'Photosynthesis in Intact Plants' Univ. of Illinois

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PROGRESS REPORT

Progress in the two years since the last renewal application has been excellent. The 13 papers and 2 Ph.D. thesis published, in press or submitted during the past year (together with several others in the pipe-line), reflect not only a high productivity in the current year, but also our ability to exploit the instruments developed and the experience gained in previous years. We have made substantial contributions on both main fronts of the project, and are particularly happy with the progress of our research on intact plants. The approach of basing our field work on a sound foundation of laboratory studies has enabled is to use methods which provide unambiguous assays of well characterized reactions. We have also made excellent progress in several laboratory studies which will have direct applications in future field work, and have introduced to the laboratory a range of molecular genetics techniques which will allow us to explore new options in the attempt to understand function at the level of molecular structure.

(References in square brackets are to work supported from this grant published, in press, or submitted for publication since the previous renewal. References in parentheses are to the bibliography.)

1) Instrumentation

a) Portable flash kinetic fluorimeter [1].

A newly-developed field-portable multi-flash kinetic fluorimeter for measuring the kinetics of the microsecond to millisecond reactions of the oxidizing and reducing sides of photosystem II (PS II) in leaves of intact plants is described and demonstrated. The instrumental technique is a refinement of that employed in the "double-flash" kinetic fluorimeter (1-3) where a low-intensity short-duration light pulse is used to measure the fluorescence yield changes following saturating single-turnover light pulses. The present instrument uses a rapid series of short-duration (2 μs) pulses to resolve a complete microsecond to millisecond time-scale kinetic trace of fluorescence yield changes after each actinic flash. Differential optics, using a matrix of optical fibers, allow very high sensitivity (noise levels between 0.05% to 0.15% F_{max}) thus eliminating the need for signal averaging, and greatly reducing the intensity of light required to make a measurement. Consequently, the measuring pulses have much less actinic effect and a multi-point trace of seven points excites less than 1% of the reaction centers in a leaf. In addition, by combining the actinic and measuring pulse light in the optical fiber network, the tail of the actinic flash can be compensated for, allowing measurements of fluorescence yield as rapidly as 20 μs after the actinic flash.

b) Portable kinetic spectrophotometer [2].

A highly sensitive, portable spectrophotometer for use in measuring flash-induced absorbance changes in intact leaves has been developed, and its utility extended by addition of a facility for continuous actinic illumination at relatively high intensity. The spectrophotometer uses trifurcated light guides to deliver measuring and actinic beams to two comparable areas of the leaf. The measuring beam is provided by a series of short, relatively intense light pulses from a xenon flashlamp in place of the constant weak measuring beam used in conventional machines, allowing for a high signal-to-noise ratio (noise levels of 10^{-5}A) without significant actinic effects. The time resolution of the instrument is 2 μs and the noise level is independent of the

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experimental time range. The new facility is an illumination source provided by an annulus of high intensity red LEDs fitted to provide light to the same area of leaf as illuminated by the actinic flash.

c) Fluorescence Video Imaging [3].

The instrument has been refined by software development which enhances the range of options available for measurement and analysis. We have also developed software to allow images captured in the fluorescence video apparatus to be transferred to an SGI 4D/20 workstation for graphical manipulation, analysis, textual labeling, etc. We are currently upgrading the flash facility on the instrument to permit activation by saturating actinic flashes. Together with the existing weak (measuring) flash, this will make possible experiments similar to those with the flash kinetic fluorimeter, except that an image of areas in the field with a high fluorescence yield at the time of the measuring flash will be recorded. We are also adding to the apparatus a filter turret to allow different filters to be rotated into the measuring and actinic path; this will make it possible to record reflectance spectra, conventional images, images in selected wavelength bands, etc.

2) Research on intact plants

a) Activation of ATP-ase in intact leaves [4].

The activation of the ATP-ase in intact cucumber leaves has been studied, using the portable kinetic spectrophotometer described above. The flux through the ATP-ase was measured by using the decay of the electrochromic shift at 515 nm as an indicator of the flux of protons across the thylakoid membrane. Plots of the rate of decay of the electrochromic shift against its amplitude were used to determine the threshold for activation of the ATP-ase. A rapid phase of decay of the electrochromic shift was found above a certain threshold amplitude in control leaves. This rapid phase was eliminated after treatment of the leaves with dicyclohexylcarbodiimide (DCCD), indicating that it was associated with flux through the active ATP-ase. The threshold amplitude of the electrochromic shift was lower in light-adapted leaves than in dark-adapted leaves indicating a lower threshold amplitude for the activation of the ATP-ase. The lowering of the threshold amplitude by light-adaptation was eliminated by treatment of the leaves with methyl viologen, which blocks electron flow to the thioredoxin system. These results are interpreted in terms of a previous model (4) to explain results from isolated systems. The reoxidation kinetics of the γ-subunit of the ATP-ase were followed by observing the extent of the slow decay phase of electrochromic shift. These kinetics showed a lag time which was dependent on the amount of light adaptation, and a recovery, the kinetics of which did not appear to be dependent on the amount of light adaptation. The kinetics are interpreted in terms of a model consisting of a large redox buffering pool with a midpoint potential somewhat more negative than that of the sulfhydryl groups of the γ-subunit of the ATP-ase, in equilibrium with a smaller pool equipotential with the γ-subunit sulfhydryls. Some possible characteristics of this pool are discussed.

b) Effect of prolonged drought on photosynthesis and ATP-ase activation [5].

The diurnal cycling of leaf water potential in field-grown sunflower was used to investigate the cause of water-deficit limitation of net photosynthesis. Daily mid-afternoon decreases in leaf water potential of up to 1.5 MPa, and in net photosynthesis of up to 50% were typical for irrigated sunflower

during seed filling. These mid-afternoon leaf water potentials were lowered an additional 0.6 to 0.8 MPa by prolonged drought treatment. There was a nearly linear relationship between the decline in net photosynthesis and reduction in leaf conductance over the course of the day. Thus, it was unexpected to find that the low, mid-afternoon rates of photosynthesis were associated with the highest intercellular CO_2 concentrations. These and other observations suggest that the daily decline in photosynthesis represents a "down regulation" of the biochemical demand for CO_2 that is coordinated with the diurnally developing need to conserve water, thus establishing a balanced limitation of photosynthesis involving both stomatal and non-stomatal factors. There were no indications that either short term or long term water deficits caused any damage or malfunction of photosynthesis, and no evidence was observed for any change in the activation of the coupling factor. Rather, both the daily declines in photosynthesis, and the nearly 25% decrease in leaf area induced by prolonged drought, appear to be well-controlled adaptive responses by field-grown sunflower plants to limited water availability.

c) **Activation of the ATP-ase is not rate limiting at low light [6].**

Simultaneous non-invasive measurements have been made of the state of activation of the chloroplast CF_1CF_0-ATP synthase, or coupling factor (CF), and of photosynthetic CO_2 fixation in field-grown sunflower (Helianthus annus) at the dark-to-light transition at sunrise. At light intensities of only 5-22 $uE.m^{-2}.s^{-1}$, the threshold pmf for activation of the CF was found to be lowered, presumably through reduction of the regulatory sulfhydryl groups of the γ-subunit of the CF. Under the same conditions, CO_2 fixation showed a linear response with light intensity until about 300-500 $uEm^{-2}s^{-1}$. When these studies were extended to chamber-grown plants, the intensity of light required to reduce half of the CF in an intact leaf was between 2-4 $uE.m^{-2}.s^{-1}$. At 4 $uE.m^{-2}.s^{-1}$, the CF was completely reduced in 3-7 sec. It is clear from these studies that CF reduction is not rate limiting under the conditions of a natural dark-to-light transition at sunrise. A model based on the redox properties of the regulatory sulfhydryls is proposed to explain the differences in light sensitivity and oxidation and reduction kinetics between the CF and other thioredoxin-modulated enzymes, and computer simulations of the light-induced regulation of three representative thioredoxin-modulated enzymes are presented. The model accounts well for the previously reported physiological regulation of the enzymes.

d) **The activation of the CF_1-CF_0-ATP-ase may be under the control of a circadian clock [7].**

By observing the slowly decaying component of the flash-induced 515 nm change using a highly-sensitive kinetic spectrophotometer, we measured the oxidation kinetics of the γ-subunit of the chloroplast coupling factor (CF) at different times in the dark-light-dark diurnal cycle in plants from a growth cabinet. A cycle of kinetic patterns was seen during the hours of illumination. In early morning, CF was reoxidized with sigmoidal kinetics: a lag and oxidation phase of about 12 min. each. From 0.5 to 5 hrs. illumination, the kinetics became more complex; a shorter lag (6 min.) was followed by two phases of oxidation (6 min. and 2 hrs.). From 5 to 11 hrs. illumination, the lag phase was replaced by a transient, almost complete oxidation, followed by a partial re-reduction and a subsequent slow reoxidation (on the order of 2 hours). At about 1 hr. before dark, and without any fluctuations in illumination, the kinetics changed to become similar to those of the early morning. The anomalous behavior an hour before the light-dark transition may indicate

that this metabolic function is under the control of a circadian clock.

e) **Oscillations in microsecond fluorescence yield measure the S-state transitions and the state of the two-electron gate in intact leaves [1].**

The new portable kinetic fluorimeter allows measurements of fluorescence yield as rapidly as 20 μs after the actinic flash with high sensitivity, and a good signal/noise ratio. This resolution makes practical the routine measurement of the microsecond turnover kinetics of the oxygen evolving complex in leaves of intact plants, in the laboratory or field, from the flash number dependency and inhibitor sensitivity of the rise and decay kinetics of flash-induced fluorescence yield changes. From these traces the period-two oscillations associated with the turnover of the two-electron gate and the period-four oscillations associated with the turnover of the oxygen evolving complex can be observed. The reaction center in the state $P^+_{680}.Q^-_A$ formed during the actinic flash is a low fluorescent state due to quenching by P^+_{680}. The fluorescence yield rise in the <50 μs range is due to re-reduction of the oxidized primary donor (P^+_{680}), while the decay over the range out to 1 ms is due to oxidation of Q^-_A. The period 4 oscillation is due to a weak equilibrium constant between $Y_Z.P^+$ and $Y^+_Z.P$, and the rereduction of Y^+_Z by the Mn-cluster with different times in the 30-1000 us range depending on S-state.

Preliminary collaborative projects.

f) **The two-electron gate in atrazine resistant *Brassica*.**

Atrazine resistant plants show a lowered efficiency of photosynthesis than their susceptible parent strains, probably because of a weaker stabilization of the secondary acceptor semiquinone, Q^-_B (see below). In collaboration with Drs. R. Wise and Don Ort, we have investigated photosynthetic electron transfer in field-grown resistant and susceptible *Brassica* biotypes, using the portable instruments discussed above. Results are presently under analysis.

g) **Direct measurement of the site of lesion in corn, wheat and barley electron transfer mutants.**

In a collaboration with Ms. Tanya Falbel and Professor Andrew Staehlin, we investigated a variety of mutant biotypes of corn, wheat and barley using the portable instruments, and the fluorescence video imaging apparatus. We were able to show directly the block in electron transfer in several mutants (b_6/f^-, and PS I^- strains), and, by using the fluorescence video imaging apparatus, we were able to follow the developmental changes in phenotype (by observing the differential fluorescence induction kinetics along the leaf) in others. This preliminary work offers several interesting possibilities for future projects.

h) **Assay of lesions in *Arabidopsis* mutants unable to grow at low light.**

In collaboration with Drs. Halina Gabrys and Don Ort, we are investigating the activation of the ATP-ase in *Arabidopsis* mutants which are able to grow at high but not low light. The rationale is that such strains will likely be deficient in photosynthetic excitation or electron transfer, ATP synthesis, or control. We have identified a number of biotypes with modified deactivation kinetics, and will be exploring these more extensively in the future.

i) **Measurement of zeoxanthin formation in leaves illuminated at high intensity.**

The role of zeoxanthin formation (5,6) in the protection of plants under high light conditions has been emphasized by Demmig-Adams (7). By adapting the portable kinetic spectrophotometer so as to provide high intensity continuous illumination, we have been able to measure the formation of zeoxanthin from the color change in intact leaves. We will characterize this phenomenon more thoroughly and use this modified instrument to investigate the protective role of the xanthophyll cycle.

3) Fluorescence video imaging of photosynthetic systems (FVIPS)

a) **Identification and characterization of mutants in *Arabidopsis* with modified fluorescence phenotype.**

In collaboration with Dr. Beverley Green and Mr. Randy Dinkins, we have used the FVIPS apparatus to screen and characterize *Arabidopsis* strains for mutants with modified fluorescence transients. Fluorescence induction curves could be easily measured from leaves a few mm in diameter, and we could readily distinguish in video frame analysis individual plants from mixed plate cultures with different induction characteristics. We were able to distinguish several classes of mutants, including some which were separately characterized as PS I deficient [16].

b) **Screening for mutants in *Synechocystis* 6803**

In collaboration with Professor Larry Bogorad and Ms. Lola Abarca, we have screened plates of *Synechocystis* 6803 containing over 400 mutant strains defective in photosynthesis. Although colonies grown on glucose containing plates had a very low variable fluorescence, we could detect significant differences between colonies. Strains grown photosynthetically showed a larger variable fluorescence, and mutational variants were easier to pick out. This preliminary work suggests that strains able to grow photosynthetically (such as herbicide resistant variants) should be resolvable using video imaging of plates in screening experiments. More work is needed to characterize the behavior of colonies grown on glucose, but it certainly seems feasible to screen for mutations with markedly different fluorescence phenotypes.

d) **Fluorescence induction in *Chlamydomonas* mutant strains resistant to inhibitors of the two-electron gate.**

Dr. Jean-David Rochaix has kindly made available to us strains of *Chlamydomonas* resistant to a variety of herbicides, or deficient in one or other of the three main enzymes of the photosynthetic chain. We have made preliminary measurements on some of these, and shown fluorescence induction curves measured with the imaging apparatus similar to those previously reported using measurement from single colonies. Fluorescence is much higher than with *Synechocystis* 6803 colonies, and the variable component is a larger fraction. There should be no difficulty in screening colonies of *Chlamydomonas* on plates for mutants with subtly different fluorescence phenotypes.

e) **Stomatal patchiness in leaves.**

In collaboration with Dr. Don Ort and Ms. Adriana Ortiz-Lopez we have used the FVIPS apparatus to monitor the segmentation of leaves. Segmentation results in areas in the leaf between which gas exchange is restricted. When a differential opening of stomata occurs in different areas, some are able to carry out photosynthesis, while others (those in which all stomata are closed) cannot. As a result, the fluorescence is high in the closed areas, and they can be readily monitored using the imaging apparatus. By using a macro lens,

the magnification can be adjusted so that an approximately 1 cm^2 area fills the field of view. With a resolution of 512 x 512 pixels, this corresponds to an area of 20 um^2 per pixel, approximately the size of a cell. With this apparatus, the fluorescence induction can be measured from a single pixel, so we can monitor the patchiness (or any other phenomenon leading to differential fluorescence behavior) with excellent spatial resolution.

4) Laboratory based research

The electron transfer chain between the photosystems

a) Redox titration of the b-cytochromes of chloroplasts, and kinetics of redox changes of the b_6/f complex under varied conditions of redox poise, pH and inhibition; the complex works through a modified Q-cycle [8,9,19].

We have re-examined the redox properties and function of the cytochrome b_6f complex in spinach chloroplasts. We found that the two b-type cytochromes in the complex had distinct midpoint potentials and pH-dependencies. Cytochrome b_H was found to have an $E_{m,7}$=-47 mV with a weak pH dependence between pH 7 and 8.75, and a possible pK on the reduced form leading to a -60 mV/pH dependence below pH 6.5; b_L was found to have an $E_{m,7}$=-147 mV and a pH dependence of about -30 mV/pH unit between pH 6.3 and 7.6, then relatively independent of pH up to pH 8.75. We have investigated the kinetic behavior of the cytochromes over a wide range of E_h values, and conditions of inhibition. The rate of reduction of cytochrome b in the presence of the inhibitor NQNO was found to be the same as the re-reduction rate of cytochrome f if the overlapping oxidation and reduction phases were deconvoluted. The results are discussed in terms of a modified Q-cycle model for the function of the cytochrome b_6f complex.

b) Cytochrome b-560; a component with $E_{m,7}$ value of 50 mV which is slowly reduced by the plastoquinone pool, but of unknown function [9,19].

In extensive redox titrations, we have noted that our titrations also included a third cytochrome species with a absorption peak at 560 nm, an $E_{m,7}$=+50 mV and a weak pH dependence (about 14 mV/pH unit) over the pH range 6.3 - 8.75. Cytochrome b-560 has a wavelength maximum (at 560.2 nm) distinct from cytochrome b-559, and the E_m value is lower than that for the low potential form of PS II-linked cyt b-559. In addition, we have shown in separate work that cyt b-560 partitions into the stromal fraction (Javier Fernadez Velasco, Irene Baroli and A.R. Crofts, unpublished results). It seems unlikely therefore that cyt b-560 is related to cyt b-559 of PS II. The function of this component is not yet known.

c) Ferredoxin donates electrons to the plastoquinone pool, rather than to the b_6/f complex, through the enzyme which is sensitive to antimycin [9,20].

We studied the reduction of b-type cytochromes on addition of NADPH in the presence of ferredoxin (Fd). It was found that the vast majority of cytochrome reduced by addition of NADPH and Fd was the species with absorption peak at 560 nm. Only a small (0.2) fraction of b_H was found to be reduced under these conditions. The same fraction of b_H and the 560 nm component could be reduced by a train of actinic flashes or constant illumination in the presence of stigmatellin and KCN. Addition of NADPH/Fd after actinic light in the presence of stigmatellin and KCN did not lead to further reduction of b cytochromes. It was found that reduction of b_H and the 560 nm component could

be separated both kinetically and by their reducibility by ascorbate; in the absence of any mediators, 5 mM ascorbate completely reduced the 560 nm component while not significantly reducing b_H. Both NADPH/Fd and light-induced b_H reduction were reversed by addition of NQNO. This probably indicates that binding of plastoquinol to the Q_C site of the b_6f complex is common to reduction of b_H by both NADPH/Fd and light, in the presence of stigmatellin and KCN. Addition of antimycin A slowed the reduction of b_H by NADPH/Fd, probably indicating the involvement of the antimycin-sensitive ferredoxin:plastoquinone oxidoreductase (FQR). The herbicide DCMU completely blocked the reduction of b_H by actinic light in the presence of stigmatellin and KCN. We suggest that reduction of b_H by NADPH/Fd under these conditions occurs though the plastoquinone pool. The small amount of b_H reduction, given the very strong reducing potential of NADPH, probably reflects the first-order equilibration between the quinol/semiquinone couple at the Q_C site and the b_H/b_H^- couple. Addition of 1,4-napthoquinone to chloroplasts treated with NADPH/Fd, led to a full reduction of b_H and a small reduction of b_L, presumably by a process different from plastoquinone reduction.

Two-electron gate of PS II

a) Physico-chemical characterization of the two-electron gate in susceptible and atrazine resistant strains of *Amaranthus hybridus* [10,11,18].

The kinetics of electron transfer from the reduced primary quinone acceptor (Q_A^-) have been measured in chloroplasts from triazine-susceptible and resistant strains of *Amaranthus hybridus* in the presence and the absence of inhibitors. The decay kinetics in the range from 50 μs to 10 ms can be described by two exponential components. We associate the most rapid component with centers transferring the electron to bound secondary acceptor quinone (Q_B) by a first order process, and the second component with centers in which an electron is transferred only after binding of secondary quinone from the pool by a second order process; a third long-lived component is associated with centers in which the electron is not transferred to an acceptor in this time range. From the kinetics and amplitudes of the two rapid components we estimate the rate constants for Q_A^- oxidation in centers initially either with plastoquinone in the Q_B-site or vacant, and the fractional population of centers in these two states; the latter value allows an estimate of the dissociation constant (K_Q) of plastoquinone from the site. We also measured, as a function of pH, the back reaction of an electron from the acceptor side to the donor side in the presence and absence of excess DCMU for both biotypes to determine the apparent equilibrium constant (K_{app}) for sharing an electron between the primary (Q_A) and secondary (Q_B) quinones of the acceptor complex. From these kinetic and thermodynamic parameters, and their dependence on pH, we have estimated a set of values for the equilibrium constants and rate constants for all processes leading to stabilization of an electron on the secondary quinone acceptor, and shown how these parameters are modified in the triazine resistant strain.

The main modifications in the resistant strain are a change in the pK value of the group stabilizing semiquinone (Q_B^-) at the Q_B-site from 8.1 in the susceptible to 7.0 in the resistant biotype, and a loss of the pK modifying the dissociation constant, K_Q, in the susceptible strain, so that in the pH range 7.0 - 8.0, the quinone binds to the Q_B-site more strongly than in the resistant strain. These two effects explain the lowered efficiency of photosynthesis in resistant biotypes.

We also measured for both biotypes the inhibition of electron transfer

from Q_A^- to Q_B in the presence of a variety of inhibitors. The resistivity is the highest against atrazine, and is the lowest against DCMU; resistivities against o-phenanthroline and a pair of cyanoacrylate derivatives, 964S and 965R, are intermediate. We discuss these changes in physico-chemical parameters for binding and electron transfer in terms of a model structure for the Q_B-site, and the residue change (Ser-264 to glycine, see section 5 below) leading to atrazine resistance in this strain.

b) **Tertiary structural model of the Q_B-binding domain of the D1 subunit of PS II from *Anacystis nidulans* [12].**

The atomic coordinates for the reaction center from *Rps. viridis* have been used as the basis for a model of the Q_B-binding domain of the D1 subunit of PS II from *Anacystis nidulans*. Construction of the model was based on homologous alignment of sequences from green plants and bacteria, and on the assumption of a similar configuration for a) the arrangement of helices in the L- and M-subunits of *Rb. sphaeroides* and *Rps. viridis*, and the D1 and D2 subunits of green plant PS II; b) the ligation of Fe by four histidines from L (D1) and M (D2) subunits; c) aligned residues at which lesions give rise to similar herbicide resistance (especially Ser-264 of D1); and conserved residues in the aligned sequences. The model provides a useful vehicle for speculation about mechanism, and a guide in planning molecular modification to the Q_B-site.

5) Molecular genetics

Development of a method for rapidly sequencing the DNA encoding the Q_B binding domain in chloroplasts and cyanobacteria using the polymerase chain reaction (PCR) and direct DNA-sequencing, and identification of the lesion giving rise to atrazine resistance in a new mutant of *Amaranthus hybridus* [12].

We have developed a method for rapid isolation, PCR amplification, and direct double-stranded sequencing of chloroplast DNA, and used it to detect mutations within the chloroplast-encoded *psb*A gene. DNA encoding the Q_B binding domain was amplified directly from total chloroplast DNA of several higher plants, and from total DNA of algae and cyanobacteria. Using this method, we have detected previously unreported sequence variations in both herbicide-resistant and susceptible strains of *Amaranthus hybridus* within a highly conserved region of the *psb*A gene in which spontaneous mutations leading to herbicide resistance have been previously reported. The resistance to atrazine in the strain sequenced was associated with a change of Ser-264 in the D1 protein of PS II to glycine, the same change as found previously in a different *Amaranthus hybridus* strain, and in several other atrazine resistant higher plants. We have also applied the same procedures to *Pisum sativum* and *Synechocystis* 6803, and been able to amplify the same span of the *psb*A gene using primers developed for *Amaranthus hybridus*. A similar method using different primers has allowed us to amplify directly, from the total DNA of *Chlamydamonas*, the segments from the chloroplast DNA encoding the Y_Z, P680-liganding, and Q_B-binding sites in exons 4 and 5 of the *psb*A gene. The ability to use PCR amplification to extract specific *psb*A coding regions from chloroplast, cyanobacterial or algal sources shows the applicability of this technique to a wide range of photosynthetic organisms. Coupled with direct sequencing, *in vitro* restriction and ligation, and transformation, these methods promise a streamlined approach to site-directed mutagenesis.

PROPOSED RESEARCH

Research is at present progressing well on a broad front, and we are involved in a number of interesting collaborative projects. With current levels of support, we will be stretched to the limit to pursue these appropriately; indeed our present progress has been possible only because of the support for personnel from the McKnight Foundation grant to the University of Illinois photosynthesis group. This latter source will terminate next year.

The general aims of the project will continue to be along the present lines. By basing our intact plant studies on research methods well characterized in parallel laboratory work, we have been able to extrapolate instrumentation, experimental rationale and methodologies to the less tractable field environment. We feel we have demonstrated the success of this approach, and laid the foundation for a promising continuation.

One of the main lessons emerging from our field work [5] has been the observation that the photosynthetic apparatus functions normally under at least one set of adverse environmental conditions. Our collaborative studies under droughting conditions have shown that the flux through photosynthesis, and the partial reactions of the electron transfer chain and photophosphorylation which we are able to probe, were the same in droughted and watered plants, and not obviously damaged in either even under extreme water stress. This is in contrast to preliminary earlier results which had suggested a role for the reactions by which the ATP-ase enzyme is activated in lowering the yield of photosynthesis under conditions of drought. We have suggested [5] that the reduced growth of droughted plants reflects a physiological diversion of resources from biomass synthesis to stress coping mechanisms; indeed the plants in our study were very successful at coping with drought conditions, and yielded quite respectable harvests. It is obviously of importance to see if this lesson can be extrapolated to other stress conditions. If so, then it will be clear that reduced growth under conditions of stress does not reflect a pathological, but a physiological response. In terms of agronomical yields, it would then seem unlikely that any improvement would result from attempts to tinker with photosynthesis.

The main limitation on photosynthesis in both watered and droughted sunflower was the daily decline in response to closing of stomata, reflecting physiological and biochemical adaptation to the diurnally developing need to conserve water. The decline represented a "down regulation" of the biochemical demand for CO_2 which established a balanced limitation on photosynthesis involving both stomatal and non-stomatal factors. In addition, at high light intensities on one very hot day, we observed a dramatic loss of photosynthetic turn-over in both types, presumably as a result of "photoinhibition". This was completely reversible, so we assume that this was also a physiological (rather than pathological) response.

In view of the above, our main effort will be directed towards an understanding of the control of photosynthesis. Classical work suggests that the rate determining step in photosynthesis is in the plastoquinol:plastocyanin oxidoreductase (b_6/f) complex, and is reflected in a rate of oxidation of the plastoquinone pool slower than its reduction, resulting in over-reduction of the pool (8). Although this is clearly the case under some circumstance, much of the evidence from work on intact plants suggests that this is not the main site of control under steady-state conditions (9-13). Instead, a complex set of interacting mechanisms comes into play which balances the redox poise of the electron transfer chain, the poise of the ATP-ase reaction and the flux through assimilatory pathways. We will explore this set of effects, paying

particular attention to the question of the rate determining step under steady-state conditions.

1) The electron transfer chain between the photosystems.

A). The rate limiting step,- does it change with the redox status of the pool or with changes in the proton motive force (pmf)?

The rate determiming step of photosynthetic electron transfer in uncoupled chloroplasts has been attributed to the oxidation of plastoquinol by the b_6/f complex (8) . This is based on kinetic measurements of the individual steps using flash activation, or relaxation from the steady-state. However, several other potential sites for control could be expected to play a role under physiological conditions.

i) We have previously pointed out that the donor side of PS II is a potential site for control (14-19). As the internal pH drops as a result of proton pumping, a strong inhibition of the oxygen evolving reactions occurs, especially for the S_3-S_0 transition, since this involves release of $2H^+/e^-$. This inhibition was observed directly by Wraight et al. (20) in early measurements of the pH dependence of O_2-yield as a function of flash number, and has been studied extensively in this laboratory through the dependence on pH, and development of a pH gradient, of fluorescence and delayed fluorescence measured following flash or continuous illumination (14-19). Many of the physiological effects observed in intact plants and isolated chloroplasts could be explained if the rate determining step was moved to the donor side of PS II:

a) With inhibition of the S-state transitions, P^+680 reduction would be inhibited, and a transiently stable population of P^+680 would be present.

b) Since P^+680 is a quencher this would result in a lowering of fluorescence.

c) In the presence of P^+680, a rapid back reaction from the acceptor side would compete with forward electron transport, and lower the photochemical yield.

d) The inhibition on the donor side of PS II would shift the rate limiting step away from the b_6/f complex. If this were to occur, the controlling effect of the b_6/f complex on the redox state of the intermediate pools would be lost, accounting for the relatively high level of oxidation of Q_A found in the steady-state (21).

e) The mechanism of control suggested above would not necessarily lead to a markedly reduced rate of photosynthesis. Rather it would control the relative flux through PS II so as to maintain an appropriate proton gradient. Dissipation of the proton gradient through ATP synthesis and assimilation would change the balance of exciton flux to restore electron transport. Any backpressure leading to an increased proton gradient would lead to further inhibition of the donor side, increased P^+680, and increased thermal dissipation of exciton input.

All the above effects are seen in plants under steady illumination at saturating intensity, and several workers have suggested that control by the proton gradient at the PS II level could explain them (12, see 9 for a review). The hypothesis above provides a naturally and simply mechanism.

ii) Further controlling parameters arising from the development of a pH gradient as a result of coupled proton pumping might be expected to effect the acceptor side through kinetic and thermodynamic mechanisms. As the pH of the stromal phase rises, it will approach the pK (at 8.1) for protonation of the $Q_A.Q_B^-$ state; at this pH, electron transfer from $Q_A^-.Q_B^-$ to $Q_A.Q_B^{2-}$ is inhibited (22). We have previously discussed the effects of a rise in stromal pH on

the poise of acceptor side protolytic reactions (16). As the pH rises, the secondary quinone and the pool would become more difficult to reduce, favoring back-reactions, and providing an additional component of back-pressure from pmf on PS II.

iii) The half-time for QH_2 oxidation by the b_6/f complex when the pool is reduced is in the range 2-5 ms. However, under these circumstances, the oxidation of Q_A^- shows a half-time of 10 ms (23). It therefore seems likely that the rate limiting step may change with the degree of reduction of the pool. This is to be expected if the reactions of reduction and oxidation involve second-order processes, since the concentrations of the reactants Q and QH_2, and the occupancy of the catalytic sites, will change markedly as the pool becomes reduced.

We will investigate these effects by further studies of the kinetics of the individual electron transfer reactions as a function of the redox state of the pool, pH, and pmf. In the context of the hypothesis outlined in i) above, it will useful to be able to measure the individual partial reactions on the donor side of PS II. We discuss the methodology using spectrophotometric methods in section 6).C below. In order to extend this methodology to intact systems, we will need to construct a UV-responsive version of the portable kinetic spectrophotometer.

B) Competition between PS II and the b_6/f complex.

When the quinone pool is reduced, no acceptor is available for PS II, and the photosystem has to compete with the quinone reductase site of the plastoquinol:plastocyanin oxidoreductase (b_6/f) complex, and possibly with FQR (see below, section D), for oxidizing equivalents generated at the quinol oxidizing site of the complex. No studies have previously been reported on this competition, but it should be readily open for investigation using the spectrophotometric and fluorescence techniques now available to us. We will measure the rates and extents of oxidation at the two sites, from:

i) The extent and rate of redox changes of cyt b_H and cyt b_L; ii) the extent and rate of the slow electrogenic reaction; iii) the rate and extent of oxidation of Q_A^-; iv) the turn-over of PS II on a second flash; v) the turn-over of PS I on a second flash.

C) Activation energy for reduction of the pool.

It has been well established that electron transfer from Q_A^- is inhibited at low temperature (24). However,no extensive study has been made of the temperature dependence of the two partial reactions over a continuous range, measuring the reactions of Q_A^- directly. Such experiments may be of importance in understanding the mechanism of chilling damage in plants. Chilling damage shares many of the lesions seen in photoinhibition at high light intensity, but occurs at relatively low light intensity (25-28). A plausible explanation may be sought in the inhibition of turn-over of PS II at low temperature, which is associated with inhibition at the two-electron gate (24). In the light of current theory, the inhibition of electron transfer observed can be explained in a number of ways:

i) The reaction proceeds through second-order (binding of Q) and first-order (electron transfer) partial processes, and the first-order process has a high activation barrier. This is the pattern we have recently identified in reactions at the Q_Z-site of the b/c_1 complex of *Rb. sphaeroides* (29).

ii) The reaction is as in (i), but the equilibrium constant for binding of Q to the Q_B-site has a high temperature dependence, favoring dissociation at low temperature. In this case, the biphasic kinetic behavior of the system

would be expected to reveal the loss of bound Q_B, with the fast phase disappearing on lowering the temperature.

iii) The reduction of the Q_{pool} is second-order, and the slowing reflects a high activation energy for the process. In this case, the temperature dependence could potentially yield important information about the diffusion coefficient of the pool plastoquinone (30,31).

iv). The reaction is second-order, and the reactants are "frozen" apart by a change in state of the lipid membrane. This mechanism would be expected to yield a broken Arrhenius plot, with a transition at the melting point.

It is clear that a reinvestigation of the temperature dependence of the reactions of the two-electron gate will be informative, and allow for discrimination between possible hypotheses. In order to make these experiments, we will construct an apparatus similar to that of Joliot (24), but incorporating our recent improvements in kinetic fluorimeter design [1].

D) Pathway for reduction of the intermediate electron transfer chain by ferredoxin.

Ferredoxin donates electrons to the intermediate chain as a component of a cyclic electron transfer chain around PS I through an antimycin sensitive reaction (32-34). We have shown that in the presence of stigmatellin, or in CN^- treated (plastocyanin blocked) chloroplasts, reduction of cyt b_H by ferredoxin (reduced by added NADPH or through a glucose, hexokinase, ATP; G-6-PDH, NADP linked-enzyme system) occurs slowly through a pathway which is sensitive to both antimycin and NQNO. Reduction through PS II occurs at a similar rate, but is sensitive to DCMU and NQNO. Since this latter reaction certainly occurs through the plastoquinone pool, it seems likely that ferredoxin reduces the quinone pool through an antimycin sensitive enzyme, and that its reduction of the b_6/f complex is indirect (see Progress Report). Little is known of the (antimycin-sensitive) enzyme which catalyses this ferredoxin:plastoquinone oxidoreductase (FQR) activity, though Bendall and colleagues (33,34), and personal communication) have evidence suggesting that FQR and ferredoxin:NADP oxidoreductase (FNR) may be associated, or two activities of the same protein. We will explore kinetic aspects of this system more thoroughly, and the relation to two other odd phenomena:

i) The free-energy drop from ferredoxin to the quinone pool (about 500 mV) would be sufficient to generate a substantial pmf if the pathway was coupled to proton pumping. In experiments with chloroplasts, the amplitude of the slow phase of the electrochromic change observed at low redox potential suggests a stoichiometry of 1 H^+/e^- for the electron transfer associated with turn-over of the b_6/f complex. With intact algae, Joliot and Joliot (35,36) have reported an amplitude in excess of 1.5 H^+/e^-, which he has explained in the context of a semiquinone cycle for operation of the b_6/f complex. An alternative explanation would be that in the intact system, the cycle around PS I is completed through FQR, via a path which is coupled to proton pumping. In isolated chloroplasts, this complete cycle would be difficult to detect because rupture of the outer envelope would lead to loss of the ferredoxin required for completion of the cycle.

ii) The observation that the high-energy-state dependent fluorescence quenching is sensitive to antimycin at concentrations similar to those required to block the FQR activity suggests that the two processes could be related. This is discussed in the next area proposed for investigation.

2) Photosynthesis in intact plants under high light stress: mechanisms of protection.

Most experimental studies in this area have relied on measurement of fluorescence (reviewed in 37), and strenuous efforts have been made to find experimental conditions and data analysis algorithms which allow information about flux, efficiency, and dissipation to be extracted from such measurements (9,12,13,21,37-46). Several mechanism contribute to changes in fluorescence associated with the protection of PS II against high light intensities, including photochemical quenching (q_Q), non-photochemical quenching (q_{NP}), and state changes associated with phosphorylation of light-harvesting complexes. Two different components of q_{NP} have been resolved, q_E and q_{NP}^z.

A) Quenching of fluorescence associated with the proton gradient (q_E).

Fluorescence lowering associated with the high-energy state was first reported by Murata and Sugahara (47), and the relation to the proton gradient was characterized by Wraight and Crofts (14), who showed that quenching was associated with the rise of the pH gradient across the thylakoid membrane. This quenching has since been heavily implicated in the physiological protective mechanism by which plants cope with high light. The mechanism of quenching is still unclear; however, recent results suggest that q_E quenching is different from the quenching associated with zeoxanthin formation (q_{NP}^z) (see ii) below) in at least several respects: a) the onset of q_E is inhibited by antimycin (48), while q_{NP}^z is inhibited by dithiothreitol (DTT) (7,49); b) q_{NP}^z is associated with a lowering of F_0, and therefore quenching in the light-harvesting pigment bed, while q_E is probably a reaction center located phenomenon (7); c) the two types of quenching show different light intensity dependencies (Demmig-Adams, B., personal communication) ; d) q_E is readily measured in isolated chloroplasts, where q_{NP}^z is seen only in the presence of ascorbate and is much weaker than in leaves (5-7). Despite these differences, it has been suggested that the two types of quenching are related, and that q_E is enhanced by a preillumination which produces zeoxanthin (42).

An intriguing recent result has been the observation that under conditions in which q_E is maximal, there is a stimulation of delayed fluorescence at 50 us ($DF_{(50\mu s)}$) (50). Several aspects of this result deserve comment.

i) We have previously explained the stimulation of delayed fluorescence at about 1 ms ($DF_{(1ms)}$) under conditions giving rise to q_E in terms of an increased probability of back-reactions, associated with stabilization of oxidized components on the donor-side and reduced components on the acceptor-side by the pH gradient (see 15-19 and section 1) A)i) above). It is not clear that the stimulation of $DF_{(50\mu s)}$ monitored in (50) is different in mechanism from that which we have previously characterized for $DF_{(1ms)}$.

ii) The $DF_{(50\mu s)}$ is most likely associated with a back-reaction from $P^+.Q_A^-$ to $P.Q_A$; the quenching effect could therefore occur through two mechanisms:

a) P^+ is a quencher, so would quench directly at the exciton level after its formation.

b) In centers in which the back-reaction occurred, it would be at the expense of forward electron transport, or of stable reduction of Q_A, resulting in loss of photochemical yield, and loss of centers with a high fluorescence yield.

iii) The back-reaction would only occur if a significant population of centers formed P^+680. In this case, any experiments under these conditions purporting to measure the concentration of P^+700 by monitoring absorbance at 820-830 nm (51,52) would likely be contaminated by a relatively slowly relaxing contribution from P^+680 which has a similar spectrum in the near IR.

iv) A direct back-reaction could explain some of the anomalous results from analysis of fluorescence data which have led Horton and colleagues to suggest the existence of a dissipative cycle around PS II (45).

It is clearly of importance to disentangle the current controversy in this area. A significant step in this direction has been the introduction of portable devices to measure absorbance changes at 820-830 nm as a means of following the redox state of P^+700 (51,52). From the considerations above, we feel that it is important to be able to make separate measurement of fluorescence, P^+700 and P^+680, and we propose to build an apparatus similar to that of Schreiber et al. (52) to make this possible. In the meantime, we can use the portable spectrophotometer and multi-flash fluorimeter to probe some preliminary aspects of the problem. In order to be able to measure partial reactions on the donor side of PS II (S-states, Y_Z) we will need to construct a new version of the portable spectrophotometer for work in the UV range.

We will also investigate the antimycin sensitivity of q_E, to find how this effect is related to the antimycin sensitive reduction of the quinone pool by ferredoxin (FQR activity). Both reactions show a titration in the micromolar range (34,48), which precludes explanations based on the uncoupling effect of antimycin. However, Davies and Bendall (34) were able to identify only one tight binding site for antimycin, and that was associated with PS I on digitonin fractionation. We will see if antimycin is an ADRY reagent (53-55), since that would provide an alternative explanation of the effect, and assay other ADRY reagents to test their titre for inhibition of the q_E phenomenon (50), and uncoupling. We will also investigate the effects of halogenated 4-hydroxypyridines, which are alternative inhibitors of the FQR activity (56,57), on q_E. If the two effects do reflect inhibition at the same catalytic site, it might be interesting to follow up the observation that the ferredoxin:NADP reductase-binding protein is identical to the 17 kDa protein thought to be an ancillary component of the oxygen evolution enhancing complex (58), which co-purifies with PS II through several different isolation procedures.

B) Role of xanthophyll cycle; quenching associated with formation of zeoxanthin (q^z_{NP}).

Early studies showing the formation of zeoxanthin from violaxanthin under conditions similar to those leading to q_E quenching (5,6) have recently been extended by Demmig-Adams and colleagues (7,38,39,48). A strong correlation between a part of the non-photochemical quenching sensitive to inhibition by DTT (q^z_{NP}) and the formation of zeoxanthin has been demonstrated, and clearly resolved from q_E. However, it is also clear that this correlation breaks down in the decay kinetics. Zeaxanthin persist much longer than q^z_{NP}, and in such circumstances there is no clear correlation between the zeoxanthin concentration in the leaf, and any component of the q_{NP} (59,60). This indicates at least that additional parameters as yet unresolved must be involved in q^z_{NP}. A related question is the role of ascorbate; Foyer et al. (9) have discussed a possible regulatory function of ascorbate involving a cycle of oxidation in zeoxanthin formation and reduction by NADPH via glutathione, its pathway of synthesis, and its anti-oxidant function.

We have shown that we can readily measure the absorbance change at 505 nm associated with zeaxanthin formation (61,62), using the portable kinetic spectrophotometer, and we intend to pursue preliminary studies of this interesting area with this instrument, making use of the differential effects of antimycin and DTT on q_E and q^z_{NP}. However, more detailed studies will require simultaneous measurement of fluorescence. We propose to construct an apparatus to do this as an alternative optical front end to the machine discussed above.

C) Justification for applying spectrophotometric methods.

Fluorescence induction in green plants is a seductive technique; the relative ease of measurement, and the obvious richness of information have resulted in an extensive literature of patchy quality. Assuming that q_E and q^z_{NP} reflect different mechanisms, it is clear that at least six phenomena contribute to the induction kinetics, and overlap in time: those giving rise to q_Q, q_E, q^z_{NP} and state 1 - 2 transitions, the state of activation of the assimilatory pathways, and the effect of the back pmf on the differential rate of filling and emptying of the quinone pool. There is the further complication of a putative dissapative cycle round PS II (45). Despite this complexity, Weiss and Berry (12,21), building on earlier work (63-65), have had some success in extracting quantitative information from fluorescence induction curves by using a pulse of strong light superimposed on the measuring beam to probe the fractional contribution of Q_A. This allows a deconvolution of contributions from q_Q and q_{NP}, and an estimation of yield, and many groups have adopted this or similar techniques (13, see 9, 37 for reviews). Nevertheless, since only one experimental variable (fluorescence) is measured, it is clear that resolution of the variables corresponding to all the phenomena above can only be achieved by separate independent conditions of measurement which select for dominance of one of these processes at a time. It is not obvious that this has been done, and in some cases the simple analysis clearly breaks down (45). A strong case can be made for measurements based on alternative experimental parameters to supplement those from fluorescence (49-52,62). If such measurements can establish conditions in which the fluorescence measurements yield unambiguous data, then the latter methods can be used with more confidence. By combining measurements of fluorescence with simultaneous measurement of spectrophotometrically detectable changes, we anticipate that we will be able to contribute to this goal.

4) Control of photosynthesis under altered gas environments

The control of photosynthesis under steady-state conditions has received much attention recently, as reviewed by Foyer et al. (9) and Sharkey (10). A major unresolved question is the mechanism of control. The view supported by Foyer at al. (9) is that control does not depend on a thermodynamic feed-back from the poise of the ATP-ase reactants, but on a subtle interplay between several interacting control mechanisms. Sharkey (10) in contrast, suggests that the concentration of inorganic phosphate (Pi) might play an important role in determining the poise of the ATP-ase reactants, and hence the control of electron transport by back-pressure on redox and photochemical proton pumps,- effectively a thermodynamic control of photosynthesis. Although these two mechanisms appear fundamentally different, they could be reconciled if the rate determining step was shifted from the b_6/f complex to the donor side of PS II, as suggested above. In collaboration with Dr. Tom Sharkey, we will investigate some aspects of the problem using the portable instruments developed under the grant, which we will adapt to allow use of gas-exchange cuvettes. This will make possible simultaneous measurement of net photosynthesis in a controlled gas atmosphere, and the spectrophotometric phenomena associated with redox changes in the electron transfer chain, and the coupled H^+ fluxes monitored by the 515 nm electrochromic changes. We will initiate preliminary studies under the present grant, and hope to continue these.

5) Mechanism of herbicide resistance in the two-electron gate

Improvements in agricultural yield have been obtained by selecting or designing plants for the artificial environment represented by modern technology-intensive agronomy. One option realized over the last few years has been the introduction of atrazine resistant crops. The biotypes used have been developed from naturally selected strains, and have the common S264 to glycine modification in the D1 protein of PS II. In algae and cyanobacteria, some herbicide resistant strains have been found with different lesions which show a good resistance but without the penalty of an impaired two-electron gate (66). Crop plants with this phenotype might be more commercially attractive. We intend to continue our laboratory based experiments to understand the mechanism of herbicide resistance, and to explore a variety of mutants, selected or engineered, with a view to identifying factors leading to herbicide resistance without impaired function.

The attempt to understand enzymatic mechanism in terms of molecular structure is a primary goal of molecular biology. In the case of the two-electron gate, the combination of established biophysical methods for assay and analysis of reaction pathways, molecular engineering techniques for modification of the catalytic site, and plausible models, makes study of the relation between structure and function a particularly attractive proposition.

A) Biophysical characterization of mutant strains.

The full biophysical characterization of mutant strains will be both labor and instrument intensive. The following experiments will be performed on strains of interest. We will use the fluorescence methods we have previously developed (3,67-72), and supplement these with absorbance measurements using the flash spectrophotometer. This battery of experiments is the minimal necessary to characterize known parameters of the site. However, preliminary information can be obtained from simple kinetic measurements following a flash ([10,11,18],66,81), and will be used for a second screening.

a) Kinetics of electron transfer as a function of flash number.

b) Apparent equilibrium constant (K_{app}) between $(Q_A^-.Q_B)_t$ and $(Q_A.Q_B^-)_t$

The sharing of an electron between the primary and secondary quinones after a single electron transfer from the dark state is described by an apparent equilibrium constant, K_{app}, which is contributed by the equilibrium constants for electron transfer, K_E, proton binding (with three pK values depending on occunancy of the Q_B-ste), and binding of plastoquinone, K_Q (3,22,67,[17,18]).

c) K_{app} as a function of pH to find values for K_Q, K_E, and pKs.

d) Redox titration of Q_A to find E_m for Q_A/Q_A^- couple.

e) From c) and d), E_m for Q_B/Q_B^-

f) K_I for inhibitors of interest; we will chose a set of inhibitors with representatives from each class. We will use the fluorescence assays previously developed in this laboratory ([17,18]).

g) Rate constants k_{on} and k_{off} for plastoquinone and inhibitors. We will use the flash fluorescence photometer ([17,18]), and the stopped-flow apparatus to measure these parameters, and our recently developed methods.

B) Site-directed mutagenesis of the Q_B-site.

The complementary arm of this project is the development of genetically engineered mutations in the Q_B-site. Professor Lee McIntosh has kindly made available to us the strains of *Synechocystis* 6803 developed in his laboratory (73,74) which have two of the three copies of *psb*A deleted, and the vectors to allow transformation of the third copy (*psb*A2). We will collaborate with Drs.

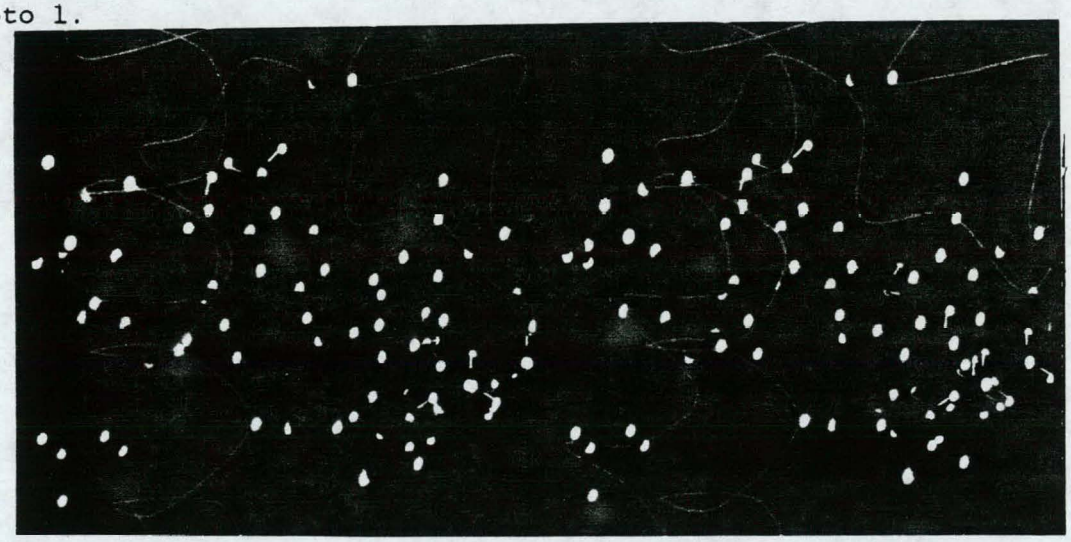
McIntosh and Hirschberg in the development of mutant strains (75-77). In Urbana, we will develop methods based on PCR (78,79,[13]) for making mutated DNA with which to transform the single-copy *Synechocystis* 6803 strain. The FVIPS instrumentation and techniques outlined above will enable us to screen mutants, and make realistic an approach based on controlled random mutagenesis. We will use a variety of PCR based techniques (80) to generate DNA in which a mixed population of mutant forms with approximately one mutation per copy are present, either at a single site, or in a defined segment, and use this to transform.

C) Screening of mutants.

Preliminary screening will be by selection for herbicide resistant strains on plates, using conventional procedures. We will also use the FVIPS method, since many interesting mutants will not show herbicide resistance, but will show a modified fluorescence induction phenotype. We have made extensive studies of fluorescence in *Anacystis* strains in order to be able to follow the kinetics of electron transfer through the two-electron gate (70). The variable fluorescence is small, but can be readily measured. We have made some preliminary measurements of *Synechocystis* 6803 colonies on culture plates using the fluorescence video imaging apparatus [3], and have been able to follow the weak fluorescence induction curves, and monitor the normalized fluorescence yield, and have demonstrated that this apparatus will be useful for screening.

D) Choice of sites for mutation.

We illustrate some choices for mutational sites through stereo models of the Q_B-site with selected side chains shown([12],83). Photo 1 shows residues around the pocket which are either modified in herbicide resistant strains, or otherwise suspected to be involved in quinone or inhibitor binding: (M214; V219, F255, G256, S264, L275 (from herbicide resistant strains; H215, H272 (Fe ligands); A263; L271 (from homology with bacterial herbicide resistance mutants)) ([12],82,83). These are obvious sites for mutation, and some have



already been targeted by Hirschberg for mutational work in *Synechocystis* 6803 (76, and personal communication). While avoiding duplication of Hirschberg's efforts, we will look at these and other residues in the site which appear from the model to impinge on the binding pocket. Some of these (L210, F211*, L218, A251*, L258, F265, F274) are shown in photo 2. A third category of potentially interesting modifications is in the pathway leading to protonation

of the reduced quinone. We have suggested that H252 may be responsible for binding a proton in the Q_B^- state. Diner and colleagues (personal communication) have made preliminary changes at this site, and shown that they interfere with forward electron transfer. H252 is one of a group of polar side chains lining the top of the Q_B-site (photo 3), which are probably involved in relaying protons into the site. In this context, our recent work on characterization of changes in binding parameters for quinone and inhibitors in a S264 to glycine mutant of *Amaranthus hybridus* are of interest (see progress report). The main parameters modified in the mutant were pK values associated

Photo 2.

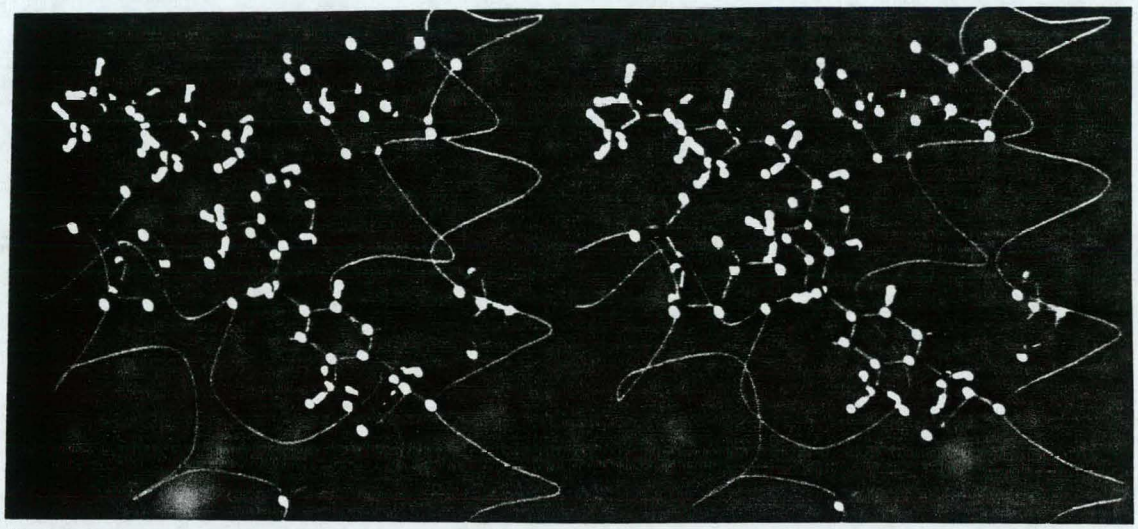
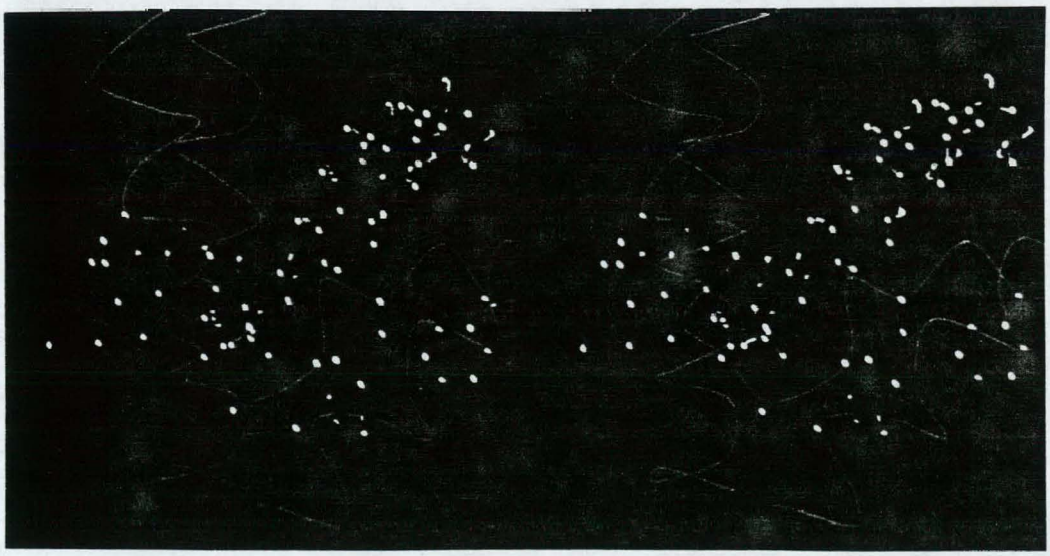


Photo 3.



Models of the Q_B-site of D1 showing groups of potential importance in binding of plastoquinone and inhibitors.

with plastoquinone binding, and with stabilization of Q_B^-. Since serine has no pK in the physiological range, it seems likely that these changes reflect a specific interaction between serine and a dissociable group. In the model, H252 is naturally placed so as to form a H^+ relay with S264. In addition, four other residues (Y262, N266*, N267, S268) are likely able to rotate so as to enter into a H-bonded network including H252. We will therefore explore this span through specific and cassette mutagenesis, and look for strains modified in forward electron transport. (Asterisked residues have recently been seen as modifications in herbicide resistant strains, 66).

E) Characterization of mutants, and site directed mutagenesis in *Chlamydomonas*.

In addition to work on *Synechocystis* 6803, we will also make some preliminary studies to find out how easy it is to do similar work with *Chlamydomonas*. Dr. Jean-David Rochaix has kindly made available to us many herbicide resistant strains of *Chlamydomonas* (81), including some which are not fully characterized, either kinetically or bt sequencing. We have made some preliminary measurements on these with the FVIPS apparatus, and are able to distinguish mutant strains on the basis of their fluorescence phenotype. We have also been able, using PCR, to pull out from whole-cell DNA the portions of exons 4 and 5 in the chloroplast-encoded *psb*A gene which code for the Q_B-site, and sequence them. The missing component of a research strategy for site-directed mutagenesis of the Q_B-site is a method for transformation of *Chlamydomonas*. At least three methods are potentially available:

a) Transformation using glass-bead shaking. Kindle (84) has recently reported a method for transformation of *Chlamydomonas* nuclear genes by shaking cell-wall deficient strains with glass beads. Although no details were given, she also stated that a modified method could be used for transformation of chloroplast genes. We have written to Dr. Kindle to ask her for more details. The simplicity of this approach would make it the method of choice.

b) Introduction of DNA into the cell using a biolistic gun. Dr. John Widholm in the Department of Agronomy at UIUC has a Du Pont gun (85); if the glass beads don't work, we will do preliminary experiments using this apparatus.

c) Dr. Rochaix has kindly given us prior to publication detailed drawings of the biolistic gun designed and constructed in his laboratory (86). He has demonstrated with this device the transformation of chloroplast genes in *Chlamydomonas* (personal communication). If this turns out to be the most effective method, we will construct our own version of this apparatus.

6) Site directed mutagenesis on the donor side of PS II.

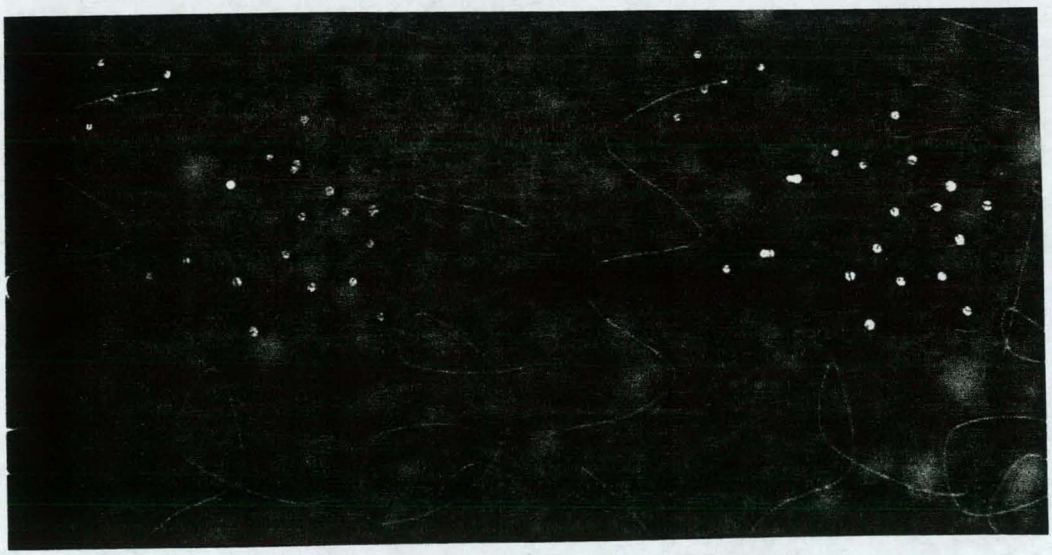
Although there appears to be no immediate prospect that molecular engineering on the donor side will be of benefit to agronomy, there seems a reasonable probability that an understanding of mechanism will be of importance in clarifying the control of photosynthesis, photoinhibition, and UV-damage. Although biophysical techniques have led to a good "black-box" picture of how the oxygen evolving complex works, further advances in our understanding will require that this biophysical skeleton be filled out with some structural flesh. The most direct line of attack is to combine molecular engineering, biophysical characterization, and biochemical resolution,- a necessarily interdisciplinary and collaborative approach.

A) Site-directed mutagenesis of D1 to probe the environment of Y_Z.

We will join Drs. Lee McIntosh and Gerry Babcock in a project using molecular engineering to change the D1 subunit of *Synechocystis* 6803 in order to probe the atomic environment contributing to Y_Z, and to the binding of Mn. Our collaborators have already made some very significant advances in this area, and developed many of the protocols needed (73,74,87). We will be concerned mainly with spectrophotometric characterization of mutant strains, and will also be generating site-specific mutations using the strain of *Synechocystis* 6803 with two copies of *psb*A deleted kindly supplied by Dr. Lee McIntosh. We will use biophysical methods to probe the donor side partial reactions, using direct spectrophotometric observation as the primary methodology (see below), complemented by EPR studies in Dr. Babcock's lab. We will follow the S-state transitions optically, or by EPR detection of S_2 through the multiline or g=4.1 signals, measure Y_D and Y_Z by optical techniques and through EPR signals II_s, and II_f, characterize the interaction of Y_Z with Mn through the EPR-saturation characteristics, and measure kinetics on the donor side through the double-flash fluorescence kinetic assay.

B) Targets for site directed mutagenesis.

In order to facilitate the choice of targets for molecular engineering, we have constructed a model of the Y_Z and Y_D secondary donor sites (73,87,88) based on a template of the *Rps. viridis* structure, using the approach outlined above for the Q_B-site model. The model shows similar environments for Y_Z and Y_D, but with a few striking differences. Both environments are rather polar, as might be expected from the need to stabilize the charge separation on oxidation of Y_Z or Y_D. The Y_Z-site includes a conserved acidic residue (D1-D170) which is replaced by phenylalanine in the Y_D-site. It seems possible that this residue is involved in binding Mn, since otherwise the uncompensated charge of the acidic residue would lower the potential of Y_Z compared to Y_D, an effect opposite to that observed. Our preliminary efforts will be to make more subtle changes to the environment of Y_Z, in order to produce mutants which still retain function. Among these will be changes to the following additional residues which in the model can be seen to contribute to the environment in the Y_Z-site (photo 4): Y161, Q165, D170, Q187, H190, M293, A294



Model of the D1 protein around Y_Z.

Mutants blocked on the donor side should show a low fluorescence yield

because of the presence of P^+680 as a quencher. We will use the FVIPS apparatus to screen for such colonies in continuous illumination. With the flash facility at present being implemented we should also be able to detect qualitatively the site of lesion by counting the number of saturating flashes needed to see a low fluorescence state(89).

C) Experimental procedures for spectrophotometric assay of the S-state transitions, and of Y_Z.

Optical measurements offer the most direct means by which the kinetics of the partial reactions on the donor side can be resolved. Our first priority will therefore be to resolve to our own satisfaction the present discrepancies between measurements of spectra from the different laboratories working in this area (90-95). We have already adapted the Joliot-type spectrophotometer to work in the UV with good kinetic resolution, but have not yet had time to test it extensively in kinetic mode. We anticipate that we will be able to measure S-state transitions and oxidation of Y_Z or Y_D in the UV, and local electrochromic effects associated with oxidation of Y_Z and Y_D in the visible. It will also be necessary to deconvolute the changes associated with semiquinone redox changes.

For the longer term aims of the present project, it is obviously important to be able to resolve the different spectral species in more or less intact green plant systems. We will therefore concentrate our initial efforts on PS II preparations from spinach (BBY (96) and KM (97) particles), as used by Dekker et al. (90), and then extend our studies to chloroplasts, algae and cyanobacteria. The different experimental conditions used by other workers to minimize the problems associated with deconvolution of the S-state spectra all have their disadvantages. It seems likely that at least some of the spectral differences observed reflect differences in conditions. Thus the spectrum of the S_0 --> S_1 transition from Saygin and Witt (93) was resolved by using NH_2OH to delay the S-states; they did not resolve this spectrum by simple deconvolution. Lavergne (94) points out the possibility that the spectrum reflects a specific intermediate associated with NH_2OH oxidation which is not seen in his own work. On the other hand, the deactivation of the S-states by FCCP in Lavergne's work could possibly have given rise to anomalous features, since, although spectra in the presence and absence of FCCP were similar, the S_0 --> S_1 spectrum could only be resolved after deconvolution under conditions in which the S-states and the two-electron gate were decoupled, requiring FCCP. We will concentrate initially on using his FCCP method to facilitate relaxation of the S-states in our own experiments, and we will use a relatively high concentration of acceptor to circumvent the poor kinetics of quinone reduction in the various Triton-X100 preparations (98, and C. Yerkes, unpublished observations). We will also investigate the use of NH_2OH to explore the spectra of S_{-1} and S_0^* (the S states after NH_2OH treatment).

It will be our goal to identify a set of wavelengths, including at least one for each component at which it contributes a major fraction of the change, and to deconvolute the different contributions by matrix analysis of kinetic changes at all wavelengths.

7) Collaborative research projects with intact plants

Our main effort in this section will be to continue and consolidate the collaborations already established, and summarized in the progress report, and the proposal above. Some additional projects discussed are outlined below:

A) Electron transfer in chill stressed plants.

Certain important crops such as tomato are sensitive to damage by chilling at temperatures well above freezing. Dr. Don Ort has been investigating the site of inhibition by chilling in tomato plants, and we have performed some preliminary experiments in collaboration in which we have looked at the rate of turn-over of PS II in chill-damaged plants. In these preliminary experiments we could see no variable fluorescence after flash activation, indicating either a complete inactivation of photochemistry, or complete inhibition of the oxygen evolving apparatus, results similar to those seen after photoinhibition (25-28) by intense illumination or UV (14). We will continue this collaboration, and investigate a wider range of chilling conditions (with and without illumination, etc.), and make use of the field spectrophotometer to investigate other electron transfer processes.

B) Electron transfer and energy conservation in leaves at different levels in the canopy.

In collaboration with Dr. John Cheeseman, we planned to use the portable spectrophotometer on a field trip to investigate canopy effects in Australian mangrove swamps, where halotolerance effects will likely accentuate the canopy stratification. Unfortunately, the instrument was damaged in transit, and could not be used. Dr. Cheeseman has plans to return to Australia, and this would provide a second opportunity for these experiments. Meanwhile, we hope to find time to investigate canopy effects in local woods, and among crops in local fields.

C) Assay of herbicidal effects under field conditions.

For herbicides which act at the level of the two-electron gate, the portable flash fluorescence apparatus developed under the grant is ideally suited to the study of various problems related to the application and efficacy of herbicides under field conditions, and the identification of herbicide resistant biotypes. The portable fluorescence video imaging apparatus will be the method of choice to study these problems.

D) Mechanism of UV-photoinhibition.

In collaboration with Dr. Christine Yerkes, we are using the instruments we have developed to investigate the mechanism of UV-inhibition. Preliminary work was started under the auspices of this grant [14]. We are following the inhibition of donor side reactions by observing the period 4 oscillations in 30 us fluorescence yield as a function of flash number with the kinetic flash fluorimeter. We will continue this collaboration in the next period, using the flash spectrophotometer to look at donor-side reactions.

Apparatus

1) **Construction of an upgraded portable kinetic spectrophotometer**

The present instruments works well in the visible, but cannot operate in the UV because of the glass and plastic used in the light path. In addition, the design of the portable computer which drives the system preceded the introduction of lap-top computers with useful display screens, data storage, I/O ports and expansion slots. We will therefore construct a second version of the field spectrophotometer which will run from a lap-top computer, and incorporate UV optics. This will enable us to study *in situ* not only the changes in the visible spectrum, but also absorbance changes in the UV due to donor and acceptor side reactions of PS II, and the quinone pool. The performance of

the instrument will be improved by incorporating multiple measuring flash lamps with rapid recharge circuitry (as developed for the flash fluorescence machine described above). This will make it possible to measure multiple kinetic points during the first millisecond after flash activation, and therefore to resolve kinetics in the microsecond range (and longer) in a single trace.

2) Fluorescence induction instrument adapted for simultaneous measurement of P^+680 and P^+700. Alternative optical front-end to measure fluorescence and 505 nm absorbance changes

The need to be able to monitor fluorescence, P^+680 and P^+700 independently (see above) requires the construction of a machine to allow this. We will experiment with a dual measuring beam configuration, using laser diodes tuned at 670 and 820-830 nm. The fluorescence will be detected around 730 nm, and the absorbance measured at both wavelengths. By modulating the beams at different frequencies and using tuned detection electronics (52), we anticipate that we will be able to follow fluorescence, and absorbance at 670 and 820-830 nm, independently, and to be able to superimpose continuous illumination. Although changes due to both P^+680 and P^+700 will contribute at both wavelengths, they will contribute differentially, and should be resolved by deconvolution. An alternative optical front-end will be built to allow simultaneous measurement of absorbance changes at 505 nm (61,62) and fluorescence. Since no LED or laser diode emitting at 505 nm is available, modulation of the beam will require a different approach. This would make it difficult to incorporate a 505 nm beam as a part of same optical system.

3) Kinetic fluorimeter linked to a cuvette with Peltier-effect rapid cooling and heating.

We wish to investigate the temperature dependence of the two-electron gate and S-state transitions by constructing a fluorimeter interfaced with a cuvette in which the contents can be rapidly cooled or heated. Joliot and Joliot (1,24) have previously used such an apparatus to study the temperature dependence of both donor and acceptor side reactions. See main proposal for further justification.

4) Portable rapid induction fluorimeter (microsecond time scale).

The present kinetic fluorimeter allows measurement from 20 μs after actinic illumination. At this time we can detect the period 4 oscillations associated with turn-over of the S-states. In order to follow these kinetics on an intermediate time-scale (0-30 μs), we need an instrument capable of measuring the kinetics of fluorescence changes during the flash. We have previously built a laboratory version of such an instrument (18), and used it in a number of studies of donor-side reactions. Advances in analog-to-digital converter and memory technology now make it possible to build a cheap and portable version of this instrument suitable for field use on intact leaves. The instrument will be used to assay the S-state transitions from the period 4 oscillation. By incorporating a second actinic flash, we will be able to assay the kinetics of turn-over of the intermediate reactions if these are inhibited, using the method developed in (89).

5) Portable version of the fluorescence video imaging apparatus.

Several companies are offering video boards to plug into a single slot on the ISA bus, and several portable computer vendors are selling portable ISA-bus (IBM PC-AT compatible) computers. This makes the construction of a portable fluorescence video imaging apparatus relatively straight forward. We have included a request for funds to construct such an instrument, which will have many obvious applications in measurement of fluorescence induction in intact plants.

PUBLICATIONS ARISING FROM GRANT, 1989-1990

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Academic Progress:

1953 Entered Denstone College, Staffordshire, England.
1957 Awarded State Scholarship. Elected Major Scholar in Natural Science, Gonville and Caius College, Universi ty of Cambridge.
1958-61 Undergraduate work in Natural Sciences Tripos Parts I and II, Honours Class 2:1 graduating in Biochemistry.
1961-64 Awarded Medical Research Council Research Assistantship for three years research to Ph.D. in the Department of Biochemistry, University of Cambridge.
1964-65 Appointed Temporary Assistant Lecturer in Biochemistry at University of Bristol.
1965 Dissertation for the Ph.D. degree accepted at the University of Cambridge.
Appointed Visiting Research Physiologist at the University of California, Berkeley.
1966-74 Appointed Lecturer in Biochemistry at the University of Bristol.
1974-77 Appointed Reader in Biochemistry at the University of Bristol.
1978- Appointed Professor of Biophysics, Department of Physiology and Biophysics, University of Illinois at Urbana-Champaign.
1978- Elected Chairman of Biophysics Division, University of Illinois at Urbana-Champaign.

Honours

1958 State Scholarship.
1958 Major Scholar in Natural Sciences, Gonville and Caius College, Cambridge.
1961 Medical Research Council Research Studentship.
1974 Reader in Biochemistry, University of Bristol.
1982 Melandri Lecture and Medal, 2nd. EBEC Meeting.
1983 Appointed Professeur du College de France (Visiting), May-June, 1983
1985 John Simon Guggenheim Fellow.
1989 University Scholar, University of Illinois at Urbana-Champaign

Membership of the Following International and National Boards and Committees:

1971-72 Editorial Board of the Biochemical Journal.
1972-77 Editorial Board of Biochimica Biophysica Acta.
1973-78 Editorial Board, Reviews on Bioenergetics.
1974 Editorial Advisory Board, Perspectives in Membrane Biology.
1975 Advisory Committee, Symposium on Bacterial Photosynthesis, Brussels, 1976.

Science Committee, Fourth International Congress on Photosynthesis, 1977.

1979-83 Editorial Board of the Journal of Bacteriology.

1980-85 Editorial Board of Archives of Biochemistry and Biophysics.

1982 Advisory Committee, University of Illinois-Rikagaku Kenkyusho Joint Solar Energy Conversion Programs.
Grant review panel, Department of Energy.

1985 Organising Committee, Table Ronde, Roussel-UCLAF on 'Proton Pumping in Photosynthesis and Respiration', Paris 1985.

1984-86 Organising Committee, 7th International Photosynthesis Congress, Providence, Rhode Island, Aug 1986.

1988 Grant review panel, Department of Energy

Additional Editorial Reviewing For: Journal of Biological Chemistry, Australian Journal of Plant Physiology, FEBS Letters, Japanese Journal of Plant Physiology, Biochemical and Biophysical Research Communications, Biophysical Journal, Photochemistry and Photobiology, Proceedings of the National Academy, Nature, Plant Physiology.

Additional Reviewing of Applications for Research Support for: National Institutes of Health, National Science Foundation, U.S. Department of Agriculture, U.S.-Israel Binational Science Foundation, and a number of private foundations.

Publications 1985-1990

Crofts, A. R. (1985). The Mechanism of the Ubiquinol:cytochrome c Oxidoreductases of Mitochondria and of Rhodopseudomonas sphaeroides, In: The Enzymes of Biological Membranes, (Martonosi, A.N., ed.), Vol. 4, pp. 347-382, Plenum Publ. Corp., New York.

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CURRICULUM VITAE

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1979 Entered University of Dayton, Dayton, Ohio

1984 University of Dayton, Dayton, Ohio, B.S. in Biology

1986 University of Dayton, Dayton, Ohio, M.S. in Cellular Biology, January, 1986.

1987 Entered Ph.D. program in Biophysics, University of Illinois, Urbana-Champaign

1990 University of Illinois, Urbana-Champain, Ph.D. thesis submitted, June, 1990, in Biophysics

Positions Held:

1976-1983 Laboratory Technician, Hamagami Laboratories, Cincinnati, Ohio. Responsibilities included clinical testing of blood samples: hematology, bacteriology, radioimmunoassays and maintaining laboratory instruments.

1984 University of Dayton Research Grant, conducted research on cell growth and organelle development in *Euglena gracilis*. Constructed a computer-interfaced instrument to measure cell and organelle volume and a kinetic fluorimeter.

1984 University of Dayton, Dayton, Ohio, Cellular Physiology Laboratory Instructor

1985 University of Dayton, Dayton, Ohio, Electron Microscopy Laboratory Instructor

1986-1987 Battelle-C. F. Kettering Research Laboratory, Yellow Springs, Ohio. Worked under Dr. D. Fleischman and Dr. R.Vallegaleti in research on higher plant and bacterial photosynthesis. Designed and

constructed an instrument for the imaging of delayed fluorescence patterns of leaves of intact plants (phytoluminography) and conducted research on environmental stresses on intact plants using this instrument. Also developed a device for fusing plant protoplants for genetic manipulation of higher plants.

1987-1988 Research Assistant. Worked under Prof. Antony R. Crofts--Designed and constructed highly sensitive field and laboratory based double-flash kinetic spectrophotometers and used these instruments to study bioenergetics of photosynethesis and the effects of stress in intact plants under laboratory and field conditions.

1988 Consultant to Battelle-C.F. Kettering Research Laboratory to aid in the development of instrumentation for detection of environmental and chemical stress in plants using fluorescence, delayed fluorescence and gas exchange techniques.

1988-present McKnight Research Fellowship, University of Illinois, Urbana-Champaign, under Prof. Antony R. Crofts. Continued work using the laboratory and field double flash kinetic spectrophotometers. Designed and constructed a highly-sensitive kinetic flash-fluorimeter for measurement of the donor and acceptor reaction of photosystem 2 in intact plants. Involved in collaborative research on the effects of stresses on photosynthesis in field-grown plants.

Other Skills:

Construction techniques: Trained on various metal and wood shop equipment.
Computer Programming: FORTH, BASIC, Fortran, Assembler, and Machine (Z80, 8088, 80286, and 6801 processors).
Electronics: First-hand experience in design of digital and analog electronic circuits, especially computer-interfaced instrumentation.

Honors:

1979 Marianist Scholarship for Academic Excellence University of Dayton, Dayton, Ohio
Hallmark Award for Fine Arts.
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Publications

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