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SUMMARY

A 3-yr research project is being carried out (under Department of Energy Contract DE-AC02-76ER03326) to investigate three different aspects of photosynthesis. Three (for the most part) different technical approaches are being used (i.e., mass spectrometry; biochemical techniques; and a combination of polarographic, spectroscopic, and fluorescence techniques).

In the first group of experiments, a specially designed mass spectrometer system was used to monitor the gas exchange in chloroplasts in response to single short flashes of light. Several gases and their isotopes (e.g., N₂, ¹⁵N₂, ¹⁸O₂, etc.) were monitored to study photosystem II reactions in chloroplasts, particularly those reactions that use donors other than H₂¹⁶O (and evolve products other than ¹⁶O₂). Our goal was to obtain a better understanding of the O₂-evolving system.

The second topic studied was the role of copper in photosynthesis. Biochemical techniques were applied to investigating the role of non-plastocyanin copper in photosystem II- and photosystem I-mediated O₂ uptake via polyphenol oxidase.

The third area of study was light harvesting and electron transport in C₄ plants. Many aspects of carbon flow in C₄ plants seem to be well understood; however, the distribution of photons and the flow of electrons in the two tissues of the leaves of C₄ plants remain enigmatic. We used polarographic, spectroscopic, and fluorescence techniques to gain an understanding of these processes.

In the following sections we describe the significant results obtained during the past 2-1/2 years on this project.
I. MASS SPECTROMETER STUDIES

During the latter part of 1978, we developed a measuring technique for mass spectrometrically monitoring the gas exchange elicited by single, short (< 5 µs), saturating flashes. In this technique, gases are admitted to the mass spectrometer via a silicone rubber membrane (supported by a metal screen). The chloroplast or algal suspension is carefully layered on the membrane and illuminated from above. Since the layered chloroplast (or algal) suspension is on one side of the membrane and mass spectrometer vacuum is on the other, the mass spectrometer can directly sample and analyze gases dissolved in the liquid phase with good time response (< 1 s).

Our primary goal of this aspect of our DOE-sponsored project was to study the PS II-mediated photooxidation of hydroxylamine (NH₂OH) and hydrazine (NH₂NH₂). Both of these components are close analogs of (two molecules of) H₂O, and studies of their system II-mediated photooxidations should provide insight into the mechanisms of the O₂-evolving process. Initially, these studies were done with chloroplasts in which the O₂-evolving system was inactivated by Tris or NH₂OH extraction (to alleviate interferences). We are currently using the results of these studies to probe the intact O₂-evolving system.

Our initial studies, which appeared in Biochimica Biophysica Acta [(1979) 546, 418-425], dealt with the PS II-mediated oxidation of NH₂OH. The summary reads as follows:

"A mass spectrometer with a special inlet was used to directly monitor the products evolved when hydroxylamine-treated chloroplasts were exposed to short saturating light flashes. We found that: (1) Molecular dinitrogen was the sole product of hydroxylamine photooxidation, and was formed in an amount equal to twice the O₂ evolved during H₂O photooxidation. (2) This reaction was driven by photosystem II, and did not involve photosystem I-generated superoxide or peroxide. (3) In the presence of 3-(3,4-dichlorophenyl)-1,1-dimethyl urea, N₂ was evolved only on the first flash. These results suggested that N₂ was formed by the combination of two single-electron oxidation products of hydroxylamine."
Our more recent studies have focused on the processes involved during the photooxidation of hydrazine. We found that, as in the case of hydroxylamine, molecular dinitrogen is the only stable product of hydrazine photooxidation. However, hydrazine (NH\textsubscript{2}NH\textsubscript{2}) photooxidation can also involve a series of secondary reactions with superoxide (O\textsubscript{2}^{-}); these secondary reactions can be eliminated by the addition of superoxide dismutase.

A manuscript describing this work was recently accepted for publication in *Biochimica et Biophysica Acta* (copy attached). The summary is as follows:

"Mass spectrometric techniques were used to directly monitor the products evolved during the course of hydrazine (NH\textsubscript{2}NH\textsubscript{2}) photooxidation by chloroplasts exposed to short saturating flashes or continuous high light. We found that:

(1) Molecular N\textsubscript{2} was the sole volatile product of hydrazine photooxidation. Isotopic studies showed that the N-N bond remained intact during the NH\textsubscript{2}NH\textsubscript{2} \rightarrow N\textsubscript{2} transformation. Under conditions in which spurious side reactions were minimized (see item 3 below), the N\textsubscript{2} yield was equal to the O\textsubscript{2} yield during H\textsubscript{2}O photooxidation.

(2) In the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea, N\textsubscript{2} was evolved, but only on the first flash, suggesting that N\textsubscript{2} was formed by the combination of single electron oxidation products of hydrazine.

(3) In addition to its production by Photosystem II, N\textsubscript{2} can also be generated by a series of secondary reactions mediated by superoxide. This "extra" N\textsubscript{2} evolution can be eliminated by the addition of superoxide dismutase.

Our results indicate that hydrazine can be used as a reliable probe of Photosystem II provided that a) N\textsubscript{2} evolution (rather than O\textsubscript{2} uptake) is monitored, and b) precautions are taken to minimize spurious side reactions. Under conditions in which the participation of superoxide is minimized, N\textsubscript{2} evolution accurately reflects the photooxidation of hydrazine by Photosystem II."

The technical advances made during the course of these studies prompted us to reexamine the long-standing question of whether CO\textsubscript{2} or
HCO\textsuperscript{3} directly participate in the O\textsubscript{2}-evolution process. Earlier, using a different apparatus, we found that the O\textsubscript{2} evolved after reactivation of CO\textsubscript{2}-depleted chloroplasts with HCO\textsubscript{3} reflected the isotopic composition of the H\textsubscript{2}O and not the HCO\textsubscript{3} [Stemler, A. and Radmer, R., (1975) Science 190, 457-458]. However, in these experiments we could not rule out the possibility that a small pool of HCO\textsubscript{3}, slowly exchanging with the medium, was the immediate O\textsubscript{2} precursor. Consequently, we undertook a series of studies of photosynthetic O\textsubscript{2} evolution with \textsuperscript{18}O-labeled substrates. A paper describing this work was published in FEBS Letters [(1980) 110, 57-61]. We reported the following:

1) When CO\textsubscript{2}-depleted chloroplasts were reactivated with \textsuperscript{18}O-labeled bicarbonate, all the O\textsubscript{2} evolved had the isotopic composition of the H\textsubscript{2}O rather than the CO\textsubscript{2}. These results support the idea that neither \textsuperscript{12}C bicarbonate nor CO\textsubscript{2} is the immediate source of photosynthetically evolved O\textsubscript{2}.

2) When these chloroplasts or normal non-depleted chloroplasts were flashed in the presence of H\textsubscript{2}\textsuperscript{18}O added in total darkness, all of the O\textsubscript{2}, including that evolved in the first few flashes, had the isotopic composition of the added H\textsubscript{2}O. This result indicates that the S\textsubscript{1} state does not contain a bound intermediate oxidation product of H\textsubscript{2}O.

3) Although CO\textsubscript{2} depletion did not affect the O\textsubscript{2} evolution system directly, it drastically decreased the magnitude of the ferri-cyanide-induced "anomalous double hit," probably due to an inhibition of reactions on the acceptor side of system II. This inhibition may provide a mechanism for regulating cyclic electron flow in vivo."

Our earlier proposal (August, 1978) also described experiments using our mass spectrometer technique to study the uptake of O\textsubscript{2} and CO\textsubscript{2} by algae elicited by single flashes of light. To carry out and interpret such experiments, a prior knowledge of the kinetics and active species (CO\textsubscript{2} or HCO\textsubscript{3}) in the carbon assimilation process was required. Accordingly, we used our continuous-light mass spectrometer system to study several aspects of carbon assimilation and O\textsubscript{2} reduction, and found that we were able to infer the uptake of HCO\textsubscript{3} and the degree to which this process supplemented CO\textsubscript{2} uptake. A manuscript describing this work appeared in Plant Physiology [(1980) 65, 723-729]. The abstract was as follows:
"Mass spectrometric techniques were used to study several aspects of the competition between oxygen and species of inorganic carbon for photosynthetically generated reducing power in the green alga Scenedesmus. (1) In contrast to the wild type no appreciable light-driven O₂ uptake was observed in a mutant lacking photosystem I. Thus, we conclude that the carbon-cycle-independent reduction of O₂ occurs at the expense of photosystem I-generated reducing equivalents. (2) The commonly observed differences between CO₂-grown and air-grown Scenedesmus with respect to CO₂ uptake and glycolate formation cannot be ascribed to differences in their capacity for light-driven O₂ uptake. We found no intrinsic differences in O₂ uptake capacity between the two physiological types under conditions in which CO₂ was saturating or CO₂ uptake was inhibited. It was only under CO₂-limited conditions that pronounced differences between the two physiological types were observed. This fact suggests that differences in O₂ metabolism and sensitivity between the two types really reflect differences in their capacity to assimilate inorganic carbon; in this respect they are analogous to C₃ and C₄ plants. (3) We tested the hypothesis that air-grown Scenedesmus can assimilate HCO₃⁻ by directly monitoring the time course of dissolved CO₂, O₂ uptake, and O₂ evolution in illuminated algal suspensions at alkaline pH. Since our measuring technique was fast compared to the non-enzymatic equilibration of the inorganic carbon species, we were able to determine the degree to which the CO₂ concentration deviated from equilibrium (with the other inorganic carbon species) during the course of illumination. The observed kinetics in air-grown and CO₂-grown algae in the presence and absence of carbonic anhydrase, and a comparison of these kinetics with theoretical (computer-generated) time courses, supported the idea that air-adapted algae are able to actively assimilate HCO₃⁻ at a high rate. Our data suggest that these algae preferentially assimilate CO₂, and supply the balance of their needs by taking up HCO₃⁻. Since (unlike C₄ plants) these algae have no special "CO₂ pump," and thus have a relatively low affinity for CO₂, HCO₃⁻ assimilation will be the major carbon uptake process at alkaline pH even when the total CO₂ is present in millimolar concentrations."

We recently began studies of the PS II-mediated photooxidation of NH₂OH and NH₂NH₂ (and their analogs) for systems in which the O₂-evolving system is intact. This preliminary work is described in the accompanying proposal.
II. BIOCHEMICAL STUDIES:
COPPER PROTEINS IN CHLOROPLASTS

Although a large amount of information has been collected on the amount, structure, and function of the copper protein plastocyanin, the existence of a second, lamellar-bound, copper-containing protein in the chloroplast has only recently been given serious attention. When plastocyanin was first described, Katoh et al. [Arch. Biochem. Biophys. (1961) 94, 136-141] found that the copper content of the isolated protein accounted for only one half of the total copper in the chloroplast. They suggested two possibilities for the other half: 1) it represented plastocyanin that was so tightly bound to the membrane that it resisted extraction under the conditions used, or 2) it was derived from another, as yet undiscovered, copper-containing protein. Katoh et al. determined that there were 130-160 moles of chlorophyll per gram atom of copper, or about 3 atoms of copper per electron transport chain, if a ratio of 400 chlorophyll molecules per reaction center were assumed. Since one half of this copper is present in plastocyanin, from 1 to 2 moles of copper per reaction center remained unaccounted for.

In light of the large number of suggested copper proteins in the chloroplast* and the small amount of available chloroplast copper to accommodate these proteins (3 gram atoms Cu/400 chlorophylls, half of which is plastocyanin), we reexamined the copper content of spinach chloroplasts. Our early results showed some degree of variability in the copper content -- we measured from 2.7 to 5.3 Cu/400 chlorophylls. Using the technique devised by Plesnicar and Bendall [Biochim. Biophys. Acta (1970) 216, 192-199], we found some variability in spinach chloroplast plastocyanin, but the amount present usually accounted for one half or less of the available copper. On the average, we found 2 plastocyanin molecules/r700 in spinach chloroplasts, a number that agrees with data reported by Haehnel [Biochim. Biophys. Acta (1977) 459, 418-441].

Subsequent work on this topic focused on two areas: 1) photosystem II, and 2) polyphenol oxidase. To detect a Cu protein in PS II, we assayed for Cu and determined the mechanism of inhibition of photosynthetic electron transport reactions by the copper chelator, salicylaldoxime. [1) salicylaldoxime inhibits electron flow at or near a site in PS II, 2) earlier chelator studies implied the existence of a metalloprotein (other than manganese) on the water-to-silicomolybdate pathway, 3) a copper-manganese pigment protein complex implicated in the water-splitting mechanism has been isolated from Phacodactylom tricornumtrum]. We found that salicylaldoxime caused 1) an irreversible loss of the manganese associated with the water-splitting mechanism, and 2) a reversible effect, which we ascribed to suppression of charge separation within the photosystem II trap. We found, however, no evidence to suggest that either mode of inhibition was due to interaction with a copper-containing protein in photosystem II.

A paper describing these findings was published in Archives of Biochemistry and Biophysics [(1980) 202, 458-466]. The abstract reads as follows:

"Salicylaldoxime (1-10 mM) inhibits chloroplast electron transport reactions by a reversible and an irreversible modification of photosystem II. The irreversible inhibition correlates with removal of the loosely bound pool of manganese associated with the water-splitting mechanism. The reversible inhibition is characterized by 1) a suppression of artificial donor reactions, 2) a high initial fluorescence yield, and 3) a decline in the amplitude of the flash-induced electric field across the membrane. After removal of the inhibitor, the initial fluorescence yield declines to near-control levels, but the variable portion of the fluorescence rise remains missing. Addition of an artificial donor restores the variable fluorescence yield and normal electron transport rates to 2,6-dichlorophenolindophenol. Characteristics of the reversible inhibition suggest that salicylaldoxime causes suppression of photochemical charge separation in photosystem II."
We also succeeded in isolating two distinct copper enzymes containing polyphenol oxidase activity (PPO I and PPO II) from the thylakoid membranes of spinach chloroplasts. We initially hypothesized that these enzymes could play a role in electron transport. A paper describing the results of these investigations recently appeared in *Plant Physiology* [(1981) 67, 977-984]. The abstract reads as follows:

"Thylakoid-bound polyphenol oxidase (E.C. 1.14.18.1) has been isolated from osmotically-shocked spinach chloroplasts and purified to homogeneity by ammonium sulfate precipitation, gel filtration, and ion exchange chromatography. The newly isolated enzyme has a molecular weight of 158,000 ± 7,000 on Sephacryl S-200 but, on aging or further purification, partly dissociates to a molecular weight of 42,500 ± 1,500. On SDS polyacrylamide gels, purified polyphenol oxidase exhibits a molecular weight of 42,500.

Sonication releases polyphenol oxidase from the membrane largely in the latent state. C18 fatty acids, especially linolenic acid, are potent activators of the enzymatic activity. In the absence of added fatty acids, the isolated enzyme spontaneously, but slowly, activates with time.

Purified polyphenol oxidase utilizes o-diphenols as substrates and shows no detectable levels of monophenol or p-diphenol oxidase activities. The $K_m$'s for 3,4-dihydroxyphenylalanine and oxygen are 8.5 and 0.065 mM, respectively. Suitable substrates include chlorogenic acid, catechol, caffeic acid, pyrogallol, and dopamine; however, the enzyme is substrate-inhibited by the last four at concentrations near their $K_m$'s.

The large seasonal variation found in the polyphenol oxidase content of spinach chloroplasts is not consistent with a role as an electron transport intermediate in photosynthesis."
III. LIGHT HARVESTING AND ELECTRON TRANSPORT IN $C_4$ PLANTS*

A. EXPERIMENTS USING CORN

In 1978, Hardt and Kok reported that bundle sheath chloroplasts of corn were capable of high rates of $O_2$ evolution [Plant Physiol. (1978) 62, 59-63]. Shortly thereafter, Walker and Izawa reported similar findings [Plant Physiol. (1979) 63, 133-138]. The impetus for this work was an earlier project in which we succeeded in obtaining good rates of $O_2$ evolution in glutaraldehyde-treated spinach chloroplasts (in which the reaction chain between the photoacts is inhibited) by using lipophilic electron acceptors, such as p-phenylenediamine, which presumably penetrate membranes and react directly and rapidly with the primary and/or secondary acceptors of photosystem II. Since most of the literature concerning corn bundle sheath tissue reports low rates of $O_2$ evolution and NADPH reduction, and disconnected reaction chains between the photoacts, we felt that the bundle sheath and glutaraldehyde-treated spinach might be analogous. Furthermore, the distribution of enzymes between mesophyll and bundle sheath did not seem reasonable if the latter tissue were unable to generate photosynthetic reducing power (and thus evolve $O_2$ from water).

Efforts for this project were devoted to three related tasks: 1) finding an unambiguous assay to determine the percent of cross contamination between mesophyll and bundle sheath chloroplasts in a given preparation, 2) developing mild preparatory procedures that would allow isolation of largely intact chloroplasts, and 3) adopting a variety of assay procedures for photosystem II, some of which would measure photosystem II centers that were unable to evolve oxygen.

* This project was primarily under the supervision of Dr. Bessel Kok until his death in April 1979.
We obtained identical results using bundle sheath chloroplasts isolated by non-enzymatic and enzymatic techniques: our studies failed to corroborate the findings of Hardt and Kok. Consistent with the early report of Anderson et al. [Biochim. Biophys. Acta (1971) 245, 253-258], our corn bundle sheath chloroplasts contained only ~20% of the photosystem II activity of mesophyll chloroplasts. We concluded that the amount of photosystem II activity in a given bundle sheath preparation, above a "basal" level of oxygen evolution (150-200 μeq/mg Chl • hr), correlates with the fraction of contaminating mesophyll chloroplasts.

A paper describing these findings was presented at the Fifth International Congress on Photosynthesis, Halkidiki, Greece, on September 7-13, 1980, and will be published in the Proceedings of the Fifth International Congress on Photosynthesis; a copy of the manuscript is enclosed with this report. The abstract reads as follows:

"Several groups have recently argued that mesophyll and bundle sheath chloroplasts of Zea mays have similar photochemical activities (Hardt, H. and Kok, B. (1978) Plant Physiol. 62, 59-63; Walker, G.H. and Izawa, S. (1979) Plant Physiol. 63, 133-138). Since these reports are at variance with earlier work that showed bundle sheath chloroplasts to be severely deficient in oxygen-evolving ability (see Edwards, G.E. et al. (1976) in CO₂ Metabolism and Plant Productivity, pp. 92-108, Burris, R.H. and Black, C.C. eds. Baltimore: University Park Press), we undertook a careful reassessment of the photosystem II content of these two tissues. Our objective was to 1) find an unambiguous assay to determine the % cross contamination of tissues in a given preparation, 2) develop mild preparatory procedures that allow isolation of chloroplasts with a high degree of demonstrated integrity, and 3) adopt a variety of assay procedures for photosystem II, some of which measure photosystem II centers that are unable to evolve oxygen. The experiments utilized chloroplasts isolated by both enzymatic and non-enzymatic techniques. The purity of the chloroplast preparations was determined by fluorescence microscopy, visible microscopy, and chlorophyll a/b ratios. We confirmed by visible and scanning electron microscopy that chloroplasts, and not chloroplast particles or fragments, had been isolated. After the initial characterization, as many assays as possible were performed on each preparation to prevent prep-to-prep variations from biasing the results. Assays included 1) measuring oxygen evolution using phenylendiamine and 2,5-dimethylquinone as electron acceptors, 2) determining variable fluorescence yields with water and tetraphenylboron as electron donors, and 3) spectrophotometrically
assaying the individual reaction centers by measuring the pH change induced by a single flash. In the latter measurement, all photosystem II centers that are photochemically active are included, even if they are unable to evolve oxygen. Our findings indicated that bundle sheath chloroplasts in young leaves of Zea mays have only 20-30% of the photosystem II content of mesophyll chloroplasts. In agreement with most other studies, we found the photosystem I content of bundle sheath to be 1.5-2 times that of mesophyll chloroplasts. The photosystem II/photosystem I ratio varied from 0.19 to 0.30 in bundle sheath chloroplasts and from 1.1 to 1.4 in mesophyll chloroplasts. We conclude that the number of reaction centers (I & II) in the two tissues, per chlorophyll, is nearly the same: the decreased number of photosystem I traps correlates with a complementary increase in photosystem I.

B. PHOTOSYSTEM II ASSAYS

In conjunction with these studies of C₄ electron transport, we undertook parallel studies related to two important aspects of bundle sheath chloroplasts, namely 1) reliable assays of PS II activity, and 2) the cyclic operation of PS I.

Even when chloroplasts have lost their water-oxidizing ability because of harsh treatments, e.g., with Tris, or by aging, the PS II reaction centers are still able to oxidize the artificial electron donor tetraphenylboron (TPB). Because of this property, we used TPB as an assay for PS II in our corn studies. In the course of this work, we made some intriguing observations concerning the secondary reactions of PS II, and the study of TPB effects has gradually evolved into more than a subsidiary project. A paper describing this work recently appeared in FEBS Letters [(1981) 126, 272-276]. Its gist is as follows:

"I describe measurements of flash-illuminated chloroplasts that indicate that the donor side of PS II acts, as required, as a 2-electron acceptor in the oxidation of TPB (oxidation does not occur with every flash). The reactions involve cytochrome b 559, which appears to be alternately oxidized and reduced by PS II in the presence of TPB and a carotenoid. The carotenoid is appreciably oxidized during the reduction of cytochrome b 559."

One of the findings reported in the manuscript is immediately relevant to TPB usage in PS II assays: the assumption of monotonous oxidation, i.e., the idea that every turnover of PS II leads to the repetition
of essentially one series of events, proved to be incorrect. Instead, this process appears to involve "charge accumulation." Each individual PS II center performs two alternate series of secondary reactions: in one series, it oxidizes a TPB molecule and extracts two electrons simultaneously; in another series, it oxidizes no TPB but stores the photo-generated oxidation equivalent and thereby prepares itself for the oxidation of a TPB molecule by a subsequent photoact. Since our estimates of PS II in different chloroplast preparations, i.e., corn bundle sheath and mesophyll chloroplasts, and spinach chloroplasts, were equally affected by this phenomenon, our conclusion, that the PS I/PS II ratio of bundle sheath chloroplasts is considerably larger than 1, remains valid.

C. STOICHIOMETRY AND THE CYCLIC OPERATION OF PHOTOSYSTEM I

For many years, a clear concept of the mechanism of a supposed cyclic electron flow around PS I was lacking, and there was considerable reason for skepticism about the importance of cyclic flow as an in vivo process. This situation has changed, however, over the past few years. In particular, the low PS II/PS I ratio in plants such as corn bundle sheath chloroplasts points to some type of cyclic electron flow.

It currently seems that cyclic flow can be understood, in principle, by a modest extension of linear flow concepts. This development is only partially due to evidence obtained about cyclic flow per se. Equally important is the evolution of our insight into the general aspects of electron flow in the plastoquinone region. Over the past contract period, we made three contributions in this field.

Studies on the mechanism of electron flow between plastoquinone and plastocyanin were described in a paper published in the Proceedings of the National Academy of Sciences [(1979) 76, 2765-2769]. The abstract reads as follows:

"With dark-adapted chloroplasts in which the plastoquinone was oxidized, a partial reduction of cytochrome b₆ was obtained."
upon illumination with a pair of short saturating flashes. The second flash of the pair was much more effective than the first, and the reduction was inhibited by the system II inhibitor diuron. When the plastoquinone pool was reduced, both the reduction and the oxidation of cytochrome $b_6$ were accelerated. The cytochrome $b_6$ oxidation appeared to proceed in association with the reduction of cytochrome $f$, although these cytochromes are not simply connected in series. From these observations it is inferred that electron flow to the secondary donors of system I alternately causes the reduction and the oxidation of cytochrome $b_6$. An interpretation is offered that also accounts for the transmembrane proton translocation that is associated with the oxidation of plastohydroquinone.


Another paper ["Electron and Proton Transfer Events in Chloroplasts During a Short Series of Flashes," FEBS Lett. (1980) 115, 167-170] describes some observations concerning the proton translocation and the electrical field generation by chloroplasts, which demonstrate that the cytochrome $b_6$–$f$ complex is involved in electron flow toward PS I when the plastoquinone pool is largely oxidized. It is clear from this and our other recent work that many phenomena (e.g., cytochrome $b_6$ turnover and secondary electrical field generation) that had been considered as specific for cyclic flow in fact reflect processes that play an equally large part in linear flow. It seems that few reactions are truly unique to the cyclic process. Thus, to gather information specific for cyclic flow, the type and conditions of measurements must be chosen very carefully.


