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Progress Report

During this last granting period, a part of our continuing work has been focused on 1) Photosystem II and O_2 evolution and 2) the reaction chain between systems I and II. The development of a new rapid glass pH electrode has enabled us to measure H^+ changes resulting from single turnovers of the photosynthetic system. This technique has been utilized very successfully in both of the above problems.

In addition our work has been extended to direct observations of light-induced electric fields in chloroplasts. The high sensitivity of our technique has permitted measurements of the magnitudes of the electric field as a function of flash number.

In the following pages we briefly summarize the results of various aspects of these studies.

A. Oxygen Evolution

I) Deactivation and Distribution of S_0 and S_1 States in Darkness

During the last year or so we have tried to chemically alter the O_2 evolving system. We approached this problem from two different points of view; (1) changing the rate of deactivation -- presumably by altering the redox state of the electron donor "R", and (2) changing the relative distribution of the S_0 and S_1 states in the dark.

We have developed a polarographic technique which is usable in the presence of external oxidants and reductants and which, unlike the bare electrode, does not impose a reducing environment on the chloroplasts.

The electrode is covered by a silicon rubber membrane and a Millipore membrane holds the chloroplasts on the electrode. A rapid-change flow system is used to expose the sample to selected chemical environments. Some of our more recent observations have been reported at last summer's Gordon Conference; they have not yet been reported in a more formal form. In general, the results to date have been somewhat frustrating, mainly because the "O₂ box" seems to be rather impenetrable. However, we plan to continue our experiments in this direction, since, if ultimately successful, the reward may be substantial. Below is a brief summary of our progress to date:

1) The process of "deactivation" involves the loss of S₂ and S₃ states and the concomitant formation of the S₁ state, presumably via reaction of the type $S_3 \xrightarrow{R} S_2 \xrightarrow{R} S_1$. To interpret our earlier data, Forbush et al⁽¹⁾ had to ascribe the loss of S₂ and S₃ to an endogenous chloroplast reductant "R", having about the same concentration as photoreductant Q. This "R" is not identical to the primary photoreductant Q since deactivation can take place even under conditions in which Q is presumably fully oxidized. When Q is reduced deactivation becomes very rapid, S₃ disappearing with half-time of less than a second.⁽²⁾ This clearly implies the existence of another path--a shortcircuit--in addition to the "normal" reaction with "R".

During the past year we have attempted to slow down deactivation by oxidizing "R" with exogenous oxidants, including very strong ones such as bromine water and potassium iridic chloride. Because these strong

oxidizing agents tend to inactivate the chloroplasts rather rapidly, experiments to date have been rather qualitative. The results show that "R" is not oxidized, even under strongly oxidizing conditions. This suggests that it is somehow shielded from the external environment, probably as part of the " O_2 box."

In the converse experiment, we have tried to reduce R by external reductants. This experiment is more difficult to do "correctly," since R must be reduced without reducing Q ($E_m \sim 0.0$ volt), which would open a second back-flow path for electrons. Phenazine methosulfate proved to be the only reagent which showed a simple behavior. Its reduced form accelerated deactivation, while its oxidized form was ineffective. Benzoquinone did not significantly affect deactivation in either its oxidized or reduced form, while DCPIP accelerated deactivation even in its oxidized state (in the presence of excess ferricyanide). Thus, deactivation does not seem to be simply related to oxidation and reduction, but seems to depend more upon specific molecular properties. In this respect, we might refer to Renger's ⁽³⁾ observations concerning the specific action of what he calls "Adry" reagents (e.g. CCCP). These agents seem to induce a cyclic flow in the system with halftimes as short as 1/10 sec.

2) An entirely different problem -- at least apparently -- concerns our attempts to reduce the S_1 state to the S_0 state in the dark by external reductants, and to reverse this process by adding chemical oxidants. Bouges ⁽⁴⁾ reported that ferricyanide could convert S_0 to S_1 in the dark.

If true, this would have the (unattractive) implications that the reaction $S_1 + e \rightarrow S_0$ has quite low midpoint potential, and that the first of the four light-induced transitions ($S_0 \rightarrow S_1$) needed for O_2 evolution puts very little energy into the system.

Our attempts to vary the S_1/S_0 ratio in the dark by changing the redox potential of the flow medium have confirmed Bouges' observation but not her conclusion. After a pretreatment with 3 flashes, the ratio Y_3/Y_4 is quite low initially, suggesting a low initial ratio of S_1/S_0 . A 30-60 min. exposure to ferricyanide raised this ratio considerably. However, after still longer exposures (up to 24 hrs. in ferricyanide and, in some cases, in potassium iridic chloride) this ratio reverted again to a quite low value. In several experiments we have observed a temporary rise of the S_1/S_0 ratio after a pre-flash pre-treatment in the absence of ferricyanide, simply by keeping the chloroplasts for an hour in sucrose buffer medium. Very long exposures, however, always lead to loss of S_1 states. We have, therefore, tentatively concluded that the increase of S_1 states in darkness may not be a direct effect of chemical oxidation of S_0 by ferricyanide or oxygen, but instead may reflect the utilization of energy somehow stored in the previous exposure to light.

The nature of the stable S_1 state has always been a puzzling aspect of our proposed scheme. Using various reductants we have tried, but never succeeded, in setting the system entirely in the S_0 state. Under mildly reducing conditions, the system readily goes to a low ratio of $Y_3/Y_4 (S_1/S_0)$. However, with increasingly stronger reducing conditions (or longer exposures) a further decline of Y_3 is accompanied by a rise of Y_5 (reflecting the formation

of "S₋₁"?) instead of Y₄. It thus seems that beyond a certain point pairs of electrons enter into the S₁ centers and no further single electron reductions occur. In these same experiments Y₄ also decreases, and this decrease is accompanied by a rise of Y₆, again as if two electrons enter the S₀ trapping centers, resulting in the formation of "S₋₂" states.

These results are similar to those reported by Bouges⁽⁵⁾, who studied O₂ flash yield sequences in the presence of hydroxylamine. She also found a displacement of the oscillation by two, Y₅ and Y₆ (rather than Y₃ and Y₄) being the highest.

We have found that this two electron shift of the oscillation pattern can be brought about by many reducing agents, such as reduced viologen, methylene blue, and pyocyanin. However, concentration and reaction times are very variable, depending upon the particular batch of chloroplasts or their pretreatment. Of the many reagents so far tested hydrazine and hydroxylamine should be singled out; they readily and quite reproducibly induce the "2 electron shift," and thus seem to have easy access to the "O₂ box."

II) Synchrony Between O₂ and H⁺ Release in Photosynthetic O₂ Evolution

We studied the reaction: $2\text{H}_2\text{O} \rightarrow \text{O}_2 + 4\text{H}^+ + 4\text{-e}$

It is known that System II requires the sequential accumulation of four oxidizing equivalents before O₂ is released. We asked the question whether a single proton is released in each oxidation step or whether four

protons are released simultaneously with an O_2 molecule. The following results were obtained:

1) In dark adapted chloroplast suspensions, containing uncoupling concentrations of gramicidin d or methylamine, the flash yield of proton release oscillated in the same way as the flash yield of O_2 . With dark equilibrated chloroplasts a periodicity of four was observed - the yield of the third flash being maximal.

2) Based upon the similar behavior of the flash yields of proton and O_2 liberation we conclude that, following the accumulation of 4 oxidizing equivalents, water is decomposed in a final concerted reaction.

3) Because these oscillations can only be seen in the presence of uncouplers, we conclude that the O_2 evolving system is located inside the thylakoid membrane.

4) In contrast to the relaxation of System II and O_2 release, which occur in < 1 msec after a flash, the efflux time of the protons released in the decomposition of water was ~ 80 msec. From this we conclude that even in the presence of gramicidin or methylamine, there is a diffusion barrier to the movement of these protons.

III) Photoactivation of O_2 Evolving Centers

Previously we published a number of papers describing kinetic analysis of the photoactivation of O_2 evolving centers in Mn deficient algae and NH_2OH extracted algae. We asked if photoactivation might be a more general phenomenon. It had been generally accepted that certain algae cultured heterotrophically in absolute darkness were capable of synthesis of the entire photosynthetic apparatus including the O_2 evolving centers.

We obtained the following results:

1) Such cells lack O_2 evolving centers and the Mn pool associated with such centers but possess fully active System II and I trapping centers and interconnecting electron transport chains.

2) The kinetics of photoactivation of such cells proved entirely similar to those previously described for Mn deficient algae.

3) The capacity to evolve O_2 was paralleled by incorporation of Mn into the O_2 evolving centers.

4) It was concluded that photoactivation of the Mn-containing O_2 yielding catalyst is a fundamental process in all photosynthetic O_2 evolving tissue. Recent data ^(6, 7, 8) from other laboratories tend to confirm this conclusion.

IV) Recombination of Charges by Dark Reactions Within DCMU System II Traps.

Figure I below represents an oversimplified scheme often appearing in the literature depicting separation of charges within a System II trap by absorption of a quantum and subsequent recombination of charges via unknown dark process leading back to the initial ground state level.

Fig. 1

The back-recombination of charges (Z^+ with Q^-) has been reported to be inhibited by NH_2OH ⁽⁹⁾, CCCP^(10, 11), antimycin A^(10, 11), tetramethylphenylenediamine (TMPD)⁽¹⁰⁾ and tetraphenylboron (TPB)⁽¹²⁾. Bennoun⁽⁹⁾

postulated that the inhibition of the back reaction by NH_2OH was a consequence of the reduction of Z^+ by an electron from NH_2OH thus leaving the high fluorescent state (Z-Chl Q^-). A similar explanation was offered by Homann⁽¹³⁾ for his observed effects obtained with TMPD and TPB. On the other hand, Cramer⁽¹¹⁾ attributed the inhibitory effects of NH_2OH , FCCP and Antimycin A to a conversion of high potential cyt 559 to low potential cyt 559, thus implying that cyt 559 (high potential) is a secondary quencher of fluorescence and participating in a cyclic path between Q^- and Z^+ . Implicit in Cramer's hypothesis is that the low potential cyt 559 cannot participate in this back reaction, thus resulting in the locked-in state $\text{Z}^+-\text{Chl}-\text{Q}^-$.

We had noted that the back-recombination of charges was severely inhibited in cells without O_2 evolving centers. We examined the rate of the back-reaction (fluorescence decay in the presence of DCMU) as a function of the concentration of O_2 centers by growth of *Chlorella* in darkness, by extraction of autotrophic cells with NH_2OH (10^{-5} to 2×10^{-3} M) and by autotrophic growth in a manganese depleted medium. Concentration of O_2 centers then was increased via photoactivation and the changes in rate of fluorescence decay was measured along with photoactivation timecourses. We obtained the following results with whole cells of *Chlorella* and/or *Scenedesmus*:

- 1) Extraction of cells with increasing concentrations of NH_2OH (10^{-5} M to 10^{-3} M) followed by exhaustive washings to remove NH_2OH resulted in increasing losses of O_2 evolving centers. Both the initial rate and final extent of the fluorescence decay measured over the range of 50 msec to 30 sec (in the presence of DCMU) were diminished equivalently by NH_2OH extraction.

The rates of fluorescence decay and quantum yields of O_2 evolution proved to be linearly correlated. Results entirely similar to those above were obtained with *Scenedesmus* cells cultured autotrophically with increasing levels of Mn depletion. Culturing of *Chlorella* in darkness with glucose as carbon source led to increasing loss of O_2 evolving centers and increasing inhibition of the fluorescence decay. Again the rate of fluorescence decay and quantum yield of O_2 evolution proved to be linearly correlated. These results indicate a close correlation between concentration of O_2 evolving centers and the rate of fluorescence decay. The above treatments are known to deplete the Mn we associate with O_2 evolving centers.

2) We then inquired whether the increase in rate of fluorescence decay paralleled the increase in concentration of O_2 centers induced by photoactivation. A single exponential time-course was obtained for both parameters. Moreover, compounds which inhibited the photoactivation of O_2 evolution also yielded inhibition of the photoactivation of fluorescence decays.

3) From studies with chloroplasts in which NH_3 was used to inhibit O_2 evolution⁽¹⁴⁾ we arrived to the same conclusion--namely, inhibition of O_2 yielding centers also yielded parallel inhibition of the fluorescence decays. To our knowledge (literature and experimental), NH_3 is not an artificial electron donor to System II, does not extract Mn and does not result in an irreversible loss of O_2 evolution. Moreover, artificial electron donors like tetra-methyl-phenylenediamine, p-phenylenediamine or hydroquinone did not retard DCMU fluorescence decays in chloroplasts in which the O_2 centers

were not perturbed. On the other hand, hydrazine, semicarbazide and other carbonyl reagents which also are reducing agents proved to abolish the fluorescence decays.

All our results thus far clearly reveal that the rate of fluorescence decay (Q^- oxidation?) in DCMU poisoned cells or chloroplasts is a function of the presence of functional O_2 centers and governed, at least in the above studies, by the Mn pool of the O_2 center.

Publication of these results on rates of fluorescence decays as a function of active O_2 evolving centers has not appeared simply because we cannot offer a single specific hypothesis to satisfy all our data or those appearing in the literature. A general explanation for these results (accepting Bennoun's hypothesis⁽⁹⁾) is that the primary electron donor of System II is shielded from reaction with electron donors (either endogenous or artificial) other than $H_2O(OH^-)$ by the manganese containing enzymes responsible for H_2O oxidation. Implicit in this hypotheses is that compounds such as hydroxylamine and hydrazine, in contrast to most System II donors, can equilibrate with the enzymes of O_2 evolution. This general explanation would be consistent with Vernon and Shaw's studies⁽¹⁵⁾ with diphenylcarbazine as an electron donor to System II. They showed that diphenylcarbazine would only react with those System II traps whose associated O_2 yielding centers had been inactivated. This explanation may indicate that our NH_3 data on 1) fluorescence decays and 2) flash yields and quantum yields of O_2 , might imply that this compound, in contrast to NH_2OH , simply causes a reversible dissociation of the O_2 center from the trapping center and thereby explain the (specific) inhibitory effect of NH_3 on O_2 evolution⁽¹⁴⁾.

V) Rate of Photoinhibition as a Function of Functional
 O_2 Centers.

Another part of our research effort during this granting period has been directed towards quantitating the relationship between rate of photoinhibition as a function of concentration of inactive (Mn depleted) O_2 centers. Previously⁽¹⁶⁾ we had noted that cells depleted of Mn were very susceptible to photoinhibition. From more recent observations^(14,17,18) it appears that any inactivation of the O_2 centers leads to a high susceptibility to photoinhibition⁽¹⁹⁾.

In our studies on cell-free photoactivation we encountered considerable variability in our results and we suspected that photoinhibition might be contributing. We have therefore studied the kinetics, action spectrum and quantum yield of photoinhibition varying the concentration of inactive O_2 centers and the concentration of a number of artificial electron donors to System II⁽¹⁷⁾. We felt this information was needed to eliminate at least one variable from our studies on cell-free photoactivation. Briefly, the following results were obtained:

1) Photoinhibition of such tissue saturates, with respect to intensity, like photosynthesis. This contrasts to photoinhibition studies^(19,20) on normal O_2 evolving tissue.

2) The quantum yield profile is similar to that for photosynthesis-- the process is sensitized by System II.

3) Kinetics are second-order with respect to light; the process shows a normal temperature coefficient ($Q_{10}=2.3-2.4$); and the photoinhibition

destroys only events associated with System II. In contrast, the previous studies with normal O_2 evolving tissue and extremely intense light (10 to 2500 times saturation) indicate a first-order, temperature independent process leading to inactivation of both Systems I and II trapping centers⁽²⁰⁾. Clearly, entirely different mechanisms are operating in these two systems.

4) The rate of photoinhibition is proportional to the number of inactive O_2 evolving centers; apparently, there is limited cooperation among units in this photoinhibition.

5) The protection against photoinhibition afforded by artificial electron donors is proportional to their rate of reaction with the oxidant generated photochemically by the System II trap.

6) The loss of ability of System II to sensitize photooxidation of artificial electron donors parallels the loss of variable fluorescence induced photochemically or chemically (in the presence of DCMU) suggesting that the photoinhibition may cause destruction of Q and the System II trap.

B. Kinetics and Interaction Between Photosystems I and II

The interaction between Systems I and II concern both quantum distribution and the movement of electrons from System II to System I. Despite numerous studies on the movement of electrons between the two photosystems, a number of points have remained unresolved: 1) what is the underlying basis for the kinetic inhomogeneity of the two electron pools connecting the photoacts?; 2) what is the electron capacity of the two pools; 3) what is the rate-limiting step of electron transport and what mechanism is involved in this rate limitation; 4) what is the relation between electron and H^+ movement?; and 5) is electron movement obligately linked to H^+ movement and does H^+ movement per se limit electron throughput?

Our continuing work on the above problems has led to the following results during this reporting period. Some but not all of these results have been published.

1. Electron Movement Between Photosystems

- 1) Both the A_1 pool (high K_{equil}) and A_2 pool (low K_{equil}) contain about 5 electron equivalents. The primary acceptor (Q) of System II and the A_2 pool behave kinetically as one identity--possibly two molecules of plastoquinone. The variability in the literature of apparent pool sizes probably is a reflection on a) partial losses of O_2 yielding centers; and b) interaction between chains via cross-transfer between Q- A_2 pools.
- 2) Conditions in which the System II acceptor (Q) is reduced leads to about a 20 fold increase in rate of deactivation of the S_3 precursor state of O_2 . This result implies a short-circuiting

of O_2 evolution by a recombination of charges ($S_3 + A_2^- \rightarrow S_2 + A_2$) and that α , the miss factor in our model of O_2 evolution, is a function of extent of Q- A_2 reduction.

- 3) These conclusions were reached by kinetic analyses of O_2 evolution with chloroplasts in the absence of an added electron acceptor. They were subsequently confirmed and extended in studies in which Hg^{++} was employed to inhibit plastocyanin, an electron carrier component of the A_1 pool.
- 4) With our sensitive glass electrode technique we measured the proton influx associated with passage of an electron from System II to I. The half-time of this influx was ~ 30 msec, independent of the ionophore, gramicidin d. Curiously, the H^+ influx is many times slower than the half-time of electron transfer.
- 5) We also measured the proton uptake ($t_{1/2} \sim 0.3$ sec) ascribed to the reoxidation of the endogenous photoreductant of System I by O_2 . This half-time, viewed by H^+ , proved consistent with our measurements viewed by O_2 .

In addition to the above points (described in more detail in the attached manuscript) we have discovered that protons are not released to the interior of the thylakoid on every flash, but seem to move in pairs. In the presence of an uncoupler the proton release (following a monotonic uptake after each flash) oscillates with a period of 2. Presumably this reflects the movement of electrons in pairs from plastoquinone to System I. Recent evidence obtained by Bouges⁽²¹⁾ and in our laboratory, supports this picture (see page 16). In addition to the information this gives us about proton transport, it also has distinct implications concerning apparent equilibrium constants and kinetics of electron transport between photosystems.

C. Electric Fields Generated by Photoevents in Chloroplasts

The existence of light-induced electric fields across photosynthetic membranes have rested mainly on indirect arguments: 1) Mitchell's concepts of energy conservation for phosphorylation; and 2) Junge and Witt's⁽²²⁾ explanation that the light induced 515 nm absorption change of green cells is of electro-chromic nature.

During this reporting period, we developed methodology which permitted the first direct measurements of an electric field generated by photoevents in photosynthesis. Studies with chloroplasts on the electric field phenomenon yielded the following results:

- 1) Illumination of structurally intact and photochemically active spinach chloroplasts with a short non-saturating flash generates a transient dipole field. Disorganization of membrane structure by physical or chemical means results in loss of signal.
- 2) The rise time of the transient proved extremely rapid ($<1 \mu\text{sec}$), reflecting early photochemical events. In the time range studied ($\sim 2 \mu\text{sec}$ to msec), the decay time of the field was inversely proportional to the conductance of the suspending medium and independent of ion or electron transport.
- 3) With increasing flash intensity, the amplitude of the transient goes through a maximum which implies that the electric field results from two oppositely directed polarizations.
- 4) To explain these observations, we suggest that the electric field arises from the paired membranes of the chloroplasts. The photo-traps are arranged within the membrane, perpendicular to the plane of each membrane. The donors of Photosystems I and II are located on the "interior" of the membrane and the acceptors are located on

the "outside." Excitation of the trapping centers results in movement of electrons to the "outside" of the membrane thus resulting in a polarization of the two membranes in opposite direction. Each Photosystem contributes equally at steady-state to the transient electric field signal.

- 5) The relation of the electric field phenomenon to photo-phosphorylation is as yet unclear. However, Witt⁽²³⁾ has shown an acceleration of the electric field decay by valinomycin. Some of our future planned experiments are directed towards clarification of the extent of contribution of the electric field to photo-phosphorylation. These and related experiments are outlined in the accompanying proposal.

REFERENCES

1. B. Forbush, B. Kok and M. P. McGloin, *Photochem. Photobiol.* 14, 307 (1971).
2. R. Radmer, and B. Kok, *Biochim. Biophys. Acta.* 314, 28 (1973).
3. G. Renger, *Naturwissenschaften* 56, 370 (1969).
4. B. Bouges-Bocquet, *Biochim. Biophys. Acta.* 292, 772 (1973).
5. B. Bouges-Bocquet, *Biochim. Biophys. Acta.* 234, 103 (1971).
6. R. Remy, S. Phung Nhu Hung and A. Moyse, *Physiol. Veg.* 10, 269 (1972).
7. R. Remy, *Photochem. Photobiol.* 18, 409 (1973).
8. M. Margulies, *Biochim. Biophys. Acta* 267, 96 (1972).
9. P. Bennoun, *Biochim. Biophys. Acta.* 216, 357 (1970).
10. P. Homann, *Biochim. Biophys. Acta.* 245, 129 (1971).
11. W. A. Cramer and H. Böhme, *Biochim. Biophys. Acta.* 256, 358 (1972).
12. P. Homann, *Biochim. Biophys. Acta.* 256, 336 (1972).
13. P. Homann, *Biochim. Biophys. Acta.* 162, 545 (1968).
14. S. Izawa, R. L. Heath and G. Hind, *Biochim. Biophys. Acta.* 180, 388 (1969).
15. L. P. Vernon and E. R. Shaw, *Biochem. Biophys. Res. Commun.* 36, 878 (1969).
16. G. M. Cheniae and I. F. Martin, in: *Energy Conversion by the Photosynthetic Apparatus*, Brookhaven Symp. in Biology, 406 (1966).
17. T. Yamashita and W. L. Butler, *Plant Physiol.* 44, 1342 (1969).
18. M. Itoh, K. Yamashita, T. Nishi, K. Konishi and K. Shibata. *Biochim. Biophys. Acta.* 180, 509 (1969).
19. J. Myers and G. Burr., *J. Gen. Physiol.* 24, 45 (1940).
20. L. Jones and B. Kok, *Plant Physiol.* 41, 1027 (1966).
21. B. Bouges-Bocquet, *Biochim. Biophys. Acta.* 314, 250 (1973).
22. H. T. Witt, in: *Fast Reactions and Primary Processes in Chemical Kinetics (Nobel Symposium V)*, (Ed. S. Claesson), p. 261, Almqvist and Wiksell, Stockholm (1967).
23. H. Witt, *FEBS Letters* (1974), in press.