely simulates the natural conditions than do hydroponic solutions, and oxygen around the roots may be controlled and made readily available. In fact, by connecting the root chamber to a gas analysis system, it should be possible to monitor root gas exchange.

Tests are now being conducted to determine rates of movement of various osmotic solutes and inorganic nu-
trients through the membranes and their uptake from the fibers into plants. Comparisons of plants growing hydroponically with those growing in hemodialyser-root chambers will be made with regard to nutrient and osmotic solute uptake, growth rates, and gas exchange.

References

ENZYME POLYMORPHISM IN THE BUTTERFLY Colias:
SELECTION ON METABOLIC PHENOTYPES
George B. Johnson

INTRODUCTION

In the last decade it has become abundantly clear that levels of genetic variability detected by electrophoresis are very high in animal populations. However, the evolutionary significance of this variation is not clear. The difficulty is in understanding why there is so much of it. Most natural populations seem to be polymorphic at around a third of their enzyme loci, and over 10% of individuals are heterozygous at a typical enzyme locus. This is far more genetic variability than theory had led us to expect. The disparity from expectation is not unlike Diogenes searching for one honest man—and finding hundreds! Among population geneticists there has been a lively discussion concerning the possibility that these very high levels of polymorphism are simply "noise," with no adaptively important differences between alleles. Allozymes are viewed as reflecting small changes in protein structure large enough to change migration in electrophoresis, but too small to alter enzyme activity significantly. Because selection cannot differentiate between two alleles with the same activity, the polymorphism is spoken of as "selectively neutral."

Many workers, however, have not accepted this hypothesis; they argue instead that each of the polymorphic variants is functionally different in activity and that these activity differences affect fitness in such a way that the polymorphism is maintained by selection.

I will not review the history of this argument here. It has been treated in depth in a variety of recent articles representing a spectrum of opinion (Crow, 1972; Johnson, 1973; Selander and Johnson, 1973; Harris et al., 1974; Lewontin, 1974). My personal judgment is that the weight of the evidence favors a selective view, but a clear and unambiguous general case has not yet been made.

EXPERIMENTAL APPROACH:
A SINGLE LOCUS STUDY

To assess the adaptive significance of genetic variation is not a trivial matter. It is not enough to simply observe patterns of allele frequency which correlate with some aspect of the natural environment, as many factors other
than adaptation may generate such patterns: Migration, founder effect, genetic drift in small populations, linkage to other loci under selection, all may have important effects. What is required is a well-defined empirical question designed to contrast adaptive values directly. To study polymorphic variation at a single gene locus, an ideal system would involve at least five factors: (1) The organism should be typically polymorphic (e.g., heterozygosity at enzyme loci of between 10% and 20%). (2) The organism should lend itself to formal genetic analysis, so that the allelic nature of variants may be verified. (3) The chosen locus should be that of a single enzyme whose physiological function is clearly understood. (4) The chosen physiological function should be influenced by a discrete quantifiable habitat factor. (5) The organism should occur in natural populations where this habitat factor differs. The investigator may then directly ask whether different alleles function differently, whether the habitat variation affects that difference, and whether the functional difference may in any way be construed as an adaptive response to the habitat variation. An approach like this, although focused on only one locus, is very powerful because the investigator knows and can measure all the important experimental factors.

Such a single locus approach has been applied to lactate dehydrogenase in fish (Merritt, 1972), alcohol dehydrogenase in Drosophila (Day et al., 1974; Clarke, 1975), and α-glycerophosphate dehydrogenase in Colias butterflies (Johnson, 1975a). The approaches and results of the three analyses are similar; I will describe the α-GPDH system in Colias, the subject of my work, as typical. I have collected experimental data on this system for five years, and over the course of the last year have analyzed these data and published them as CIW-DPB publication 559 (Johnson, 1976a; CIW-DPB publication 562 (Johnson, 1976b); and CIW-DPB publication 568 (Johnson, 1976c).

The α-GPDH system has all the properties of a good single-locus experimental system:

1. In five Colias species, mean heterozygosity varies from 11% to 24%, based upon examination of 14 loci in samples exceeding 100 individuals.

2. The function of α-GPDH is well understood in insects, where it plays much the same role that LDH does in vertebrates—It modulates cell redox potential \((\text{NADH} / \text{NAD}^+)\) so as to regenerate NAD\(^+\) during prolonged flight (Sacktor, 1970). Thus, null mutants for this enzyme locus in insects are flightless.

3. Formal genetic analysis of α-GPDH variants may be carried out routinely, if not conveniently. Variants segregate in crosses in a simple Mendelian fashion.

4. Flight in Colias is influenced by a discrete and measurable habitat factor, temperature. The interaction has been elegantly investigated by Watt (1968), who implanted thermistors in Colias butterflies and asked under what conditions they fly. They fly only within a narrow range of body temperature. Because this critical flight temperature is usually higher than ambient, the butterflies raise their body temperatures by solar heating. This behavior is unmistakable in the field. When a cloud covers the sun, all flying Colias drop to the ground; when the sun comes back out, they warm up again in a few minutes and resume flying. By recording solar flux with a pyrothermograph, one may quantitatively characterize a habitat in terms of available flight windows for Colias. Thus habitat temperature, particularly solar flux, seems a promising choice for an environmental factor importantly affecting the functioning of α-GPDH. Temperature has proven a fortunate choice, as the biochemical behavior of enzyme alleles of α-GPDH in Colias is indeed differently affected by reaction temperature (Johnson, 1976a). By marking the wings of live individuals, one may carry out mark-release-recapture studies in a straightforward manner,
and thus learn the size and genetic structure of natural populations.

5. Finally, the available habitats of the Colorado Colias encompass many very different thermal environments. The alpine species C. meadii is typically found on tundra, above timberline (>12,000 ft). The montane species C. alexandra occurs in montane open valleys at elevations of about 9500 ft. C. scudderii lives in montane habitats of 9000–11,000 ft in conjunction with willow (the other species are restricted to legumes). The lowland species complex C. philodice-C. eurytheme occurs as an agricultural pest in lowland farmland from 5000 to 8000 ft. Transitional populations occasionally occur in which populations of the lowland complex occupy montane meadows, or in which the alpine species occurs in montane habitat.

**Polymorphism at the α-GPdH Locus in Colias** (Summary of CIW-DPB Publication 562)

Polymorphism at the α-GPdH locus was examined in 18 populations over a period of five years. Two variant forms were detected by analysis of population samples on 7% polyacrylamide gels (there is reason to believe that additional "hidden" alleles exist which are not detected by this approach). Chemical characterization of the two allozymes indicates that the same homologous alleles occur in each of the five species examined. The two alleles segregate in crosses in a Mendelian manner, and heterozygous individuals can be shown to possess three electrophoretic bands (the middle band being a heterodimer or hybrid molecule). The substrate-binding kinetics of the two forms are significantly different. In particular, the faster-migrating variant enzyme binds substrate more effectively at 10°C (has a lower $K_m$), while the slower-migrating variant is more effective at 30°C. As this corresponds roughly to the thermal range of the butterfly habitat during the Colias flight season, this difference between the alleles is likely to be of adaptive significance.

When polymorphism for α-GPdH is compared for the 18 populations, a significant pattern is evident (Table 20): All nine montane populations are quite polymorphic (heterozygosity greater than 10%), while alpine or lowland populations are far less variable. This is true within species as well as between them. Thus alpine populations of C. meadii are not variable at this locus, while montane populations are. The result seems quite general over the five species: For 162 alpine individuals, heterozygosity was 35%; for 174 lowland agricultural individuals, average heterozygosity was 7%.

This pattern of genetic variability is consistent with what we know of the thermal nature of these habitats. While the montane valleys may get quite cold at night, they offer warmer habitats during the day than the windswept open tundra of the high alpine populations. It is in the colder high altitude populations that the fast allele predominates, and it is the fast allele which is the most effective binder of substrate at low temperature. Montane environments are consistently less predictable in their thermal extremes than are alpine areas, so that the pattern as well as the range of the two habitats differ.

The general results are thus quite consistent with the hypothesis that enzyme polymorphism at the α-GPdH locus reflects adaptation to a heterogeneous thermal environment. This hypothesis makes a clear and testable prediction: When single populations occupy diverse habitats, different portions of the population should experience very different selection. Thus, for example, a number of populations of C. meadii are known which occur right at timberline. Portions of these populations live in alpine habitats, while other portions extend down into the montane. The genetic structure of one such population (Mesa Seco) has been studied intensively by Watt and co-
TABLE 20. Patterns of \(\alpha\)-Glycerophosphate Dehydrogenase Polymorphism in Several Species of *Colias*

<table>
<thead>
<tr>
<th>Species</th>
<th>Location (Altitude)</th>
<th>Habitat</th>
<th>No. of Individuals Analyzed</th>
<th>Heterozygosity of (\alpha)-GP(d)H</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. meadii</em></td>
<td>Cumberland Pass (12,000')</td>
<td>Alpine</td>
<td>48</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>Uncompahgre Peak (12,500')</td>
<td>Alpine</td>
<td>23</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>Mesa Seco (12,200')</td>
<td>Alpine</td>
<td>30</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>Upper Cement Creek (12,000')</td>
<td>Alpine</td>
<td>36</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>Upper Queen Basin (12,100')</td>
<td>Alpine</td>
<td>18</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>Copper Creek (10,200')</td>
<td>Montane</td>
<td>16</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>Los Pinos Pass (10,500')</td>
<td>Montane</td>
<td>20</td>
<td>0.45</td>
</tr>
<tr>
<td><em>C. scudder</em></td>
<td>Upper Cement Creek (11,700')</td>
<td>Alpine</td>
<td>7</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>Lower Cement Creek (9,800')</td>
<td>Montane</td>
<td>21</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>Taylor Park (10,500')</td>
<td>Montane</td>
<td>32</td>
<td>0.17</td>
</tr>
<tr>
<td><em>C. alexandra</em></td>
<td>East River (9,500')</td>
<td>Montane</td>
<td>76</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>Brush Creek (9,400')</td>
<td>Montane</td>
<td>40</td>
<td>0.45</td>
</tr>
<tr>
<td><em>C. philodice</em></td>
<td>Slate River (9,100')</td>
<td>Montane</td>
<td>46</td>
<td>0.39</td>
</tr>
<tr>
<td></td>
<td>Lower Cement Creek (9,400')</td>
<td>Montane</td>
<td>22</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>Hotchkiss (5,000')</td>
<td>Agricultural</td>
<td>89</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>St. Louis, Mo.</td>
<td>Agricultural</td>
<td>40</td>
<td>0.05</td>
</tr>
<tr>
<td><em>C. eurytheme</em></td>
<td>Lower Cement Creek (9,200')</td>
<td>Montane</td>
<td>19</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>Los Banos, Calif. (500')</td>
<td>Agricultural</td>
<td>45</td>
<td>0.02</td>
</tr>
</tbody>
</table>

workers, and it is known from their mark-release-recapture studies that the population is genetically continuous, with at least a few individuals passing along its entire length each generation. Thus, migration within the population would be expected to render it genetically uniform—unless very strong differential selection were acting upon the two alleles.

When \(\alpha\)-GP\(d\)H polymorphism is examined along a transect from alpine to montane within the Mesa Seco population, the transect is not uniform (Table 21). A pronounced cline in heterozygosity is seen: The alpine sites are essentially monomorphic for the fast allele, as observed previously, but the slow allele becomes increasingly more common as lower sites are examined. Only in the highly heterozygous lower sites are the two alleles in Hardy-Weinberg equilibrium; the higher sites show large deficiencies in the slow homozygote.

Again, this result seems quite general. When the same population was sampled again two years later, an identical cline was seen. Two other timberline populations, geographically quite distant, exhibit similar alpine-montane clines in \(\alpha\)-GP\(d\)H heterozygosity.

Thus variation at this locus is in all respects consistent with an adaptive hypothesis. One cannot, of course, rule out the possibility that selection actually is occurring on some other locus that we don't know about, and that \(\alpha\)-GP\(d\)H is simply linked to it. However, if linkage forms the basis for the observed \(\alpha\)-GP\(d\)H polymorphism, it is remarkably fortunate that it has produced such a functionally suitable distribution of alleles!

**Variation at Other Loci—The Multi-Locus Problem**

While a single-locus approach such as described above accounts reasonably well for the genetic polymorphism seen at the \(\alpha\)-GP\(d\)H locus, the result need not be general. Species of the genus *Colias* exhibit high levels of genetic variability at many enzyme loci, and...
TABLE 21. $\alpha$-GPdH Polymorphism along a Transect through the Mesa Seco Population

<table>
<thead>
<tr>
<th>Year</th>
<th>Site No.</th>
<th>Altitude of Site</th>
<th>No. of Individuals Analyzed</th>
<th>Observed Frequency of $\alpha$-GPdH Heterozygotes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1971</td>
<td>13</td>
<td>12,200</td>
<td>21</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>13a</td>
<td>12,000</td>
<td>9</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>11,500</td>
<td>24</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>11,000</td>
<td>21</td>
<td>0.43</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>10,800</td>
<td>5</td>
<td>0.80</td>
</tr>
<tr>
<td>1973</td>
<td>13</td>
<td>12,200</td>
<td>40</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>11,500</td>
<td>40</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>11,300</td>
<td>40</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>11,000</td>
<td>40</td>
<td>0.43</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>10,800</td>
<td>20</td>
<td>0.47</td>
</tr>
</tbody>
</table>

we have accounted for only one. What of the others? To account for the generalized occurrence of enzyme polymorphism, one of two hypotheses is usually advanced. They are both fundamentally single-locus hypotheses. One is the hypothesis we have used to account for the $\alpha$-GPdH variation: a heterogeneous environment selecting for different alleles under different circumstances. Similarly contrasting environmental influences are known to produce a polymorphism for sickle-cell hemoglobin in man and have been implicated in lactate dehydrogenase (AdH) polymorphism in Drosophila. However, if such single-locus explanations provide the basis for most of the polymorphic enzyme variation that is being reported, then we shall have to do a great deal of work to document this fact!

An alternative hypothesis is that of molecular overdominance: Hybrid enzyme molecules (formed from subunits of both parental types) are viewed as intrinsically more stable or kinetically superior. This functional superiority produces a direct heterosis, and because heterozygotes are always at an advantage, high levels of polymorphism result. This hypothesis has great difficulty, however, in accounting for polymorphisms at loci of monomeric enzymes without multiple subunit structures.

Thus, neither of these single-locus hypotheses is particularly satisfactory. I believe that the reason for this situation lies less with the hypotheses than with the question they address. The key is in realizing that $\alpha$-GPdH, LdH, AdH, and hemoglobin were selected for study because each involves a discrete physiological function directly affected by environmentally imposed reaction conditions; it is not unreasonable that a single-locus hypothesis would be satisfactory in these cases. However, this is true of few of the other polymorphic loci of Colias. Most of the polymorphic enzymes are intimately involved in intermediary metabolism, and a change in the activity of one may influence the functioning of many others. Thus a change in hexokinase, which generates glucose-6-phosphate, cannot help but affect the reactions of phosphoglucomutase, phosphoglucoisomerase, and glucose-6-phosphate dehydrogenase, all of which use glucose-6-phosphate as a substrate. It seems likely that only a multi-locus hypothesis will be able to account for variation among such loci. In this regard it is worth noting that polymorphic variation occurs primarily at regulatory (rate-limiting) steps in intermediary metabolism. This is a pattern one would expect only if selection were acting on the integrated metabolic phenotype rather than on individual loci per se.

One multi-locus hypothesis which seems to me very attractive is that enzyme polymorphism is selected at regu-
latory loci so as to buffer these reactions from environmental perturbation. To maintain metabolic integration in a variable environment is of major evolutionary importance, as many metabolic control systems are interrelated. Yet critical reactions may respond quite differently to changes in temperature, etc. It may be of significant adaptive advantage to be able to maintain a constant relationship among critical regulatory reactions.

It is easy to envision molecular mechanisms that would produce such a homeostasis. If the activities of two alleles respond differently to a habitat variable such as temperature (and such differences are well documented for tissue-specific isozymes), then the functional displacement with respect to temperature can buffer the coordination of metabolism from the effects of a temperature change. Any one allele of a regulatory enzyme can exhibit a low $K_i$ (e.g., bind substrate well) only over a relatively narrow temperature range. This thermal sensitivity is an inevitable result of the requirement that regulatory enzymes be structurally flexible enough to be sensitive to allosteric "effectors" (low molecular weight molecules such as ATP whose binding acts as a metabolic signal). Over a broad temperature range a regulatory reaction cannot maintain a constant affinity for substrate and a constant binding affinity for effector molecules. As a result, it is difficult to maintain coordinate regulation with respect to other pathways catalyzed by different proteins responding differently to the change. A heterozygous individual, however, has two allelic forms present in each cell. For alternative alleles $\alpha$ and $\beta$, the $\alpha$ allozyme may have the stronger binding affinity for substrate (lower $K_i$) at lower temperatures. When substrate concentrations are low, which is typically the case, only the $\alpha$ form will bind substrate at low temperature, and it will determine the reaction rate. Were it the only form present, the rate of binding would change at higher temperature. However, if there is a functional displacement of the $\beta$ allele, the $\beta$ allele has the lower $K_i$ at higher temperatures. As a result, in heterozygotes it is the form which binds the substrate at these temperatures—and the realized binding affinities have not changed over the broad range of temperatures!

This model suggests that heterozygotes are not overdominant so much as conditionally hemizygous, and that it is the very difference between the alleles which produces the adaptive advantage. Polymorphism is seen as a genetic strategy for maintaining metabolic integration in the face of environmental heterogeneity.

MULTIPLE LOCI IN Colias
(SUMMARY OF CIW-DPB PUBLICATIONS 559 AND 568)

The highly coordinated nature of intermediary metabolism suggests that if polymorphic alleles at regulatory enzyme loci are functionally different, then the particular allele present at one locus will importantly affect the activity of many other reactions. Thus if an individual possesses a "low-temperature" allele at one locus rather than a "higher temperature" form, then it makes a difference which functional forms are present at other regulatory loci. If a network of related regulatory enzymes are all optimally suited to low temperature, then one may speak of a metabolic phenotype adapted to these conditions. It is at this level that selection would be most reasonably expected to act on the expressed phenotype of individuals rather than upon individual loci. Because the particular functional variant occurring at each regulatory locus influences the physiological state of the individual, selection on metabolic phenotypes implies selection on allozymic genotypes. To understand enzyme polymorphism, then, it will be necessary to characterize simultaneously a variety of loci in each sampled individual of a population.
Such a study is best carried out within a single population to eliminate the possibility that differences in allele frequencies arise from demographic complications. If different loci sampled from the same individuals exhibit different patterns of allele frequency, then the result may not be attributed simply to migration or habitat selection by mobile adults.

For such a genotypic comparison, 12 loci of *C. meadii* were characterized, each butterfly being tested for all 12 loci. The Mesa Seco population was selected for the study, and samples collected at each of five sites along a transect from alpine to montane: an alpine site (#13), timberline (#12), montane forest (#11), montane forest-meadow boundary (#10), and open montane meadow (#9). The total distance traversed by the transect was about 2 miles, and the elevational differences about 2000 ft. The population size was known from previous mark-release-recapture studies to exceed 1000 individuals (one generation per year) and seemed to maintain approximately the same numbers from year to year. Individuals are seen to exchange between adjacent sites at a frequency of about 10%, although little or no exchange is seen between the more distal sites.

The general result of this survey is that despite extensive migration, a variety of different patterns of allele frequency are seen at different loci sampled from the same individuals. There is no way in which migration alone may be evoked to account for these results.

What then of the genotypes? The results from site #9 are typical. Of 20 individuals, fully nine appear to have highly organized genotypes: Five individuals have identical alleles at each of seven of the 12 loci; four others are identical at six loci. The odds that an individual would exhibit such coordination by chance are very low. For the first group, the common genotype and associated allele frequencies are:

<table>
<thead>
<tr>
<th>Locus</th>
<th>α-1-PG</th>
<th>EST-1</th>
<th>EST-2</th>
<th>AK-1</th>
<th>AK-2</th>
<th>G6PDH</th>
<th>HK-1</th>
<th>HK-2</th>
<th>ME</th>
<th>PGM</th>
<th>FUM</th>
<th>MSH-1</th>
<th>MSH-2</th>
<th>TP1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allele</td>
<td>B</td>
<td>A</td>
<td>C</td>
<td>A/B</td>
<td>A</td>
<td>B</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>(Genotype)</td>
<td>0.79</td>
<td>1.0</td>
<td>0.50</td>
<td>1.0</td>
<td>0.84</td>
<td>0.80</td>
<td>0.25</td>
<td>0.37</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>0.61</td>
</tr>
</tbody>
</table>

The joint probability that one individual will have this genotype is the product of the allele frequencies, \( P = 0.02 \). The probability that five individuals would possess this genotype by chance is only \((5^{12}) \times 0.02^{5}\), or \(3.66 \times 10^{-5}\)!

Two such commonly recurring genotypes are apparent in the sample from site #9, one involving six loci and one involving seven. The implication is very strong that they reflect selection for particular constellations of alleles.

When the other sites are examined, similar results are obtained: Highly organized genotypes involving more than half the examined loci repeatedly occur at high frequency. However, these genotypic combinations are different for each site! In the face of the observed adult migration, this is a remarkable result. None of the genotypic combinations common at one site are ever observed at any other. These results are summarized in Table 22. In this table, loci where more than one genotype occur among the group are symbolized by a dash.

The genotypic organization seen in the results of Table 22 clearly relates to the overall metabolic phenotype, as it involves almost exclusively regulatory as opposed to nonregulatory loci. Only those reactions which significantly affect the rate of intermediary
# TABLE 22. Genotypic Combinations Specifying at least One Half of Examined Loci

<table>
<thead>
<tr>
<th>Site</th>
<th>α-GPDH</th>
<th>EST-1 &amp; -3</th>
<th>EST-2 &amp; -3</th>
<th>AK-1 &amp; -2</th>
<th>G6PDH &amp; -2</th>
<th>HK-1 &amp; -2</th>
<th>ME</th>
<th>PGM</th>
<th>FUM</th>
<th>MdH-1</th>
<th>MdH-2</th>
<th>TPI</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>A</td>
<td>A</td>
<td>B,C</td>
<td>A/B</td>
<td>A,B</td>
<td>B</td>
<td>—</td>
<td>—</td>
<td>B</td>
<td>A</td>
<td></td>
<td></td>
<td>0.25</td>
</tr>
<tr>
<td>12</td>
<td>C</td>
<td>A/C</td>
<td>B,C</td>
<td>A/B</td>
<td>A,B</td>
<td>A</td>
<td>—</td>
<td>—</td>
<td>A</td>
<td>B</td>
<td></td>
<td></td>
<td>0.33</td>
</tr>
<tr>
<td>11</td>
<td>A</td>
<td>C</td>
<td>B</td>
<td>A,C</td>
<td>A,B</td>
<td>B</td>
<td>B/—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
<td></td>
<td>0.20</td>
</tr>
<tr>
<td>10</td>
<td>C</td>
<td>—</td>
<td>—,C</td>
<td>A/B</td>
<td>A,B</td>
<td>C</td>
<td>A/—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
<td></td>
<td>0.35</td>
</tr>
<tr>
<td>9</td>
<td>B</td>
<td>C</td>
<td>—</td>
<td>A,B</td>
<td>A,B</td>
<td>D</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
<td></td>
<td>0.15</td>
</tr>
<tr>
<td>4</td>
<td>B</td>
<td>—</td>
<td>D</td>
<td>A,C</td>
<td>A/B</td>
<td>A,B</td>
<td>D</td>
<td>—</td>
<td>—</td>
<td>A</td>
<td>B</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>B</td>
<td>—</td>
<td>—</td>
<td>A,C</td>
<td>A/B</td>
<td>A,B</td>
<td>D</td>
<td>—</td>
<td>—</td>
<td>A</td>
<td>B</td>
<td>0.20</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>B</td>
<td>—</td>
<td>D</td>
<td>A,C</td>
<td>A/B</td>
<td>A,B</td>
<td>D</td>
<td>—</td>
<td>—</td>
<td>A</td>
<td>B</td>
<td>0.20</td>
<td></td>
</tr>
</tbody>
</table>

metabolism seem to be included in the organized genotypes.

I have considered α-GPDH and the esterases separately, as their functions are individually relatable to habitat factors such as temperature or secondary plant compounds. The genotypes of these loci also appear highly correlated with the localized habitat.

The unavoidable conclusion one must draw from these results is that organized genotypes do exist in natural populations, apparently maintained by selection in the face of significant migration.

**POLYMORPHISM AS GENETIC STRATEGY**

The genotypic associations described above suggest rather strong selection. For the genotypes of site #9, the indicated fitness (expected genotypic frequency/observed) is about 0.10. This seems very strong selection, and it raises the question of how the genetic and population structure of *C. meadii* has evolved to cope with what appear to be stringent environmental constraints.

The observed properties of the *C. meadii* genetic system are: (1) It involves a very large number of small chromosomes ($2N = 62$). (2) Although each female will lay several hundred eggs during a yearly flight season, population sizes remain relatively constant (this suggests a mean zygotic fitness of the order of 0.005). (3) Mark-release-recapture studies indicate low adult mortality, suggesting that selection is primarily at the larval stage. (4) Mating appears to be panmictic within local populations. (5) Members of individual subpopulations appear to be quite sedentary; while some individuals may forage for several hundred meters, the distribution of most adult individuals appears localized to portions of the cline. (6) Unlike alpine populations of *C. meadii* studied at other localities (where there is little exchange between subpopulations), there is significant exchange between adjacent subpopulations of the Mesa Seco population.

The observed genotypic associations may be maintained in such a genetic system by at least two very different genetic strategies. One strategy is that of linkage. If the key loci are tightly linked, then the observed high-linkage disequilibrium would be an inevitable result of the low recombination fraction between them. Such a hypothesis implies that the subpopulations along the cline must be genetically isolated from one another, despite migration. Otherwise genotypes common in one subpopulation would appear in adjacent ones. To maintain the observed genotypic discontinuities would require strong selection.

The alternative hypothesis is that the genetic system of *C. meadii* is analogous to that of the plants on which it feeds—that it utilizes the great segregational power of its high chromosome number to produce in each generation a wide array of genotypes. From this varied assortment a small fraction survive to become adults at any given site. Different sites might then select for different genotypes. Such a genetic strategy is highly flexible, being capable of reorganizing the genotypic constitution of a local subpopulation yearly. Such a strategy would constitute ideal adaptation to an unpredictably variable local habitat.

In this respect it is worth noting that efficient food processing is of paramount importance to *Colias* larvae, and that the regulatory loci of Table 22 encompass many of the key points of physiological regulation of intermediary metabolism.

Both segregation and linkage strategies imply a heterogeneous habitat and strong selection within the Mesa Seco population. It is possible to distinguish experimentally between them by reexamining these sites in subsequent years. The population subdivision suggested by a linkage strategy predicts temporal stability. The local genotypic combinations should recur
from year to year. In contrast to this, a segregational strategy implies temporal as well as spatial habitat variability: The local genotypic combinations may be quite different from year to year. The data are not yet available to distinguish between these two alternatives.

It is clear that a great deal remains to be done to understand these patterns of genetic variation. To me, the most attractive conceptual framework within which to organize the findings discussed above is to view the patterns of enzyme polymorphism seen within *Colias* butterflies as adaptive strategies, which in each case match the flexibility of the metabolic phenotype to the heterogeneity of the environment. The detailed information needed to evaluate this interpretation involves both biochemical study of the differential functioning of allozymes and far more extensive surveys of natural populations.

**References**


**CHARACTERIZATION OF ELECTROPHORETICALLY HIDDEN VARIATION IN THE BUTTERFLY Colias**

George B. Johnson

Several lines of evidence have recently begun to suggest that allelic variants detected by electrophoresis may be heterogeneous—discrete electrophoretic variants actually representing a collection of alleles, each migrating to the same position on a gel. This paper describes a series of investigations carried out over the last six years employing gel-sieving analysis to detect and characterize electrophoretically "cryptic" variation. A method has been developed during the past year which permits characterization of such variation in natural populations. This approach is described in Carnegie Institution of Washington–Department of Plant Biology Publications 561 and 569, and implications of the results are discussed in Carnegie Institution of Washington–Department of Plant Biology Publications 563, 572, and 573.

**GEL-SIEVING ANALYSIS**

Electrophoretic alleles of enzyme loci are typically detected and characterized by comparing their mobilities on starch or acrylamide gels. This has provided a particularly straightforward and convenient approach to detect genetic polymorphism, as mobility classes appear clearly discontinuous in most systems. In discussing electrophoretically detected enzyme polymorphism of this sort, differences between alleles are commonly ascribed to amino acid substitutions involving charged residues. The observed discon-
continuities in gel mobility would be an integral property of such "charge state" variation.

From a theoretical point of view, such a model is overly simple. In ideal electrophoresis a protein migrates in a field at a rate (the free electrophoretic mobility) determined by its net charge. Gel electrophoresis does not conform to ideal conditions, however, as the gel and protein interact during the course of the protein's migration. Whether one visualizes the interaction as frictional (the protein "bumps into" fibers) or hydrodynamic (sheer forces are generated by the protein's movement past fibers), the shape and size of proteins should also affect their migration rates on gels.

The migration of proteins in polyacrylamide gels has been studied by physical chemists in some detail (the matter is reviewed in Chrambach and Rodbard, 1971). The theory describing how a protein migrates in polyacrylamide gel electrophoresis stems from the observation by Ferguson that a protein's mobility in gel electrophoresis is a logarithmic function of gel pore size (Ferguson, 1964). This suggests a straightforward theoretical description:

\[ R_f = \frac{M_u}{U_f} (K_r T) \]  

(1)

Where \( R_f \) = protein mobility relative to front; \( U_f \) = a buffer constant; \( M_u \) = free electrophoretic mobility, a function of net charge; \( K_r \) = retardation coefficient, a function of molecular weight and protein conformation; and \( T \) = percentage of acrylamide, which determines pore size and is inversely proportional to it.

A considerable body of theory has been developed elaborating this simple model (Fawcett and Morris, 1966; Rodbard and Chrambach, 1970, 1971; Chambach and Rodbard, 1971; Gonnene and Lebowitz, 1975). In this model the rate of migration is seen to vary not only as a function of net charge (expressed as free electrophoretic mobility, \( M_u \), corrected by a constant, \( U_f \), for the buffer employed) but also, as suggested by Ferguson, as a logarithmic function of the gel pore size (expressed as percentage of acrylamide) and of the gel-protein interaction as the protein passes through these pores (expressed as the retardation coefficient, \( K_r \)).

The approach suggested by Equation 1 permits independent characterization of the contributions of charge and of interactive effects such as size or conformational differences to the electrophoretic behavior of a protein. These parameters may be empirically estimated by the simple expedient of taking the log of both sides of Equation 1, yielding a function of linear form:

\[ \log R_f = \log \frac{M_u}{U_f} + K_r T \]  

(2)

One runs replicate samples of an individual in parallel on several gels of differing pore size (\( T \)), and determines for each gel the corresponding mobility (\( R_f \)), thus characterizing directly the degree to which reducing the pore size retards migration. Regressing \( \log R_f \) on \( T \), one obtains a linear plot with a slope of \( K_r \) and an intercept whose antilog is \( M_u \) divided by a constant. A typical result is presented in Fig. 43.

One may standardize determinations of \( K_r \) and \( M_u \) for an enzyme of an individual by expressing the values relative to the corresponding value of the (similar) internal standard determined from the same gels. Because the standard is evaluated many hundreds of times in population survey work, the procedure permits standardization of any error affecting both proteins. Details of such standardization procedures are given in CIW-DPB Publications 561 and 569.

In order to demonstrate that an enzyme characterized in one individual is
electrophoretically different from that characterized in another, it is necessary to demonstrate that \( K_r \) or \( M_o \) or both differ significantly between the two forms. In comparing estimates of \( K_r \) and \( M_o \), it is important to note that \( K_r \) and \( M_o \) pairs are determined from a single linear regression as slope and intercept, and that error in the estimation of an intercept is not independent of error in the estimation of the corresponding slope.

One simple approach to estimating the error associated with a mobility estimate independent of that associated with a corresponding \( K_r \) estimate is to express mobility in terms of the \( R_f \) observed at the mean value of \( T \). A linear regression may be considered to rotate around such a midpoint as its slope varies, the midpoint remaining unchanged despite great changes in the \( y \) intercept. The error in the estimate of this parameter is independent of the error in the estimate of the slope \( K_r \).

When sampling from a natural population, there will be an error variance in \( K_r \) and in mid-\( Y \) associated with each protein type present in the sample. To document the existence of multiple classes requires an independent estimate of experimental error. This estimate may be readily obtained from the internal standards run in the same gels. In plotting \( K_r \), mid-\( Y \) estimates from a natural population, points reflecting homologous proteins should have a distribution no greater than that seen for the standard. A significantly greater distribution is evidence of heterogeneity.

**VARIATION AT THE \( \alpha \)GPdH Locus IN Colias**

When gel-sieving analysis such as described above was carried out on individuals sampled from natural populations of butterflies, it was immediately apparent that the variation in mobility observed previously reflected more than simple charge differences. If the only source of difference is net charge (presumably produced by amino acid substitutions involving charged residues), then one would expect variants to have similar retardation coefficients, \( K_r \), and to differ primarily in free electrophoretic mobility, \( M_o \). The range of variability in their \( K_r \) values would be expected to be limited to about that observed for the hemoglobin internal standard. In fact, the distribution of \( K_r \) values obtained is very much broader than the corresponding hemoglobin distribution. Noncharge differences clearly contribute to the differences seen in mobility on 7% acrylamide gels.

This proved an important result, as it provided the basis for understanding the apparently continuous variation in mobility consistently seen in samples of natural populations. The difficulty in analysis on 7% acrylamide gels is that charge and size/conformation interact in determining mobility, and these two protein properties prove to vary concordantly—As \( M_o \) values increase, so do the absolute values of \( K_r \) (e.g. bigger or more asymmetric proteins have greater net charge). The re-

---

Summary of CIW-DPB Publication 561.
result is that the mobility functions described by Equation 2 intersect at intermediate gel pore size. The nature of the mobility variation at the α-GPdH locus is now clear (Fig. 44): A survey conducted at 5% acrylamide (equivalent to 10%–11% starch) will not discriminate between variants and will reveal a single, uniform mobility type. Such a survey would classify this locus as uniformly homozygous. A survey conducted at 5% acrylamide, as were my previous surveys, would report two segregating alleles (see, for instance, Johnson, 1972, or Johnson, 1976b), with considerable variation in the exact mobility observed. This mobility variation reflects the fact that there is a minimum of five alleles segregating at this locus.

Gel-sieving analysis thus provides direct evidence of protein heterogeneity within electrophoretic classes. Note also that there are alleles that do not differ in net charge but only in $K_r$.

The Nature and Extent of Heterogeneity

The results reported above raise a variety of interesting questions concerning the nature and extent of electrophoretically detectable protein variation. I will address myself here to four: (1) To what extent is the newly detected variation genetic? Particularly, does variation in $K_r$ have a genetic basis? (2) Does such electrophoretically cryptic variation occur in other organisms than Colias? Do intensively studied groups such as Drosophila also exhibit this class of variation? (3) How much variation of this sort is there? Is it restricted to one or a few loci, or is it typical of most loci? What is the allele frequency distribution of these electrophoretically cryptic alleles? (4) Are the many newly detected variant proteins significantly different in how they function? Particularly important, does a heterogeneous electrophoretic class of uniform (5% T) $R_r$ contain variant proteins with differing $K_r$ or $V_{max}$ values?

The Genetic Basis of Variation in $K_r$.*

When variants of the five allelic classes shown in Fig. 44 are crossed and their progeny analyzed (CIW-DPB Publ. 571), the resulting ratios are consistent with normal Mendelian segregation, indicating that the variants represent true alleles (Table 23): When two individuals presumed homozygous for the same allele are crossed, the progeny exhibit $K_r$ values for α-GPdH with standard errors of approximately 6% of the mean, very similar to the 5% standard error associated with the $K_r$ values of hemoglobin determined from the same gels. In presumptive backcrosses, involving a homozygote and a presumed heterozygote, 1:1 segregation was observed. In the single dihybrid cross, the results were consistent with a 1:2:1 pattern of segregation. It should be noted here that the Class 4 of Table 23 has proven heterogeneous upon subsequent study; this will be discussed below.

When the progeny of a gravid female homozygous for a Class 4 variant of α-GPdH are analyzed, only three clas-

* CIW-DPB Publications 561, 563 and 569.
TABLE 23. Genetic Crosses of a-GPDH Variants of Colias philodice

<table>
<thead>
<tr>
<th>Phenotype Classes of Crosses</th>
<th>Numbers (Phenotype) of Progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \times )</td>
<td>a</td>
</tr>
<tr>
<td>2/3 3/3</td>
<td>18 (2/2)</td>
</tr>
<tr>
<td>4/3 3/3</td>
<td>24 (3/3)</td>
</tr>
<tr>
<td>3/3 4/4</td>
<td>31 (3/3)</td>
</tr>
<tr>
<td>4/4 3/3</td>
<td>0</td>
</tr>
<tr>
<td>2/2 2/2</td>
<td>17 (2/2)</td>
</tr>
<tr>
<td>3/3 3/3</td>
<td>62 (3/3)</td>
</tr>
</tbody>
</table>

*aAfter Johnson, 1976a.

Heterogeneity in Drosophila*

To assess the degree to which commonly employed "homozygous" strains of Drosophila may also carry hidden heterogeneity, gel-sieving analysis has been carried out on several species.

Drosophila pseudoobscura. Gel-sieving analysis of the esterase-5 locus of D. pseudoobscura indicates that at least 14 variants are detected by gel-sieving analysis of seven presumably homozygous esterase-5 lines. These variants are not routinely detected by electrophoresis, as all analyzed individuals yield indistinguishable \( R_f \) values on 5% acrylamide gels. In all, seven allelic classes were examined, and all but one proved heterogeneous.

Drosophila aldrichi. Nineteen lines of D. aldrichi, representing five alleles of the esterase-C locus, were provided by Dr. R. H. Richardson, and their gel-sieving characteristics analyzed. Eleven distinct variants are observed, each presumptive allelic class proving heterogeneous. (Johnson, unpublished).

Drosophila mulleri. Twelve lines of D. mulleri were examined (again provided by R. H. Richardson), representing two alleles of the esterase-C locus. Five variants were detected. (Johnson, unpublished data).

Gel-sieving analysis thus appears to discriminate a significant amount of heterogeneity in each of the three Drosophila species examined to date. It seems quite likely that the finding of extensive electrophoretically cryptic variation will prove a general result in surveys of natural populations.

*CIW-DPB Publ. 563.
Evaluating Patterns of Hidden Heterogeneity*

The discovery of extensive variation among α-GPDH and esterase alleles raises the question of whether these two enzyme loci are typical in this respect. Certain loci, particularly esterases, typically exhibit far more electrophoretically detectable variation than others. Is the newly detected variation concentrated among a particular subset of enzyme loci? To address this question, gel-sieving analyses were carried out on 14 loci of the alpine butterfly *Colias meadii*. For all loci but MdH, several common variants were detected (Table 24). Most of them would not have been detected in routine 7% acrylamide gels, as the associated values of $R_f (7\% T)$ indicate. At all loci, a number of unique variants occur clearly distinct from the common forms. A typical locus, G6PDH, is illustrated in Fig. 45.

The common variants. Among the 14 loci examined, 32 variants occur at frequencies of greater than 10%. Sixteen of the 32 variants would not have been distinguished on 7% acrylamide gels.

*CIW/DPB Publication 569.

### TABLE 24. Electrophoretic Characterization of Common Variants at 14 Enzyme Loci of *Colias meadii*

<table>
<thead>
<tr>
<th>Locus</th>
<th>$R_f$</th>
<th>$K_r$</th>
<th>$M^o$</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenylate Kinase-1</td>
<td>0.20</td>
<td>-0.027</td>
<td>1.11</td>
<td>0.39</td>
</tr>
<tr>
<td>Adenylate Kinase-2</td>
<td>0.29</td>
<td>-0.041</td>
<td>1.15</td>
<td>0.23</td>
</tr>
<tr>
<td>Esterase-1</td>
<td>0.62</td>
<td>-0.083</td>
<td>1.06</td>
<td>0.13</td>
</tr>
<tr>
<td>Esterase-2</td>
<td>0.57</td>
<td>-0.082</td>
<td>1.15</td>
<td>0.26</td>
</tr>
<tr>
<td>Esterase-2</td>
<td>0.32</td>
<td>-0.058</td>
<td>1.41</td>
<td>0.32</td>
</tr>
<tr>
<td>Fumarase</td>
<td>1.6</td>
<td>-0.32</td>
<td>4.67</td>
<td>0.16</td>
</tr>
<tr>
<td>Glucose-6-phosphate</td>
<td>0.60</td>
<td>-0.086</td>
<td>1.16</td>
<td>0.54</td>
</tr>
<tr>
<td>dehydrogenase</td>
<td>0.56</td>
<td>-0.083</td>
<td>1.22</td>
<td>0.24</td>
</tr>
<tr>
<td>α-glycerophosphate</td>
<td>0.33</td>
<td>-0.065</td>
<td>1.52</td>
<td>0.24</td>
</tr>
<tr>
<td>dehydrogenase</td>
<td>0.34</td>
<td>-0.068</td>
<td>1.57</td>
<td>0.32</td>
</tr>
<tr>
<td>Hexokinase-1</td>
<td>0.21</td>
<td>-0.052</td>
<td>1.64</td>
<td>0.44</td>
</tr>
<tr>
<td>Hexokinase-2</td>
<td>0.23</td>
<td>-0.050</td>
<td>1.50</td>
<td>0.36</td>
</tr>
<tr>
<td>Malic enzyme</td>
<td>0.73</td>
<td>-0.146</td>
<td>2.31</td>
<td>0.50</td>
</tr>
<tr>
<td>Malate dehydrogenase-1</td>
<td>0.69</td>
<td>-0.068</td>
<td>0.69</td>
<td>0.43</td>
</tr>
<tr>
<td>Malate dehydrogenase-2</td>
<td>1.66</td>
<td>-0.342</td>
<td>6.21</td>
<td>0.47</td>
</tr>
<tr>
<td>Phosphoglucomutase</td>
<td>0.38</td>
<td>-0.056</td>
<td>1.18</td>
<td>0.32</td>
</tr>
<tr>
<td>Triose phosphate isomerase</td>
<td>0.34</td>
<td>-0.064</td>
<td>1.48</td>
<td>0.19</td>
</tr>
<tr>
<td>Triose phosphate isomerase</td>
<td>0.35</td>
<td>-0.068</td>
<td>1.50</td>
<td>0.21</td>
</tr>
</tbody>
</table>

*The sample represents a single collection from one natural population (after Johnson).*
Fig. 45. Electrophoretic gel-sieving survey of glucose-6-phosphate dehydrogenase (G6PdH) in a natural population of Colias meadii. Values for hemoglobin (Hb) were determined from the same gels, after Johnson, in press (a).

Of the 32 common variants, 30% differ only in charge, 10% differ only in $K_r$, and 70% differ in both $M_o$ and $K_r$. Thus fully 80% of the common variants differ significantly in $K_r$. If differences in $K_r$ reflect conformational differences, as seems likely (the matter is discussed below), then it seems quite unlikely that this widespread variation in shape does not affect the functioning of the enzymes.

The rare variants. Among the 14 loci, a total of 97 "rare" variants occur! In a sample of 20 individuals, 91 variants occur only once and 6 others occur twice. Fully 30% of the genes analyzed in this survey code for proteins that appear only once in the sample. Perhaps these variants all occur typically at frequencies of 5%; or, they may be unique alleles occurring only once. A larger sample is required to resolve this issue. The variants appear allelic: Note that Class 4 of $\alpha$-GPDH discussed earlier is heterogeneous and composed of rare variants. Class 4 variants were observed to segregate in crosses in a Mendelian fashion.

All 14 loci exhibit rare variants. Fully 70% of the rare variants are not detected on 7% acrylamide gels.

Of the 97 rare variants, 34% differ solely in charge, 21% differ solely in $K_r$, and 45% involve differences in both $M_o$ and $K_r$. Thus fully two-thirds of the rare variants involve significant differences in $K_r$. Again, conformational variation seems prevalent.

Other classes of variation. It seems likely that cryptic variation reported in the thermal stability of proteins is quite different from the sorts of variation reported here. These variants may well represent polymorphism for conservative (polar $\rightarrow$ polar; nonpolar $\rightarrow$ nonpolar) amino acid substitutions within the interior of the protein which alter hydrogen bonding and thus affect stability. It is unlikely that such variants would be detected by gel-sieving analysis.

The chances of detecting variation cryptic because of identical net charge (as described by the charge state model of electrophoretic mobility) are much better. A body of data indicate that the pK's of amino acids may be strongly influenced by an amino acid residue's position in a protein (the matter is discussed in detail in Johnson, 1975b). Two proteins with different amino acid substitutions but with the same net charge at one pH would be very unlikely to exhibit identical changes in net charge at a different pH. Thus serial analysis at two different pH's (in my laboratory, 7.0 and 8.9) should reveal any amino acid differences cryptic at one pH because of identical net charge. The finding of substantial microheterogeneity in isoelectric point within $M_o$ classes (Johnson, 1976a) reflects this same underlying heterogeneity.

Are Electrophoretic Classes Functionally Heterogeneous?

Extensive $K_r$ variation, if conformational, suggests that biochemical comparisons of polymorphic alleles may be difficult to interpret unless the alleles
are demonstrably homogeneous. The extensive literature on functional variation within alleles of *D. melanogaster* alcohol dehydrogenase perhaps reflects to some degree undetected structural heterogeneity.

**References**


---

**THE EFFECT OF DEUTERIUM OXIDE ON THE GEL-SIEVING BEHAVIOR OF PROTEINS IN ELECTROPHORESIS**

George B. Johnson

In animal and plant populations extensive genetic variation occurs at enzyme loci. Several papers in this *Year Book* describe variation in the gel-sieving properties of proteins. This variation is too small in magnitude to be ascribed to different states of subunit aggregation, and appears heritable. It is usually discussed as reflecting allelic proteins which differ in conformation, although other interpretations are possible.

Any interpretation of variation in the "conformational" retardation coefficient, \( K_r \), must account for at least three unexpected phenomena:

1. At a given locus, the spacing between the \( K_r \) values of the variants is unexpectedly uniform. For \( \alpha \)-glycerophosphate dehydrogenase (\( \alpha \)-GPDH) in *Colias*, for instance, the values obtained are:

   \[
   0.065, 0.068, 0.071, 0.077, 0.080, 0.003, 0.003, 0.006, 0.003
   \]

2. The value of the step in \( K_r \) magnitude varies from one enzyme to another and is not a function of subunit molecular weight.

3. Hybrid dimer molecules seen in heterozygous individuals are not intermediate in \( K_r \) but rather assume a \( K_r \) similar to one of the parental types.

There are three basic hypotheses that would entail genetically heritable variation in gel sieving behavior while at the same time accounting for these three phenomena:

A. Variation in \( K_r \) may reflect conformational differences. A variety of studies are consistent with this interpretation: (1) When \( \alpha \)-chymotrypsin is made asymmetric, its \( K_r \) value changes significantly (Johnson, 1976). (2) Fibrinogen is a molecule of 341,000 molecular weight which is known to be very asymmetric. Such a molecular weight would produce a \( K_r \) value of \(-0.14\) for a symmetrical molecule, but the observed value is \(-0.065\), indicating that asymmetry is easily detected by this approach. However, because asymmetry may alter \( K_r \) does not imply that all \( K_r \) variation is produced by asymmetry. Nor do conformational interpretations readily account for the uniform spacing seen between variants in the magnitude of \( K_r \).
B. Variation in $K_r$ may reflect changes in the hydration shells of the proteins. Much of the sieving behavior of proteins in electrophoresis may be hydrodynamic and could in principle be sensitive to the diameter of the hydration shell. If substituting a polar amino acid for a nonpolar one (or vice versa) on the surface of a protein altered the hydration shell diameter, then uniform spacing in $K_r$ between variants may easily be explained. This hypothesis would require that single amino acid substitutions produce rather large changes in the volume of the hydration shell (up to 20%).

C. Variation in $K_r$ may reflect alteration in the dissociation constant of dimers, $\alpha \alpha = 2 \alpha$. If the rate is fast and the equilibrium towards dimer formation, then only one band (the dimer) will be seen in electrophoresis. A slight decrease in such a rate has the effect of lessening the fraction of time the protein molecules are in the dimer form. This will decrease the sieving retardation of the protein by the gel fibers in electrophoresis. The perceived result will be a $K_r$ suggesting a smaller molecule. Uniform changes in $K_r$ might reflect polar/nonpolar substitutions in the hydrophobic patch comprising the subunit binding site. This hypothesis would suggest that the magnitude of the uniform change in $K_r$ between variants would be a function of the area of the hydrophobic subunit binding region, and therefore a function of subunit molecular weight.

It is possible to test the suitability of these alternative conceptualizations by altering the conditions of electrophoresis. This paper reports data that permit the rejection of the hydration shell hypothesis.

The hydration shell of a protein is produced largely by the hydrogen binding of uncharged polar surface amino acids with the polar solvent water. A significant increase in the polarity of the water solvent would be expected to contract the water envelope. Thus a protein in 99.8% D$_2$O rather than H$_2$O would exhibit a smaller hydration shell. If the magnitude of $K_r$ reflects the volume of the hydration shell, then electrophoresis in D$_2$O should alter $K_r$. More to the point, two proteins with similar molecular weights but with different $K_r$ values ought to respond differently to D$_2$O, one exhibiting a greater $K_r$ alteration than the other.

Two such similar protein pairs are bovine hemoglobin and Drosophila pseudoobscura esterase-5, or the same hemoglobin and Colias meadii $\alpha$-glycerophosphate dehydrogenase. The subunit molecular weights are 64,500, 66,000, and 65,000, respectively. For each pair the effect of D$_2$O was examined by running eight gels for each individual: 5%, 6%, 7%, and 8% acrylamide in H$_2$O, and 5%, 6%, 7%, and 8% acrylamide in 99.8% D$_2$O. For the D$_2$O runs, all gel reagents and running buffers were made up in 99.8% D$_2$O. The two sets of four gels were run in parallel simultaneously under controlled temperature (10°C), assayed, scanned, and $K_r/M_a$ values calculated for the matched treatments. Data for the two protein pairs are presented in Tables 25 and 26 (with the dissimilar standard protein ferritin).

In Table 27 the differences in $K_r$ produced by D$_2$O are summarized. For both Hb/esterase and Hb/$\alpha$-GPdH, the responses of the two proteins to D$_2$O are precisely parallel, despite very different $K_r$ values. This relationship is illustrated in Fig. 46 for Hb/$\alpha$-GPdH, where all points fall about the axis of symmetry. When the $\alpha$-GPdH results are standardized to the hemoglobin run in the same gels ($\alpha$GPdH) to reveal any pattern of difference from hemoglobin, very little difference is seen as a result of D$_2$O. These results are not consistent with the hypothesis that the $K_r$ differences between the protein pairs reflect hydration shell differences.

It is important to note that changes in $K_r$ do occur, both enzyme and hemoglobin standard exhibiting $K_r$ values 10%–20% more negative. This result is consistent with a conformational interpretation if the initial differences in $K_r$ are produced by strong...
<table>
<thead>
<tr>
<th>No.</th>
<th>Standard</th>
<th>Hb</th>
<th>Esterase</th>
<th>EST</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.055 ± .019</td>
<td>0.203 ± .060</td>
<td>0.080 ± .023</td>
<td>0.081</td>
</tr>
<tr>
<td>2</td>
<td>0.050 ± .008</td>
<td>0.206 ± .055</td>
<td>0.075 ± .015</td>
<td>0.062</td>
</tr>
<tr>
<td>3</td>
<td>0.055 ± .016</td>
<td>0.210 ± .059</td>
<td>0.079 ± .021</td>
<td>0.061</td>
</tr>
<tr>
<td>4</td>
<td>0.054 ± .004</td>
<td>0.205 ± .022</td>
<td>0.078 ± .006</td>
<td>0.060</td>
</tr>
<tr>
<td>5</td>
<td>0.051 ± .008</td>
<td>0.201 ± .033</td>
<td>0.074 ± .004</td>
<td>0.079</td>
</tr>
<tr>
<td>6</td>
<td>0.055 ± .007</td>
<td>0.217 ± .046</td>
<td>0.076 ± .006</td>
<td>0.079</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>No.</th>
<th>Standard</th>
<th>Hb</th>
<th>Esterase</th>
<th>EST</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.052 ± .011</td>
<td>0.201 ± .030</td>
<td>0.075 ± .018</td>
<td>0.080</td>
</tr>
<tr>
<td>2</td>
<td>0.060 ± .005</td>
<td>0.205 ± .024</td>
<td>0.072 ± .009</td>
<td>0.079</td>
</tr>
<tr>
<td>3</td>
<td>0.061 ± .001</td>
<td>0.212 ± .027</td>
<td>0.081 ± .009</td>
<td>0.078</td>
</tr>
<tr>
<td>4</td>
<td>0.060 ± .011</td>
<td>0.235 ± .098</td>
<td>0.083 ± .016</td>
<td>0.079</td>
</tr>
<tr>
<td>5</td>
<td>0.050 ± .008</td>
<td>0.219 ± .062</td>
<td>0.076 ± .007</td>
<td>0.080</td>
</tr>
<tr>
<td>6</td>
<td>0.060 ± .009</td>
<td>0.235 ± .090</td>
<td>0.085 ± .013</td>
<td>0.081</td>
</tr>
</tbody>
</table>

*Numbers represent different individuals. $K_r$ values are determined from four gels of 5%, 6%, 7%, and 8% acrylamide. EST values represent point-to-point standardization of esterase to hemoglobin (this Year Book). Hb: Hemoglobin; Fe: Ferritin.

### TABLE 26. The Retardation Coefficient of α-Glycerophosphate Dehydrogenase from *Colias meadii* Determined in Parallel in H_{2}O and in 99.8% D_{2}O*

<table>
<thead>
<tr>
<th>No.</th>
<th>Standard</th>
<th>Hb</th>
<th>αGPdh</th>
<th>αGPdh</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.049 ± .001</td>
<td>0.202 ± .031</td>
<td>0.060 ± .003</td>
<td>0.068</td>
</tr>
<tr>
<td>2</td>
<td>0.051 ± .008</td>
<td>0.195 ± .015</td>
<td>0.062 ± .009</td>
<td>0.068</td>
</tr>
<tr>
<td>3</td>
<td>0.055 ± .007</td>
<td>0.214 ± .021</td>
<td>0.067 ± .004</td>
<td>0.068</td>
</tr>
<tr>
<td>4</td>
<td>0.053 ± .010</td>
<td>0.193 ± .042</td>
<td>0.063 ± .007</td>
<td>0.066</td>
</tr>
<tr>
<td>5</td>
<td>0.050 ± .003</td>
<td>0.207 ± .055</td>
<td>0.065 ± .010</td>
<td>0.072</td>
</tr>
<tr>
<td>6</td>
<td>0.055 ± .008</td>
<td>0.209 ± .047</td>
<td>0.057 ± .040</td>
<td>0.057</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>No.</th>
<th>Standard</th>
<th>Hb</th>
<th>αGPdh</th>
<th>αGPdh</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.063 ± .004</td>
<td>0.245 ± .089</td>
<td>0.073 ± .016</td>
<td>0.067</td>
</tr>
<tr>
<td>2</td>
<td>0.055 ± .005</td>
<td>0.224 ± .083</td>
<td>0.068 ± .016</td>
<td>0.071</td>
</tr>
<tr>
<td>3</td>
<td>0.061 ± .008</td>
<td>0.231 ± .061</td>
<td>0.075 ± .016</td>
<td>0.071</td>
</tr>
<tr>
<td>4</td>
<td>0.060 ± .011</td>
<td>0.231 ± .059</td>
<td>0.071 ± .010</td>
<td>0.068</td>
</tr>
<tr>
<td>5</td>
<td>0.060 ± .003</td>
<td>0.236 ± .086</td>
<td>0.069 ± .004</td>
<td>0.067</td>
</tr>
<tr>
<td>6</td>
<td>0.057 ± .008</td>
<td>0.232 ± .067</td>
<td>0.071 ± .011</td>
<td>0.070</td>
</tr>
</tbody>
</table>

*Numbers represent different individuals. $K_r$ values are determined from four gels of 5%, 6%, 7%, and 8% acrylamide. EST values represent point-to-point standardization of esterase to hemoglobin (this Year Book). Hb: Hemoglobin; Fe: Ferritin.
TABLE 27. Effect of D

<table>
<thead>
<tr>
<th>Hb No.</th>
<th>Standard</th>
<th>Standard Esterase</th>
<th>EST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drosophila pseudoobscura</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>-0.05</td>
<td>-0.01</td>
<td>-0.06</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>-0.01</td>
<td>-0.04</td>
</tr>
<tr>
<td>3</td>
<td>0.09</td>
<td>0.01</td>
<td>0.08</td>
</tr>
<tr>
<td>4</td>
<td>0.11</td>
<td>0.03</td>
<td>0.06</td>
</tr>
<tr>
<td>5</td>
<td>-0.02</td>
<td>-0.01</td>
<td>0.03</td>
</tr>
<tr>
<td>6</td>
<td>0.10</td>
<td>0.02</td>
<td>0.12</td>
</tr>
<tr>
<td>Colias meadii</td>
<td>αGPDH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.19</td>
<td>0.21</td>
<td>0.22</td>
</tr>
<tr>
<td>2</td>
<td>0.08</td>
<td>0.15</td>
<td>0.10</td>
</tr>
<tr>
<td>3</td>
<td>0.11</td>
<td>0.08</td>
<td>0.12</td>
</tr>
<tr>
<td>4</td>
<td>0.13</td>
<td>0.20</td>
<td>0.13</td>
</tr>
<tr>
<td>5</td>
<td>0.20</td>
<td>0.14</td>
<td>0.19</td>
</tr>
<tr>
<td>6</td>
<td>0.08</td>
<td>0.11</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.23</td>
</tr>
</tbody>
</table>

*Data are presented as the fractional difference ρ observed in K, e.g., K, (H₂O) + K, (H₂O) • ρ = K, (D₂O).

localized binding forces. It is also consistent with a subunit dissociation hypothesis: As mol wt's are similar, the size of the subunit binding sites should also be similar and therefore equally sensitive to the polarity of the solvent.

The effect is variable from one Colias individual to another. This variability is of some interest because the standard hemoglobin also varies in its response to D₂O. When run alone (no extract), there is no significant variation in the effect of D₂O on hemoglobin. When run with Colias extracts, on the other hand, hemoglobin exhibits great variation in the magnitude of its response to D₂O. Clearly, some physiological variable is important in the D₂O effect.

Finally, D₂O is seen to produce large positive changes in Mᵣ. Such a relationship is expected, as isotope effects alter hydrogen ion dissociation constants. The effect is to increase the net negative charge in alkaline buffer and thus to increase Mᵣ. While hemoglobin and α-GPDH have different Mᵣ values, the effect of D₂O is the same for both molecules because D₂O acts on all charged residues and not simply on the small fraction that constitutes the net difference in charge.

These experiments with D₂O permit rejection of the hypothesis that variation in Kᵣ reflects variation in the hydration shell of proteins. They do not, however, serve to distinguish between the conformational and subunit dissociation hypotheses. Entirely different lines of experimentation will be required to do this. The most obvious approach is to obtain purified protein from each of several Kᵣ variants and to determine whether varying the protein concentration affects the magnitude of Kᵣ. A subunit dissociation hypothesis would predict a significant dependence, while a conformational hypothesis would not. Such experiments are currently being initiated.

**Reference**

Studies of P700-chlorophyll-protein complexes have continued this year, and the results may be divided into two sections. The first describes some preliminary attempts to isolate these complexes from different kinds of algae. The second deals with fluorescence and light-induced absorbance changes of a highly enriched P700 complex from spinach chloroplasts and leads to a new interpretation of electron transfer through the photosystem I reaction center.

**ISOLATION OF CHLOROPHYLL-PROTEIN COMPLEXES FROM DIVERSE ALGAE**

Among the green algae, *Chlamydomonas* has been a frequent choice of study because it has a known sexual life-cycle which makes genetic analysis possible and because it is easily cultured and broken. CPI\(^*\) has been isolated from this alga (Kan and Thornber, 1976) and appears to be similar to that from higher plant leaves. Chlorophyll-proteins enriched in P700 have also been isolated from various blue-green algae (Thornber, 1975).

In the present study, various microalgae were grown in liter batches under appropriate conditions, harvested by centrifugation, washed and broken with a French pressure cell. The fragments were washed to remove much of the soluble protein, pelleted, and stored in a freezer. If cells are harvested and frozen before breaking, phaeophytinization of some of the chlorophyll may occur during early steps of the following procedure and prevent recovery of the reaction center.

The method of Shiozawa et al. (1974) was followed for the preparation of CPI except that 20 mM sodium ascorbate was omitted from the washing solutions. A pellet containing a measured amount of chlorophyll was homogenized in sufficient 1% Triton X-100 to give a Triton-to-chlorophyll ratio of about 75 (w/w). CPI was eluted in 0.2 M sodium phosphate, pH 7.5, except for *Euglena* as noted below.

The yellow-green algae, which contain only chlorophyll \(a\) of the photosynthetic pigments, have been little studied. After some trial and error, we chose from this group two species that grow readily in batch culture and can be broken by one passage through a French pressure cell. These species are *Bumilleriopsis filiformis* (No. 309, Culture Collection of Algae, Indiana University) and *Botrydiopsis* sp. (AB-2A-ADN, kindly supplied by Professor Ralph Lewin, Scripps Institute of Oceanography, University of California at San Diego). Preliminary experiments with both of these species indicate that a fraction enriched in P700 can be eluted in 0.2 M phosphate. Thus far, the lowest molecular ratios of chlorophyll to P700 obtained were 41 for *Bumilleriopsis* and 80 for *Botrydiopsis*. It is to be expected that both these ratios can be lowered by further experimentation.

Considerable effort was spent in attempting to isolate a P700-chlorophyll \(a\)-protein from *Euglena gracilis* (No. 752, Culture Collection of Algae, Indiana University). Eventually it was discovered that the fraction most enriched in P700 is eluted from the hydroxylapatite column by the 10 mM phosphate normally used as a washing solution and is therefore gone by the time the 0.2 M phosphate is applied. This 10 mM phosphate fraction usually had a chlorophyll to P700 ratio of 60–70, but in one experiment this ratio was lowered to 46 by a repeated chromatography on hydroxylapatite.

A second result of the *Euglena* fractionation was the finding that the 0.2 M phosphate eluate contained another chlorophyll \(a\)-protein (CP\(a_1\)) having the unique absorption spectrum shown in *Year Book 73*, p. 703 (1974) and

---

CPI = P700-chlorophyll \(a\)-protein.

---
without detectable P700. Not only is the appearance of a 684-nm absorption band unusual, but the steepness of the slope on the long wavelength side of this band is also remarkable. This year the corresponding fluorescence emission spectrum of this fraction was measured. The emission maximum is at 683–684 nm at 20°C and 687 nm at -196°C. At both temperatures this emission band is unusually narrow (half-band width ~ 12 nm at -196°C). This is about 60% as wide as emission bands in other comparable chlorophyll complexes in vivo. These unusual characteristics have been observed in CPα preparations from at least five separate experiments and do not seem to have any obviously trivial explanation.

Previously Shiozawa et al. (1974) reported that P700 and cytochrome f were found in equimolar amounts in CPI prepared from tobacco leaves. However, this relationship does not mean that they are associated. Difference spectra between samples oxidized by ferricyanide and reduced by ascorbate have been measured from different eluates from Euglena chromatography and with CPI preparations from spinach chloroplasts. The amounts of cytochrome f and P700 can be calculated from the differences in absorption at 552 and 697 nm. Occasionally an equimolar amount of P700 and cytochrome f was observed in spinach CPI, but usually the proportion of P700 increased and cytochrome decreased following repeated chromatography. The Euglena CPI eluted in 10 mM phosphate did not show any evidence of a cytochrome in its difference spectrum. Furthermore, measurement of light-induced absorbance changes of a spinach CPI preparation (described below) gave no evidence of a change near 550 nm characteristic of a cytochrome coupled to P700 oxidation. All of this evidence suggests that cytochrome f is probably a contaminant in CPI preparations made according to Shiozawa et al. (1974) and is not bound closely to P700 as an electron donor.

**Absorption and Fluorescence of CPI**

Fluorescence emission spectra of spinach CPI were published in last year's Report (Year Book 74, p. 779). Even at that time, considerable variability had been encountered when measuring different preparations, particularly at low temperature. We now understand some of the reasons for that variability and have improved procedures for preparing CPI routinely with a chlorophyll α to P700 ratio of 30 to 35.

The main problem seems to have been the tendency of CPI to aggregate on the hydroxylapatite column, in solution and especially during freezing. Recently it has been found that this problem can be alleviated by making the 0.2 M phosphate eluting buffer 0.02% in Triton X-100. This small amount of detergent does not modify the spectral characteristics of the chlorophyll-protein complex itself.

Figure 47 shows the fluorescence emission and Fig. 48, the absorption at -196°C of CPI preparations having different ratios of chlorophyll to P700. All samples were dilutions of 0.2 M phosphate eluates from hydroxylapatite
columns. The only difference between them was the proportion of light-harvesting or antenna chlorophyll a which had been removed from the reaction center complex by repeated chromatography. In numerous experiments CPI has been prepared from spinach chloroplasts and rechromatographed several times to give different chlorophyll to P700 ratios; so far 30 has been the lowest ratio observed.

Figure 47 illustrates the difference between CPI preparations as a function of their P700 concentration. As the chlorophyll to P700 ratio decreases from 100 to 30, a shoulder near 695 nm on the side of the 685 nm band increases until it becomes the maximum. Also, as the proportion of antenna chlorophylls to reaction centers decreases, the fluorescence yield also decreases because more of the actinic light energy is funneled directly to a reaction center.

The amount of fluorescence near 670 nm varies in different preparations and is thought to come from a small amount of highly fluorescent Triton-solubilized chlorophyll a. The longer wavelength bands around 720–730 nm may be due to lower vibrational bands of the biological forms of chlorophyll a. It can be seen in Fig. 48 that there is a small redshift in chlorophyll absorption with increased P700 concentration. The amount of this shift does not vary consistently with a change in P700 concentration, but the shoulder near 670 nm always decreases as the proportion of antenna chlorophyll decreases. Therefore, the differences in relative proportion of the long wavelength emission bands (720–730 nm) may reflect changes in proportion of the 670-nm and 684-nm forms of chlorophyll a. We have not yet been able to do detailed curve analysis as in previous years because of a change in computer hardware at Stanford University.

This rather detailed discussion of the differences in fluorescence and absorption spectra has been given to show why it is unlikely that the small differences in absorption that occur when the relative concentration of P700 is increased can account for the large increase in emission near 695 nm. It seems very probable that this emission is related in a direct way to P700. The difficulty, of course, is to determine how a pigment absorbing at 700 nm could fluoresce at shorter wavelengths.

One very reasonable explanation comes from the observation first made by Kok (1956) that the negative band near 700 nm in light-minus-dark difference spectra of various algae did not have the normal shape of an absorption band but was skewed, being steeper on the shorter wavelength side. If this band was made symmetrical and the experimental curve subtracted from it, a new absorption band with a lower extinction coefficient appeared at about 690 nm. This experiment has now been repeated with CPI, and the hypothetical absorption band occurs at 687 nm. Lozier and Butler (1974) and Visser and Rijgersberg (1975) have measured the light-minus-dark difference spectra of chloroplasts at −196°C. In both cases an increase in absorption near 690 nm was observed when P700 was photo-
oxidized. Therefore, it could be oxidized P700 absorbing near 690 nm that fluoresces near 695 nm.

If this were strictly true, one might expect to see a rise in 695-nm fluorescence that is kinetically identical to P700 bleaching. Ulrich Schreiber looked for such corresponding changes both at room and low temperature in CPI preparations. He did not see the predicted kinetic change in fluorescence but only the effect of the transmission change and reabsorption of fluorescence when P700 was bleached. It was therefore necessary to look further for an explanation of the 695-nm fluorescence.

A suggested explanation was found in the paper by Lozier and Butler (1974), who were attempting to explain both the positive absorbance change near 690 nm and the fact that only a part of the P700 absorbance change is reversible at low temperature. They proposed a formulation similar to the following:

\[
[P \cdot P] \cdot Y \xrightarrow{hv} [P^+ \cdot P^-] \cdot Y \\
[P^+ \cdot P] \cdot Y^-
\]

where \(P\) is a chlorophyll molecule in a P700 dimer and \(Y\) is an electron acceptor. The species \([P^+ \cdot P^-]\) would be the candidate for the 690-nm absorption band and, in the present case, the source of 695-nm fluorescence. This scheme can account for the lack of fluorescence yield change in parallel with the light-induced bleaching and also for a low extinction coefficient of the 690 absorption band because one of the pair of chlorophylls is oxidized or bleached.

Recently Katz et al. (1976) found strong evidence from line-width studies of the EPR signal caused by the photooxidation of P700 that the reaction center must indeed be a dimer or, as they prefer to say, a "special pair" of chlorophyll molecules. They also suggest a formulation for the electron transfer from P700 to the acceptor \(Y\) that is almost identical to the above.

The only major difference is that the Argonne group believes there may be a water molecule bound between the chlorophyll molecules in the "special pair." However, an equally valid hypothesis is that it is the electron acceptor \(Y\) and the protein which serve to bind and orient the chlorophyll dimer in a particular way that is necessary to make the charge separation energetically feasible.

The natural electron acceptor is probably a type of bound ferredoxin or iron-sulfur protein (Malkin, 1975). Nelson et al. (1975) have also been studying the relationship between P700 and the iron-sulfur protein in reaction center complexes using EPR spectroscopy. They were able to measure the signal from P700 oxidation after the signal from the iron-sulfur protein had disappeared following special treatment. This experiment indicated that the iron-sulfur protein is not the primary electron acceptor. The above formulation also accounts for their results, since in this case one of the chlorophyll molecules is the primary acceptor.

**Absorption Change Kinetics**

Absorption change kinetics in the millisecond time range were measured in a CPI sample having a chlorophyll to P700 ratio of about 40. These experiments were performed in collaboration with David Fork, using equipment described in Year Book 71, p. 183. We did not average a series of signals as we did last year because the dark reduction of the photooxidized P700 by ascorbate was very slow \((t_d \approx 1 \text{ min})\). The change from a single flash was displayed on an oscilloscope and then transferred to an X-Y recorder.

A light-minus-dark difference spectrum was measured using a saturating actinic light of 1.4 sec duration and 2-min dark time between exposures. The sample contained approx. 30 mM sodium ascorbate but no other artificial electron donors or acceptors. The dif-
ference spectrum was similar to that reported last year (Year Book 74, pp. 779–783) with negative peaks at 700 and 430 nm, typical of P700. However, there was no shoulder near 420 nm or change near 550 nm that would have indicated the photooxidation of cytochrome f in this CPI preparation (see discussion of cytochrome f in Euglena section above).

While measuring the difference spectrum, we again noticed biphasic recovery kinetics that last year were attributed to some involvement of cytochrome f. However, further investigation has now revealed that the proportion of the rapid recovery phase \( t_1 \sim 35 \) msec depends upon the length of the light exposure. The curves at the top of Fig. 49 show that after a 60-msec exposure, 82% of the P700 was reduced rapidly, whereas after 680 msec only 23% was reduced rapidly. The percentage of rapid recovery was measured for a number of different exposure times, and these results are plotted on the lower half of Fig. 49. It is apparent that after about 100 msec, the rapidly recovering component begins to disappear.

An extension of the same formulation shown above to explain the fluorescence results can also be used for these kinetic measurements:

\[
\begin{align*}
[P+] \cdot Y & \xrightarrow{hv} [P^+ \cdot P^-] \cdot Y \rightarrow [P^+ \cdot P^-] \cdot Y^- \xrightarrow{Q_o} [P^+ \cdot P] \cdot Y \\
\end{align*}
\]

We assume that in this CPI preparation the natural electron acceptor, \( Y^- \), can react back with \( P^+ \) with a half-time of about 35 msec. But during a longer exposure time, \( Y^- \) is oxidized by \( O_2 \), and then \( P^+ \) can only be reduced by ascorbate. Malkin (1975) reported the light-dependent electron transfer from ascorbate to \( O_2 \) with the half-time of \( \sim 1 \) min by a similar CPI preparation.

This model formulation suggests several experiments to test the hypothesis further, e.g., to test the effect of anaerobiosis and viologen dyes upon the kinetics of the chlorophyll absorption changes. It also predicts that the light-dark difference spectrum measured after a flash too short for the transformation of \([P^+ \cdot P^-]\) to \([P^+ \cdot P]\) would show only the symmetrical band of bleached P700 and not be skewed by the 690 band.

The model that Lozier and Butler (1974) originally proposed accounts for the partial reversibility of the absorbance change at \(-196^\circ C\) by the back reaction of \( P^+ \) and \( P^- \). It would also be expected that the difference spectrum of the reversible change would show only the symmetrical bleached band, whereas the spectrum of the irreversible change would be skewed by the
formation of the 690 band. Such spectral differences were actually observed by Mayne and Rubinstein (1966) when they measured absorption changes in *Anacystis nidulans* at -196°C.

The new data presented here on the spectral characteristics of P700–chlorophyll α-protein, as well as the reinterpreted data of several other experimenters, provide strong support for the concept that the reaction center of photosystem I is a chlorophyll dimer. Light energy may effect a charge separation within this special pair of chlorophyll molecules; one becomes oxidized and the other reduced. The reduced chlorophyll may in turn reduce another electron acceptor that is probably a bound iron-sulfur protein. In vivo, the oxidized chlorophyll of the dimer is probably reduced by an electron from photosystem II passed through plastocyanin and cytochrome f, but in CPI preparations this electron must come from an artificial donor such as ascorbate.

**References**


**TEMPERATURE DEPENDENCE OF CHLOROPHYLL α FLUORESCENCE IN ALGAE AND HIGHER PLANTS IN RELATION TO CHANGES OF STATE IN THE PHOTOSYNTHETIC APPARATUS**

*David C. Fork*

**INTRODUCTION**

Measurements made last year (Year Book 74, pp. 766–776; Murata et al., 1975; Murata and Fork, 1975) of fluorescence as a function of temperature in the blue-green alga *Anacystis* treated with DCMU to eliminate the influence of photosynthetic photochemical reactions on the fluorescence yield revealed maxima in fluorescence yields that changed depending upon the temperature used to grow the algae. These maxima were found to reflect a change in the physical phase of the lipids in the photosynthetic membrane and occurred at approximately 13° or 24°C in the organisms grown at 28° or 38°C, respectively. The activation energies of several photosynthetic reactions were found to change at the temperature of phase transition.

In this investigation fluorescence versus temperature curves were measured over a wide range of temperatures and for a number of algae and higher plants adapted both to warm and to cool growth conditions and for chloroplasts and a chlorophyll-protein preparation. The resulting curves were complex and varied widely, depending upon the growth temperature of the organism in question. Some parallel measurements of the photochemical
activity versus temperature were also made, and correlations could be seen between the changes of fluorescence yield and photosynthetic activities.

**TEMPERATURE DEPENDENCE OF CHLOROPHYLL a FLUORESCENCE IN ALGAE**

The thermophilic unicellular alga *Cyanidium caldarium* inhabits acidic hot springs where it has been found growing at temperatures up to 56°C. This organism grows best at a pH between 2 and 3. Although this alga contains the chromoprotein phycocyanin, as do the blue-green algae, it nevertheless has a morphological and cytoplasmic organization more like a red alga (Doemel and Brock, 1971).

Figure 50 shows the fluorescence versus temperature (F-T) curve measured for *Cyanidium* grown at 45°, 39°, and 21°C. In these and other measurements of the F-T curves, 10⁻⁵ M DCMU [(3-(3',4'-dichlorophenyl)-1,1-dimethylurea)] was added to prevent the effect of photosynthetic photochemical reactions on the fluorescence yield. For cells grown at 45°C, broad maxima were seen near 37.5° and 10°C upon lowering the temperature. These peaks were much more pronounced when the temperature was immediately increased after attaining 1°C and were separated by a clear minimum near 27.5°C. Increasing the temperature still further gave rise to a broad shoulder centered around 47°C and minima at 60° and 65°C, a small maximum near 63.5°C, and a large peak in fluorescence emission at 67°C. Above this temperature the fluorescence decreased sharply.

Upon lowering the temperature of *Cyanidium* grown at 39°C, a broad maximum was seen near 9°C; and upon increasing the temperature, conspicuous maxima appeared at 8°, 30°, 62°, and 67°C and minima at 21.5°, 57°, and 65°C. The F-T curve for cells grown at 21°C shows less distinct maxima around 2°, 22°, 55°, and 65°C and minima near 19° and 38°C, as well as at

![Fig. 50. Temperature dependence of chl a fluorescence in *Cyanidium caldarium* grown at 45°, 39°, and 21°C upon excitation of chl a with 440 nm actinic light (500 ergs cm⁻² sec⁻¹). Fluorescence at 685 nm was measured continuously as the temperature was decreased and increased at the rates of 0.5° and 1°C/min, respectively. The sample was held in a 5-mm thick brass cuvette whose top was covered by a Lucite window and bottom surface soldered to a 100 ml insulated brass container through which circulated 100 ml of coolant fluid DCMU, 10⁻⁵ M.](image)
Heating cells above the high-temperature fluorescence peak leads to irreversible loss of fluorescence variations as a function of temperature. Figure 51 shows an example of this for *Cyanidium* grown at 39°C and heated to 67°C and then cooled immediately to 39°C. It can be noted that the *F-T* curve upon cooling lacks the previously seen maxima and minima.

The *F-T* curves for *Anacystis* grown at 39° and 22°C are given in Fig. 52. Fluorescence maxima appeared near 13°C when the temperature was lowered and around 19°C when the temperature was raised. In the culture grown at 22°C the maximum appeared at 6°C upon lowering the temperature and 10°C upon increasing the temperature. Maxima appeared near 55°C for both cultures. Similar results were reported previously for *Anacystis* grown near these temperatures. (*Year Book 74*, pp. 766–776; Murata et al., 1975).

The *F-T* curve of a high-temperature strain of *Chlorella* (Sorokin and Myers, 1953; Sorokin, 1967), which has its optimum temperature for growth near 39°C, showed a maximum, as did *Anacystis*, around 55°C (Fig. 52). Unlike *Anacystis*, however, it did not show maxima near 13° or 20°C. It seems that a fluorescence maximum will occur at temperatures somewhat below 0°C. Cells of the high-temperature strain of *Chlorella* that had been heated to 60°C and cooled again produced a flat fluorescence versus temperature curve with none of the previously seen maxima or minima.

A number of *F-T* curves were measured for intertidal marine algae. The red alga *Iridaea* had a complex curve with maxima at 37°, 46°, and 51.5°C and minima at 30°, 43.5°, and 48°C. The green alga *Ulva* did not show the complicated curve of *Iridaea* but had a
steep decline of fluorescence with increasing temperature, with a shoulder around 32°C. The brown alga Laminaria also showed a steep decline of fluorescence with increasing temperature, with an inflection near 38°C.

The red alga Porphyra perforata was used to compare the behavior of fluorescence and photosystem II activity. This alga inhabits the upper reaches of the tidal zone and undergoes repeated cycles of drying and rehydration with tidal changes. It was found previously (Year Book 72, pp. 384–388) that dried Porphyra lost its photosystem II activity upon drying but still retained measurable photosystem I activity (P700 oxidation). However, the photosystem II activity was restored immediately when the dried alga was rehydrated with seawater.

The ratio of variable fluorescence to maximum fluorescence yield ($F_v/F_{max}$) was measured in Porphyra as a function of increasing temperature. This ratio has been proposed (Duysens and Sweers, 1963; Butler and Kitajima, 1975) to be a measure of the photochemical activity in photosystem II. Figure 53 shows a plot of $F_v/F_{max}$ and $F_{max}$ versus temperature in Porphyra treated with $10^{-5} \text{M} \text{DCMU}$. The $F_v/F_{max}$ ratio (Fig. 53B) decreased with increasing temperature above 20°C. At temperatures above 37.5°C the $F_v/F_{max}$ ratio declined steeply and the $F_{max}$ curve (Fig. 53A) increased rapidly. The $F_v/F_{max}$ ratio reached zero when the $F_{max}$ curve had attained its maximum.

Figure 53C illustrates that features of the $F-T$ curve seen previously were lost when a dried thallus was measured. After reconstitution of the thallus with seawater, the $F-T$ curve showed features noted previously (Fig. 53D).

Temperature Dependence of Chlorophyll a Fluorescence in Higher Plants

The $F-T$ curves measured for a number of higher plants leaves are presented in Fig. 54. A leaf from "winter" spinach had minima at 31° and a maximum at 42.5°C (Fig. 54A). Chloroplasts prepared from spinach leaves by contrast showed only an inflection at 31°C and a faint shoulder around 43°C (Fig. 54B). Chloroplasts prepared from winter spinach were used to measure the photochemical ac-
activity of photosystem II as a function of temperature from 10° to 46°C as the $F_o/F_{max}$ ratio described above. The activity of photosystem II declined slowly with temperature to about 30°C and declined steeply thereafter. This decline corresponded to the beginning of the steep fluorescence increase with temperature of $F_{max}$ (Fig. 54C). In another experiment the ratio of $F_o/F_{max}$ in a spinach leaf harvested in the spring after freezing conditions had ended showed an abrupt change of slope at a somewhat higher temperature (35°C).

In sunflower leaf (Fig. 54 D) the minimum appeared at 41.5° and a maximum at 46°C, with a small inflection around 52°C. In a pea leaf (not shown) an indistinct minimum was seen near 38°C and a peak near 46°C. In tomato (Fig. 54 E) fluorescence increased as the temperature was lowered to 5°C. Increasing the temperature from this point resulted in a decline of fluorescence to a minimum at 35.5°C and a subsequent increase to a maximum at 46°C. Cooling the leaf again after passing over the 46°C peak produced a fluorescence to temperature curve that increased with increasing temperatures and did not show the characteristic features seen previously. Although no low-temperature fluorescence maximum appeared in the tomato curve, it appears that a maximum could be seen at slightly lower temperatures, perhaps near or below 0°C.

Chlorophyll a Protein from Spinach

The temperature dependence of fluorescence was measured for a chlorophyll protein preparation made from spinach that contained a chlorophyll a/P700 ratio of about 50. Some characteristic properties of this preparation are described elsewhere in this Report. The temperature dependence of this preparation is shown in Fig. 55. Unlike spinach chloroplasts, it has a broad minimum near 40°C and a maximum around 63°C.

This survey of the variation of fluorescence as a function of temperature in a number of plants having distinct pigmentation and growing under different conditions reveals several common features.

The fluorescence maxima seen at 13°C upon decreasing and at 19°C upon
increasing the temperature in *Anacystis* grown at 22°C were attributed last year (Year Book 74, pp. 766–776; Murata et al., 1975; Murata and Fork, 1975) to a thermal transition of the physical phase of membrane lipids. The maxima occurred near the temperatures where the transition of the phase of membrane lipids changed from the liquid crystalline to the mixed, solid–liquid crystalline states. In *Anacystis* we found that the Arrhenius plots of reduction rates of P700, of oxygen evolution, and of the configurational change of the thylakoid membrane that are related to the state 1 and state 2 shifts (Murata, 1969, 1970; Bonaven­tura and Myers, 1969) were composed of two straight lines with breaks near the phase transition temperatures as determined by electron spin resonance or by the appearance of fluorescence maxima. Activation energies were always lower above the transition temperature and higher below the transition point.

The fluorescence maxima seen in *Cyanidium* near 10°C for cells cultured at 45°C, and near 8°C for cells cultured at 39°C, and from 0° to 5°C in cells cultured at 21°C also appear to represent similar phase changes in this alga. As is the case with *Anacystis*, the lipid composition of *Cyanidium* changes with growth temperature. The ratio of unsaturated to saturated fatty acids has been found to decrease threefold with increasing growth temperature. Cells cultured at 20°C contained 30% of their fatty acids as linoleic acid. This fatty acid was not found in cells grown at 55°C (Kleinschmidt and McMahon, 1970a, 1970b). Studies are in progress with *Cyanidium* to see if, as in *Anacystis*, fluorescence maxima represent lipid phase changes that can also be correlated to changes of photosynthetic reactions.

It was expected that the high temperature strain of *Chlorella* might show a phase change like those seen for *Anacystis* cultured at 39°C. Perhaps the phase change as detected by the appearance of a fluorescence maximum may occur at temperatures near or slightly below 0°C, and the lipid composition of *Chlorella* adapted to high temperatures will be found to be different from that of *Anacystis* or *Cyanidium*.

Most of the plants examined exhibited a high-temperature fluorescence maximum. At temperatures above this maximum irreversible effects were produced such that the previously observed variations of fluorescence with temperature were lost. Lavorel (1969) has observed similar maxima in fluorescence yield in the green algae *Chlorella* and *Scenedesmus* heated at a rate of 0.7°C/min. The rise began at about 42°C, peaked near 52°C, and then declined at higher temperatures.

The high-temperature fluorescence peak was not invariably seen, however. It could not be detected in *Ulva* and was seen only as a shoulder in *Laminaria*. It was likewise indistinct in pea leaf, and chloroplasts from spinach showed only a minor inflection where a peak was seen in the leaves. It may be, as discussed previously (Year Book 74, pp. 751–759), that the high-temperature fluorescence peak, particularly as seen in leaves, represents some alteration in the scattering or absorption properties produced by heat damage of cells and chloroplasts of the leaves. This explanation, however, does not explain the 55°C maximum
seen in the blue-green alga *Anacystis*. These organisms, like the bacteria, have a primitive cellular organization. They lack vacuoles and do not have lamellae organized into grana stacks as do higher plants but have them distributed throughout the entire cell. It may be that the fluorescence peak seen at high temperatures represents the irreversible denaturation of a pigment-protein complex. The disappearance of variations of fluorescence as a function of temperature after heating above the high-temperature fluorescence maximum is consistent with this idea. In addition, the system I chlorophyll-protein preparation that has only a trace of lipid (Thornber, personal communication) also showed a high-temperature fluorescence peak. The maximum at 63°C seen in this preparation is consistent with many observations that system I is more resistant to high temperatures than is system II.

The high-temperature fluorescence maximum did not change much with growth temperature in *Anacystis* or *Cyanidium*; however, the smaller satellite peak at temperatures below the high-temperature fluorescence peak did vary with growth temperature in *Cyanidium*.

Most of the leaves examined had a high-temperature peak around 46°C, as did leaves of *Atriplex sabulosa*, which is native to a cool habitat (*Year Book* 74, pp. 751–759). Exceptions to this observation were seen in winter spinach (maximum at 42.5°C) and in *Tidestromia oblongifolia* native to a hot habitat (peak near 57°C) (*Year Book*, 74, pp. 751–759).

In this study the loss of photosystem II activity as revealed by the steep drop in the $F_F/F_{max}$ ratio in the red alga *Porphyra* and in spinach chloroplasts seemed to be correlated with the beginning of the steep rise of fluorescence to its high-temperature maximum. This was also seen earlier in a leaf of *Atriplex*; but in *Tidestromia*, damage to photosynthesis was seen already around 46°C, whereas the beginning of the high-temperature fluorescence rise occurred near 53°C (*Year Book* 74, pp. 751–759).

A decline of photosynthetic activity with increasing temperature was observed by Mukohata *et al.* (1973), who showed that ferricyanide reduction in spinach chloroplasts was inhibited at temperatures higher than about 40°C. Krause and Santarius (1975) noted that the rate of rise of variable fluorescence in the light was slowed down by mild heat treatment. This rate dropped rapidly at temperatures above 40°C, as did the activity of cyclic photophosphorylation.

Santarius (1974) found seasonal changes in plant membrane stability to heat treatment. In thylakoid membranes isolated from frost-hardy spinach plants harvested in November, the decline of the Hill reaction measured as ferricyanide reduction began at temperatures above about 40°C. Surprisingly, the Hill reaction and cyclic photophosphorylation in thylakoid membranes isolated from summer spinach were found to be more sensitive to heat treatment, the decline in activity of both beginning around 30°C, similar to that observed here for the $F_F/F_{max}$ ratio.

**Acknowledgment**

The chlorophyll protein preparation used in this study was kindly provided by Dr. Jeanette S. Brown.

**References**


**CORRELATION BETWEEN GROWTH TEMPERATURE AND HEAT-INDUCED CHLOROPHYLL FLUORESCENCE CHANGES IN Scenedesmus obliquus**

Ulrich Schreiber

**INTRODUCTION**

Chlorophyll fluorescence is a sensitive intrinsic probe to determine the efficiency of quantum conversion in photosynthesis. The fluorescence yield in chloroplasts or algae is primarily determined by the rate of charge separation at photosystem II reaction centers. But substantial yield changes can also be effected by changes in other radiationless de-excitation processes, such as energy transfer, including that to photosystem I (spillover), quenching by molecular oxygen, and heat dissipation. Both PS II reaction centers and antenna pigments are embedded in the fluid mosaic of the thylakoid membrane. The properties that determine fluorescence yield are correlated with the state and intactness of this membrane. Murata et al. (1975) and Murata and Fork (1975) have demonstrated a correlation between maximum fluorescence yield in *Anacystis nidulans* and a thylakoid membrane fluidity change in the 10°–25°C region, adaptable to growth temperature. We have reported previously on large increases (up to 400%) in fluorescence yield with a jump from room temperature to 45°–55°C in a variety of plants (Schreiber et al., 1975; Schreiber et al., 1976). In those studies, the heat-induced fluorescence increase was most pronounced in plants from a low-temperature environment, suggesting a correlation between heat stability and heat-induced fluorescence increase.

In the present study, a unicellular alga, *Scenedesmus obliquus*, was grown at 20° and 28°C. It was found that fluorescence properties at 45°–55°C are substantially altered by a change in growth temperature. Included are changes in fluorescence yield with slow heating and changes in fluorescence induction with a temperature jump (T-jump). It will be shown that there is a close correlation between these fluorescence changes monitored at high temperatures and heat damage in the vicinity of PS II centers, assayed after returning the algae to room temperatures.

**METHODS**

*Scenedesmus obliquus* (Indiana Culture Collection) was grown either at
samples were dark-adapted for 2 hr at room temperature (20°-23°C) before the experiments.

The apparatus for rapid temperature changes (95% of a 25°C change in 2 sec) and fluorescence measurements was essentially the same as described earlier (Schreiber et al., 1976). The method was improved by using solenoid valves to regulate cold and hot water flow through the cuvette. Usually, very dilute cell suspensions were used with approximately 5 x 10³ cells/μl. Fluorescence curves were corrected for stray excitation light reaching the photomultiplier.

RESULTS

Slow Heating Curves

Temperature and temperature adaptation can be expected to affect a great number of photosynthetic partial reactions, all of which will more or less directly determine fluorescence yield. In order to study the direct effect of temperature and temperature adaptation on the state of Chl a and PS II reaction centers in the thylakoid membrane via fluorescence measurements, it is necessary to eliminate the influence of photosynthetic dark reactions on fluorescence. Such influences are minimized in two extreme situations. The first is that of all PS II centers being closed, e.g., in the presence of DCMU in continuous strong light, with fluorescence displaying its maximum level, Fₘₐₓ. The second is that of all PS II centers being open, e.g., in an extremely weak fluorescence measuring beam, yielding minimum fluorescence, F₀.

In practice it is difficult to assure complete inhibition of fluorescence quenching in Fₘₐₓ measurements. Even in the presence of DCMU there is fluorescence quenching via molecular oxygen, spillover, and the back-reaction at PS II reaction centers. It can be expected that these quenching mechanisms show some temperature dependency. For example, it is known that the PS II back-reaction is stimulated substantially at higher temperatures (Bennoun, 1970). On the other hand, it becomes irreversibly blocked at 45°-55°C (see section below). To exclude this factor NH₄OH, which prevents the back-reaction, was added for Fₘₐₓ measurements. In F₀ measurements it is difficult to keep Q fully oxidized with extended periods at elevated temperatures, where O₂ uptake is stimulated and an endogenous electron donor, possibly related to hydrogenase, is induced when conditions become anaerobic (Gaffron, 1944; Schreiber et al., 1976). Use of extremely dilute algal suspensions retards the development of anaerobic conditions.

With these precautions, Fₘₐₓ and F₀ changes with slow heating of Scenedesmus cells grown at 20° or 28°C are shown in Fig. 56. Both Fₘₐₓ and F₀ curves display a correlation with growth temperature. The characteristic yield changes are shifted by approximately 1°C in the 28°C cells, relative to the 20°C cells. This shift is particularly clear in the F₀ curves, which have a main rise phase (at 46°-52°) well separated from a minor rise at T < 46° and a decay at T > 52°. The Fₘₐₓ curves show a less conspicuous main decay phase extending over the whole temperature region, with some characteristic slope changes. The significance of these slope changes will become clearer from an analysis of the Fₘₐₓ T-jump curves to be shown below. It appears that the F₀ curves are a sensitive indicator for a dramatic change within a relatively narrow temperature interval of a parameter essential in regulating fluorescence yield. The same change causes an inverse effect on Fₘₐₓ, but as far as slow-heating curves are concerned, the information from F₀ seems to be more specific.

The fact that F₀ and Fₘₐₓ slow-heating curves are shifted with growth temperature indicates that beyond expected changes in the enzymatic dark reaction part of photosynthesis, temperature adaptation has also affected
some parameter associated with the pigment system and PS II reaction centers. Slow-heating curves as such do not reveal which parameter is affected.

**T-Jump Curves**

With a T-jump the time course of the heat-induced fluorescence changes can be monitored, giving additional information on the nature of changes. Figure 57 shows T-jump curves of $F_0$ and $F_{\text{max}}$ for the 20° and 28° cells. As with the slow-heating curves, there is a clear correlation with growth temperature. Heat-induced changes are more pronounced in the 20° cells. Temperatures for roughly equivalent fluorescence time courses are shifted by approximately 1°C upward for the 28° cells. At a given temperature, 28° cells have to be heated about twice as long as 20° cells to give an equivalent fluorescence increase (e.g. compare curves for 47°C). The T-jump curves reveal several phases, suggesting that there are several mechanisms by which heat induces fluorescence changes. A detailed analysis for $F_0$ curves has been presented elsewhere (Schreiber et al., 1976). A new finding is the inverse relationship between the time courses of the $F_0$ and $F_{\text{max}}$ curves, particularly pronounced in the slower part of the curves. At present no definite explanation can be given for this phenomenon. However, it appears unlikely that it is based on changes in quenching at PS II centers or changes in de-excitation rate constants of Chl $\alpha$ in the pigment system. Such changes should affect $F_0$ and $F_{\text{max}}$ in the same direction. As a working hypothesis it may be proposed that the process that leads to the increase in $F_0$ and the decrease in $F_{\text{max}}$ affects the energy distribution favoring PS I in the $F_0$ state and PS II in the $F_{\text{max}}$ state. Any disturbance of such an active distribution mechanism would cause an increase in $F_0$ and a decrease in $F_{\text{max}}$.

**Reversibility of the Light-Induced Fluorescence Increase in the Presence of DCMU**

In the sections above it was shown that *Scenedesmus* grown at different
temperatures displays consistent differences in heat-induced fluorescence changes. It remains to be shown that these differences relate to a difference in thermal stability. It has been shown previously that heat treatment inhibits the back reaction at PS II centers (Schreiber, 1971). When a sample is illuminated in the presence of DCMU, a high steady-state fluorescence yield reflects complete reduction of $Q$. In a following dark period $Q$ normally becomes reoxidized, as indicated by a reproducible fluorescence rise curve with subsequent illumination. Heat treatment prevents this reoxidation. Figure 58 compares fluorescence rise curves in the presence of DCMU in a nonheated control and a sample heated for 1 min at 56°C. In Fig. 58A dark-adapted samples are compared; heating causes only a small increase in $F_0$. Figure 58B demonstrates that there is no reoxidation of $Q$ in the heat-treated sample following preillumination. The light-induced fluorescence rise in the presence of DCMU is irreversible. This behavior is identical to that in the presence of NH$_2$OH, which has been shown to inhibit the back-reaction at PS II centers by keeping the donor Z-reduced (Bennoun, 1970).

When a sample is only partially heat damaged, only part of the PS II reaction centers will be inactivated in their back-reaction, and part of the DCMU fluorescence rise is reversible. The degree of irreversibility can be considered a measure of heat damage.

In Fig. 59 the term $(F_{\text{max}} - F_0)/F_{\text{max}}$ is plotted as an expression of still active centers versus heating temperature for the 20° and 28° cells. Inhibition of the back-reaction occurs in the same temperature region where slow-heating and $T$-jump curves showed characteristic fluorescence yield changes. The inhibition characteristic is shifted by about 1°C toward higher temperatures for the 28° cells.

Heat damage in higher plant chloroplasts has been correlated with changes in $F_0/F_{\text{max}}$ by Berry et al. in this laboratory (Year Book 74, p. 751, and elsewhere in this Report). New aspects suggested by the present study are the importance of controlled preillumination and the similarity of the heat damage to the action of NH$_2$OH. As with NH$_2$OH, heat damage does not block the PS II primary reaction as such, because without preillumination a close-to-normal fluorescence rise is observed (see Fig. 58). By analogy with the effect of NH$_2$OH, heat damage appears to prevent reoxidation of $Q$ via electron transfer to Z$^+$ at the PS II donor side (Bennoun, 1970). To date, it is difficult to distinguish between two alternate hypotheses. Heat damage possibly causes the breakdown of a barrier that normally prevents reduction of Z$^+$ by endogenous electron donors. This barrier may be provided...
Fig. 58. Effect of heat treatment on reversibility of fluorescence rise-curve measured at 25°C in the presence of DCMU. Solid lines, unheated control; broken lines, sample heated 1 min at 56°C. A, samples before preillumination; B, samples preilluminated for 1 sec. Curves measured following subsequent 1 min dark time. $5 \times 10^{-6} \text{M DCMU}$ was added in the dark 2 min before measurement of curves (a). 20° cells; light intensity, 10^6 erg/cm²/s.

Fig. 59. Reversibility of DCMU rise-curve in dependency of heating temperature in 20° and 28° cells. Samples were heated for 1 min at given temperature; DCMU was added in the dark; after 2-min incubation, 1-sec preillumination was given; the DCMU rise-curve was measured after subsequent 1-min dark time; $F_{\text{max}}$ and $F_0$ values determined as indicated in the inset. The inset shows the remaining rise in 20° cells for 47°C treatment (broken line) as compared to a nonheated control (solid line). Conditions as in Fig. 58.

by an intact membrane. Alternatively, heat damage could cause denaturation of the water-splitting enzyme. Both hypotheses account for a block of the PS II back-reaction and a block of $O_2$ evolution.

There is close similarity between $F_0$ slow-heating curves (see Fig. 56) and the deactivation curves in Fig. 59. One may conclude that irreversible heat damage and the $F_0$ increase measured at elevated temperatures are related. Deactivation of the PS II back-reaction or the water-splitting enzyme system as such should not cause the dramatic increase in $F_0$. Both phenomena appear
to be caused by a common drastic change in the photosynthetic apparatus in a critical temperature region. This critical temperature region is higher in plants grown at higher temperatures, suggesting the capacity for partial adaptation. The common cause may be a fluidity change in the thylakoid membrane, affecting energy transfer and distribution as well as lipid-protein interaction. Adaptation to growth temperature could involve a change in the ratio of unsaturated to saturated fatty acids, as is known for blue-green algae (Holton et al., 1964).

CONCLUSIONS

The above results demonstrate a correlation between growth temperature, heat damage, and heat-induced fluorescence changes in Scenedesmus obliquus. Similar experiments were carried out with other unicellular algae (Chlorella pyrenoidosa and Anacystis nidulans) and gave similar results. It can be concluded that these algae can partly adapt their photosynthetic apparatus to their thermal environment. Chlorophyll fluorescence, particularly in $F_0$ measurements, appears to be a sensitive indicator for heat-induced damage and, consequently, for assaying heat sensitivity in plants.

REFERENCES


DARK UPTAKE OF $\text{HCO}_3^-$ BY WASHED CHLOROPLAST GRANA

Alan Stemler

Bicarbonate ions play an important role in both the light and the dark reactions in photosynthesis. Of the two light reactions, $\text{HCO}_3^-$ is necessary for photosystem II activity. Evidence suggests (Stemler and Govindjee, 1973) that $\text{HCO}_3^-$ is taken up by broken chloroplasts as a prior condition to activation of oxygen evolution during a Hill reaction. A quantitative estimate of $\text{HCO}_3^-$ uptake by broken chloroplasts is therefore desirable to allow insight into possible modes of action of this ion. To obtain such an estimate, competitive binding studies were done using $\text{H}^4\text{CO}_3^-$ and maize chloroplast fragments.

Chloroplasts were isolated by a procedure described elsewhere (Stemler and Govindjee, 1973). During isolation, they were subjected to an osmotic shock, after which they were washed, frozen, and then thawed before use. Such chloroplasts normally cannot assimilate $\text{CO}_2$ in the usual fashion and cannot evolve oxygen without an added Hill oxidant such as ferricyanide. Nevertheless, under certain conditions such chloroplast fragments can bind large amounts of $\text{HCO}_3^-$ in the dark.

When grana are provided with $\text{H}^4\text{CO}_3^-$ and increasing concentrations of cold $\text{HCO}_3^-$, competition develops for a limited number of binding sites on or within the thylakoid membranes. After correction for "trapped" $\text{H}^4\text{CO}_3^-$ (i.e., unbound $\text{HCO}_3^-$ associated with the chloroplasts because of their finite vol-

DARK UPTAKE OF $\text{HCO}_3^-$ BY WASHED CHLOROPLAST GRANA

Alan Stemler

Bicarbonate ions play an important role in both the light and the dark reactions in photosynthesis. Of the two light reactions, $\text{HCO}_3^-$ is necessary for photosystem II activity. Evidence suggests (Stemler and Govindjee, 1973) that $\text{HCO}_3^-$ is taken up by broken chloroplasts as a prior condition to activation of oxygen evolution during a Hill reaction. A quantitative estimate of $\text{HCO}_3^-$ uptake by broken chloroplasts is therefore desirable to allow insight into possible modes of action of this ion. To obtain such an estimate, competitive binding studies were done using $\text{H}^4\text{CO}_3^-$ and maize chloroplast fragments.

Chloroplasts were isolated by a procedure described elsewhere (Stemler and Govindjee, 1973). During isolation, they were subjected to an osmotic shock, after which they were washed, frozen, and then thawed before use. Such chloroplasts normally cannot assimilate $\text{CO}_2$ in the usual fashion and cannot evolve oxygen without an added Hill oxidant such as ferricyanide. Nevertheless, under certain conditions such chloroplast fragments can bind large amounts of $\text{HCO}_3^-$ in the dark.

When grana are provided with $\text{H}^4\text{CO}_3^-$ and increasing concentrations of cold $\text{HCO}_3^-$, competition develops for a limited number of binding sites on or within the thylakoid membranes. After correction for "trapped" $\text{H}^4\text{CO}_3^-$ (i.e., unbound $\text{HCO}_3^-$ associated with the chloroplasts because of their finite vol-
ume), results are expressed in a Scatchard plot (Scatchard, 1949) shown in Fig. 60. Where a single binding site is involved, a Scatchard plot will yield a straight line with the intercept of the abscissa indicating the concentration of the binding site. The plot in Fig. 60 shows instead a curved line, indicating the presence in chloroplast grana of more than one binding site for $HCO_3^-$. The sharp drop in the left portion of the curve shows a small pool of binding sites having a relatively high affinity for $HCO_3^-$. A rough estimate of the maximum size of this pool can be obtained by a line tangent to the upper section of the curve and intersecting the abscissa (Klotz and Hunston, 1971). The dashed line so drawn in Fig. 60 intersects the abscissa at $3.5 \mu M$, and this represents the upper limit of the concentration of this binding site (or sites, as the pool may be heterogeneous) when the chlorophyll concentration is $1.0 \mu M$.

The long tail of the Scatchard plot (Fig. 60), which approaches the abscissa very obliquely, indicates that there also exists in grana another quite large pool of relatively low affinity $HCO_3^-$ binding sites. The size of this pool is difficult to determine, since it cannot be saturated on account of the limited solubility of $HCO_3^-/CO_2$ at the pH at which the experiment was done (6.5). However, the size must be greater than $160 \mu M$ when the chlorophyll concentration is $1.0 \mu M$, since this amount was actually observed bound under a nonsaturating concentration of $HCO_3^-$. The conditions of this experiment, however, may not have been optimal for $HCO_3^-$ binding, so conclusions concerning the actual number of both high- and low-affinity sites can only be tentative until other parameters such as temperature, illumination, and incubation time can be studied.

An example of a very critical factor controlling the amount of $H^{14}CO_3^-$ bound to grana is the pH of the suspension. This relationship is plain in Fig. 61. Chloroplasts take up very little $H^{14}CO_3^-$ above pH 7.0, but show nearly a tenfold increase in bound $H^{14}CO_3^-$ when the pH is lowered from 7.0 to 5.6. As other factors that may influence binding are examined, a better idea of the size and function of the binding sites should emerge.

With our present limited knowledge, we can only guess the significance of the two pools of bound $HCO_3^-$ in grana.

![Fig. 60. Scatchard plot for the binding of $HCO_3^-$ to chloroplast grana. The suspension contained 1.0 mg chlorophyll ml$^{-1}$ (i.e., 1.0 mM), 0.1 M Na phosphate, pH 6.5, 0.2 m NaCl, 1.2 $\mu$ Ci Na$^{14}CO_3$. The concentration of unlabeled NaHCO$_3$ was varied from 0.0 to 50 mM. The grana were incubated for 5 min at 30°C after addition of $H^{14}CO_3^-$.](image)
Fig. 61. Binding of $^{14}$CO$_3^-$ to chloroplast grana as a function of pH. The suspension contained 384 $\mu$g chlorophyll ml$^{-1}$, 0.1 M Na phosphate, 0.2 M NaCl, 33 $\mu$M NaHCO$_3$ (1.2 $\mu$Ci ml$^{-1}$). The pH was varied by addition of appropriate amounts of NaOH.

We know, for instance, that depleting chloroplasts of HCO$_3^-$ has two distinct effects (Stemler et al., 1974): About half the photosystem II reaction centers become totally inactive, while those still able to evolve oxygen have dramatically reduced recovery rates. Assuming both pools of bound HCO$_3^-$ are indeed functional in photosystem II activity, each may control one of the above observed effects.

Of the two pools of bound HCO$_3^-$, the larger is already more strongly implicated in oxygen evolution. The small pool is saturated by addition of about 1 mM HCO$_3^-$, while 10 mM HCO$_3^-$, a concentration needed to fill the larger pool substantially, is required to restore oxygen evolution fully in HCO$_3^-$ depleted chloroplasts. Both pools, however, may be equally important, and will be studied in detail.

References


**BIBLIOGRAPHIC INFORMATION RETRIEVAL**

J. S. Brown

In *Year Book 72*, p. 407, a computer-based system for the storage and retrieval of bibliographic information from the photosynthesis literature was described. Also, the Thesaurus (list of categories) under which the references in the "Plantbio" file are listed was printed out. Currently, the file size has increased to more than 6000 entries, and the following new categories have been added to the Thesaurus: C-3, ecological; C-4, solar energy; D-6, xanthophylls; G-14, temperature effects; G-15, oxidation/reduction; G-16, pH effects; H-14, superoxides; L-3, superoxide dismutase; R-6, N$_2$ fixation.

Unfortunately, the cost of storing the file in a way that makes on-line searching possible at any time has become prohibitive (about $10.00 per day) unless justified by a large number of users. An attempt was made to form a Users' Club by sending out information about the file to approximately 150 photosynthesis workers around the world. Many were interested, but few could support it financially.

Therefore, we now have a new procedure whereby we can unload the complete file on to tape and restore it again when we choose for about $20.00. This means that we can search and update the file only during specified periods, but on-line storage costs are greatly reduced.

However, we have found another way in which the file can be useful. It is easily possible to search and print out all of the references in a particular category alphabetically by first author for a reasonable cost ($5.00 for categories having less than 300 references). We have already sent several of these cat-
category listings to other laboratories, and we shall be glad to provide more specific information such as category size and costs upon request. Inquiries should be addressed to "The Librarian."

BIBLIOGRAPHY

Anderson, J. M., see Boardman, N. K.
Berry, J. A., see Mooney, H. A.
Björkman, O., see Boardman, N. K.; Ehleringer, J. R.; Mooney, H. A.


Briggs, W. R., see Britz, S. J.; Song, P.-S.


559 Fork, D. C., see Murata, N.


556 Johnson, D. J., see Boardman, N. K.

555 Grimme, L. H., see Boardman, N. K.


562 Johnson, G. B., Polymorphism and predictability at the a-glycerophosphate dehydrogenase locus in Colias butterflies: Gradients in allele frequency within single populations, Genetics, in press, 1976.


571 Mackenzie, J. M., Jr., see Britz, S. J.


Mooney, H. A., see Ehleringer, J. R.


Nultsch, W., see Britz, S. J.


564  Pfau, J., see Britz, S. J.


SPEECHES

Berry, Joseph A., James Ehleringer and Harold A. Mooney, Death Valley Days. Meeting of the Bay Area Biosystematists, University of California Marine Laboratory, Bodega Bay, California, March 20, 1975.


Boynton, John E., The Genetics of Chloroplast Ribosome Biogenesis. Seminar, Department of Biology, University of California, Davis, California, December 5, 1975.

Boynton, John E., Transmission, Segregation and Recombination of Chloroplast Genes. Seminar, Department of Genetics, University of California, Davis, California, February 2, 1976.

Boynton, John E., The Genetics of Chloroplast Ribosome Biogenesis. Seminar, Department of Biology, University of California, Los Angeles, California, May 17, 1976.


Briggs, Winslow R., Blue Light–Induced b-Type Cytochrome Reduction in Membrane Fractions of Corn and Neurospora. Symposium on Sensory Transduction in Microorganisms, Gordon Conference, Santa Barbara, California, December 30, 1975.


Briggs, Winslow R., Studies on the Phototropic Photoreceptor. Seminar, Department of Biology, University of California, Los Angeles, California, May 10, 1976.

Briggs, Winslow R., Blue Light Photoreceptor in Higher Plants and Fungi. Meeting of the
American Society of Biological Sciences, San Francisco, California, June 8, 1976.
Briggs, W. R., see Brain, R. D.
Brown, Jeanette S., see Fork, David C.
DeVay, J. E., see Rogler, Charles.
Dohrmann, Ulrike, In vitro Binding of Auxin to Membranes from Corn Coleoptiles and Membrane Receptors. Seminar, Department of Biology, San Diego State University, San Diego, California, May 31, 1976.
Ehleringer, James, see Berry, Joseph A.
Fork, David C., Pigments and Energy Harvesting. Department of Biological Sciences, Stanford University, Stanford, California, October 13, 1975.
Fork, David C., and Jeanette S. Brown, The Use of Rapid Light-Induced Absorbance Changes of Chlorophylls and Carotenoids to Distinguish between Algae of Different Pigment Composition (presented by Jeanette S. Brown). Meeting of the Physiological Society of America/AIBS, Corvallis, Oregon, August 18, 1975.
Freeberg, J., see Brain, R. D.
Johnson, George, Enzyme Polymorphism and Adaptation in Alpine Butterflies. Missouri Botanical Garden Symposium, St. Louis, Missouri, October 18, 1975.
Johnson, George, Enzyme Polymorphism in Alpine Butterflies. Seminar, Department of Biology, San Diego State University, San Diego, California, November 17, 1975.
Johnson, George, Do Most Electrophoretically Detected Enzyme Polymorphisms Involve Differences in Charge? Seminar, Department of Biology, University of Indiana, Bloomington, Indiana, December 10, 1975.
Johnson, George, Evaluation of Kimura and Ohata's Stepwise Mutation Model of Electrophoretic Mobility: Gel Sieving Analysis of Esterase-5 Alleles of Drosophila pseudoobscura. Seminar, Department of Biology, University of California, Santa Cruz, California, January 20, 1976.
Johnson, George, Molecular Studies of Enzyme Polymorphism: Cryptic Alleles in Colias. Seminar, Department of Evolutionary Biology and Ecology, University of Kansas, Lawrence, Kansas, March 3, 1976.
Johnson, George, Selection on Metabolic Phenotypes in Alpine Butterflies. Seminar, Department of Genetics and Ecology, University of Aarhus, Denmark, May 10-14, 1976.
Johnson, George, Selection on Integrated Metabolic Phenotypes. Seminar, Department of Genetics and Ecology, University of Aarhus, Denmark, May 12, 1976.
Mooney, H. A., see Berry, Joseph A.
Pratt, L. H., see Mackenzie, John.
Rogler, Charles E., Characterization of Plasmid DNA from Agrobacterium tumefaciens and Its Role in Crown Gall Disease. Seminar, Department of Plant Sciences, University of California, Riverside, California, March 24, 1976.
Rogler, Charles, W. F. Thompson, and J. E. DeVay, Correlation of Serological and Plasmid...


Solbrig, Otto T., The Evolution of Breeding Systems in Plants. Seminar, Department of Biology, University of California, Santa Barbara, California, February 4, 1976.


Thompson, William F., Sequence Studies on Plant DNA. Seminar, Department of Botany, San Diego State University, San Diego, California, December 5, 1975.

Thompson, W. F., see Rogier, Charles.

Weiss, C. V., see Brain, R. D.

## PERSONNEL

### Research Staff

<table>
<thead>
<tr>
<th>Name</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>Joseph A. Berry</td>
<td>Director</td>
</tr>
<tr>
<td>Olle Bjorkman</td>
<td>Director Emeritus</td>
</tr>
<tr>
<td>Winslow R. Briggs</td>
<td>Director</td>
</tr>
<tr>
<td>Jeanette S. Brown</td>
<td>Associate Director</td>
</tr>
<tr>
<td>David C. Fork</td>
<td>Associate Director</td>
</tr>
<tr>
<td>C. Stacy French, Director</td>
<td>Emeritus</td>
</tr>
<tr>
<td>William M. Hiesey, Emeritus</td>
<td>Associate Director</td>
</tr>
<tr>
<td>Malcolm A. Nobs</td>
<td>Associate Director</td>
</tr>
<tr>
<td>William F. Thompson</td>
<td>Associate Director</td>
</tr>
</tbody>
</table>

### Clerical and Technical Staff

<table>
<thead>
<tr>
<th>Name</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donna C. Atwood</td>
<td>Department Secretary</td>
</tr>
<tr>
<td>Benny Catanzaro</td>
<td>Electronic Technician</td>
</tr>
<tr>
<td>Ruth Fischer</td>
<td>Administrative Assistant</td>
</tr>
<tr>
<td>Glenn Ford</td>
<td>Laboratory Manager</td>
</tr>
<tr>
<td>Edward G. Gausden</td>
<td>Technician</td>
</tr>
<tr>
<td>Steven Graff</td>
<td>Technician</td>
</tr>
<tr>
<td>Alan Grundmann</td>
<td>Administrator</td>
</tr>
<tr>
<td>Richard W. Hart</td>
<td>Mechanical Engineer</td>
</tr>
<tr>
<td>James Johnson</td>
<td>Technician</td>
</tr>
<tr>
<td>Kathleen Koller</td>
<td>Secretary</td>
</tr>
<tr>
<td>Jan Kowalik</td>
<td>Technician</td>
</tr>
<tr>
<td>Fred Lakin</td>
<td>Technical Illustrator</td>
</tr>
<tr>
<td>Frank Nicholson</td>
<td>Senior Technician</td>
</tr>
<tr>
<td>Ernest Ramos</td>
<td>Technician</td>
</tr>
<tr>
<td>Susan Reed</td>
<td>Technician</td>
</tr>
<tr>
<td>Jonathan Walton</td>
<td>Technician</td>
</tr>
<tr>
<td>Charles V. Weiss</td>
<td>Technician</td>
</tr>
</tbody>
</table>

### Carnegie Institution of Washington Fellows

<table>
<thead>
<tr>
<th>Name</th>
<th>Fellowship</th>
</tr>
</thead>
<tbody>
<tr>
<td>John Cross</td>
<td>Fellow</td>
</tr>
<tr>
<td>Ulrike Dohrmann</td>
<td>Fellow</td>
</tr>
<tr>
<td>Algirdas J. Jesaitis</td>
<td>Fellow</td>
</tr>
<tr>
<td>George Johnson</td>
<td>Senior Fellow, Washington University, St. Louis, Missouri</td>
</tr>
<tr>
<td>Aaron Kaplan</td>
<td>Fellow</td>
</tr>
<tr>
<td>Bruce E. Mahall</td>
<td>Fellow</td>
</tr>
<tr>
<td>Norio Murata</td>
<td>Senior Fellow, Tokyo University, Tokyo, Japan</td>
</tr>
<tr>
<td>Michael Murray</td>
<td>Senior Fellow, San Jose State University, San Jose, California</td>
</tr>
<tr>
<td>Charles E. Rogier</td>
<td>Fellow</td>
</tr>
<tr>
<td>Ulrich Schreiber</td>
<td>Fellow</td>
</tr>
<tr>
<td>Alan Stemler</td>
<td>Fellow</td>
</tr>
</tbody>
</table>

### Fellows

<table>
<thead>
<tr>
<th>Name</th>
<th>Fellowship</th>
</tr>
</thead>
<tbody>
<tr>
<td>Murray Badger</td>
<td>Fellow, CSIRO, Canberra, Australia</td>
</tr>
<tr>
<td>John Boynton</td>
<td>Senior Fellow, National Institutes of Health Research Development Award, Duke University, Durham, North Carolina</td>
</tr>
<tr>
<td>Richard Carey</td>
<td>Senior Fellow, San Jose State University, San Jose, California</td>
</tr>
<tr>
<td>Jack Freeberg</td>
<td>Senior Fellow, University of Massachusetts, Amherst, Massachusetts</td>
</tr>
</tbody>
</table>

*Resigned April 15, 1976.
Retired August 31, 1975.
Resigned April 30, 1976.

*To December 15, 1976.
*To September 1, 1975.
*To January 9, 1976.