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PHOTOCHEMISTRY AND ENZYMOLOGY OF PHOTOSYNTHESIS

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ABSTRACT

In the second year of a renewal contract we propose to essentially continue the three basic tasks in progress under the present contract and described in our Proposal No. BI 78-13R, dated 1 August 1978:

1. Mass Spectrometer Studies: Measurements in Flashing Light
2. Biochemical Studies
3. Light Harvesting and Electron Transport in C₄ Plants.

In the first task, we will make use of a specially designed mass spectrometer system to monitor the gas exchange occurring in response to single short flashes of light. This apparatus will be primarily used to study photosystem II donor reactions, such as the photooxidation of hydroxylamine, hydrazine, and hydrogen peroxide. We also intend to use this technique to study the light-induced exchange of O₂ and CO₂ in algae.

The second task, biochemical studies, will focus on the role of chloroplast copper in photosynthesis. We propose to isolate, purify and characterize the chloroplast copper enzyme polyphenol oxidase, and attempt to elucidate its role in photosynthesis. These studies will be integrated with a new program (not in our original proposal) devoted to the biochemical response of the photosynthetic membrane to stress.

The third task is a series of studies on the light-harvesting and electron-transport mechanisms of C₄ plants. This program will address three basic problems: 1) the effect of different preparative procedures on various photosynthetic reactions, with particular emphasis on photosystem II reactions in corn bundle sheath chloroplasts; 2) the development and testing

of photosystem II assays; and 3) studies of the stoichiometry of electron carriers in bundle sheath chloroplasts, and whether cyclic phosphorylation could be a major pathway in this tissue.

INTRODUCTION

This combined progress report and renewal proposal describes the current status of our DOE-sponsored programs and the significant results obtained to date (for the period of November 1978 to July 1979). Some of this work was described earlier in our five-month Progress Report, April 1979. The general outline is that of our previous proposal (No. BI 78-13R, August 1, 1978).

The general format of this report/proposal follows the basic outline of our earlier proposal and the task structure of the present contract. Section numbers correspond to the three tasks, and each section contains first a report of progress to date under that task, followed by a specific proposal for work to be continued under that task. In cases where the focus of a particular task or subtask has changed little during the past year, the proposed work description is brief. For tasks that are new or have been substantially changed, there is a somewhat detailed description of the newly proposed work.

I. MASS SPECTROMETER STUDIES: MEASUREMENTS IN FLASHING LIGHT

Our recent proposal (BI 78-13R) described a technique to monitor the gas exchange elicited by a single short saturating light flash. During the past year we completed a series of studies in which we used this apparatus to study the photosystem II-mediated oxidation of hydroxylamine. A summary of these studies, which appeared recently in *Biochimica Biophysica Acta* (546, 418-425), 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 O_2 evolved during H_2O 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, N_2 was evolved only on the first flash. These results suggested that N_2 was formed by the combination of two single-electron oxidation products of hydroxylamine."

The results of these studies prompted us to reexamine the long-standing question of whether CO_2 or HCO_3^- directly participate in the O_2 -evolution process. Earlier, using a different apparatus, we found that the O_2 evolved after reactivation of CO_2 -depleted chloroplasts with HCO_3^- reflected the isotopic composition of the H_2O and not the HCO_3^- (A. Stemler and R. Radmer, *Science* 190, 457-458, 1975). However, in these experiments we could not rule out the possibility that a small pool of HCO_3^- , only slowly exchangeable with the medium, was the immediate O_2 precursor.

Using the NH_2OH -DCMU chloroplast system described in the above summary as a control, we recently were able to demonstrate that the observed O_2 was the first O_2 evolved after the addition of (^{18}O -labeled) HCO_3^- . The isotopic composition of this O_2 supports the idea that neither HCO_3^- nor CO_2 is the immediate precursor of photosynthetically evolved O_2 .

Our earlier proposal described experiments to use mass spectrometer technique to study the light-driven uptake of O_2 and CO_2 by algae elicited by single flashes of light. In order to carry out and interpret such experiments, a prior knowledge of the kinetics and active specie (CO_2 or HCO_3^-) of the carbon assimilation process was required. To this end, we recently used our continuous-light mass spectrometer system to study several aspects of carbon assimilation and O_2 reduction. An interesting aspect of these studies is that we were able to infer the uptake of HCO_3^- , and the degree to which this process supplemented CO_2 uptake. A manuscript describing this work was recently submitted to Plant Physiology, a copy of which is enclosed (Appendix A). Its abstract reads:

'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 O_2 uptake was observed in a mutant lacking photosystem I. Thus, we conclude the carbon-cycle-independent reduction of O_2 occurs at the expense of photosystem I-generated reducing equivalents. (2) The commonly observed differences between CO_2 -grown and air-grown Scenedesmus with respect to CO_2 uptake and glycolate formation cannot be ascribed to differences in their capacity for light-driven O_2 uptake. We found no intrinsic differences in O_2 uptake capacity between the two physiological types under conditions in which CO_2 was saturating or CO_2 uptake was inhibited. It was only under CO_2 -limited conditions that pronounced differences between the two physiological types were observed. This fact suggests that differences in O_2 metabolism and sensitivity between the two types really reflect differences in their capacity to assimilate inorganic carbon; in this respect they are analogous to C_3 and C_4 plants. (3) We tested the hypothesis that air-grown Scenedesmus can assimilate HCO_3^- by directly monitoring the time course of dissolved CO_2 , O_2 uptake, and O_2 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 CO_2 concentration deviated from equilibrium (with the other inorganic carbon species) during the course of illumination. The observed kinetics in air-grown and CO_2 -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 HCO_3^- at a high rate. Our data suggest that these algae preferentially assimilate CO_2 , and supply the balance of their needs by taking up HCO_3^- . Since (unlike C_4 plants) these algae have no special "CO₂ pump," and thus have a relatively low affinity for CO_2 , HCO_3^- assimilation will be the major carbon uptake process at alkaline pH even when the total CO_2 is present in millimolar concentrations."

PROPOSED WORK

We plan to continue this project basically according to the plan outlined in Proposal BI 78-13R, although the priorities have changed somewhat. As described above, we obtained significant results concerning the (lack of a) role of HCO_3^- or CO_2 in O_2 evolution. Our work in this connection, along with completing the NH_2OH oxidation studies, superseded our originally-proposed first-line studies.

We are currently studying the low- NH_2OH problem described in our earlier proposal. Subsequent photosystem II donor studies will be devoted to the photooxidation of hydrazine and H_2O_2 ; both of these topics were outlined earlier.

We plan to defer the flashing-light studies on algae until the completion of the photosystem II donor experiments (i.e., hydroxylamine, hydrazine and peroxide). The algal studies will probable be done in conjunction with continuous light experiments similar to those described in the enclosed manuscript.

II. BIOCHEMICAL STUDIES

A. Chloroplast Copper Proteins

1. RuDP-Carboxylase-Oxygenase

This aspect of the program is no longer being pursued. We are satisfied with McCurry *et al.*'s demonstration (Biochem. Biophys. Res. Commun. (1978) 84, 895-900) that Ribulose-1,5-bisphosphate carboxylase/oxygenase elutes from Sepharose 6B as a single peak and that the purified enzyme contains less than 0.2 g atoms copper per mole of enzyme.

2. Copper Chelators and Photosystem II

In the past year, we studied the effect of the copper chelator salicylaldehyde on electron transport reactions, fluorescence, and the 515 nm absorbance change in spinach chloroplasts. We found that salicylaldehyde inhibits electron transport reactions at two distinct sites in photosystem II. The first is an irreversible site which involves the loss of the loosely-bound pool of manganese from its locus in the water-splitting mechanism. The second is a reversible site which involves the ability of salicylaldehyde to rapidly interfere with reactions closely associated with primary photochemistry. Among the effects at this site are a) the suppression of artificial donor activity, b) a high initial fluorescence yield (F_i) upon illumination, and c) a decreasing amplitude of the 515 nm absorbance change with increasing concentrations of salicylaldehyde. After removing the inhibitor by washing, there is a resumption of artificial donor activity and a lowering of F_i to near-control levels. The inclusion of an efficient electron donor (NH_2OH) at this point allows a near-normal fluorescence rise curve to be established. These data suggest that salicylaldehyde rapidly and reversibly interferes with charge separation within the photosystem II trap.

We could find no evidence to suggest that salicylaldehyde inhibits a copper-containing protein in photosystem II: 1) The loss of the loosely-bound pool of manganese adequately explains the irreversible site of inhibition. We found no loss of membrane-bound (extra-plastocyanin) copper at the concentrations of salicylaldehyde used to inhibit photosystem II. 2) We were able to isolate a photosystem II particle from the membrane that was almost devoid of bound copper. We found that the inhibition of this particle resembles quite closely the inhibition observed in normal chloroplasts. The concentration of salicylaldehyde necessary to half-inhibit the artificial donor activity in this particle is ~ 2 mM, compared to ~ 3 mM in chloroplasts.

These data indicate that a copper protein can be virtually eliminated as the target site of salicylaldehyde for either mode of inhibition in photosystem II. Moreover, if a copper protein does exist in photosystem II, it must function prior to the artificial donor feed-in site ("Y₁"), close to the water-splitting mechanism.

A manuscript describing these findings is in preparation.

3. Polyphenol Oxidase

In spite of the apparent failure to locate a copper protein in photosystem II using chelators, we have succeeded in isolating a copper-containing protein from the chloroplast lamellae. Our search has led directly to polyphenol oxidase (O-diphenol:oxygen oxidoreductase) found in large amounts in spinach chloroplasts. Our studies indicate that the enzyme cannot be removed from the chloroplast by repeated washing, even in hypotonic medium, which shows that it is not simply a contaminant in the chloroplast preparation. The enzyme can be inhibited with KCN and diethyldithiocarbamate, a behavior

consistent with its assignment as a copper-containing protein. We found that the enzyme is tightly bound to the lamellae but can be released by sonication in a low ionic-strength medium. Using ammonium sulfate fractionation and sequential gel chromatography on Sepharose 6B and Sephacryl S-200, the protein can be purified to homogeneity.

On Sephacryl S-200, all activity is centered in two peaks, one at molecular weight near 140,000 and the other at or near molecular weight 260,000; the high molecular-weight peak is probably dimer of the first. SDS gel electrophoresis shows three proteins bands, and by comparison with proteins of known molecular weight, these peptides correspond to molecular weights of 130,000, 66,000 and 50,000 daltons.

Upon isolation, polyphenol oxidase shows no dopa-mediated oxygen uptake unless activated. The activation process can be accomplished with any C_{18} - C_{20} saturated or unsaturated fatty acid, as well as with trypsin. The only stipulation is that the carboxyl group of the fatty acids must be free; methyl esters of the C_{18} fatty acids are totally ineffective, as are triglycerides. Because of the low amounts of fatty acid required for activation, we suspect that trypsin activation (Tolbert, N.E., Plant Physiol. 51, 234-244) is actually due to fatty acids that are contaminants in the commercially-prepared enzyme.

PROPOSED WORK

Progress in stabilizing, activating and purifying (to homogeneity) polyphenol oxidase has allowed us to begin a physical-chemical characterization. We have recently found that the protein shows maximum stability at low pH (the half-life of the isolated protein is typically 2 weeks at pH 5.5 vs. 24 hours at pH 8.5). In the past, the rapid degradation of activity severely

handicapped our ability to work with polyphenol oxidase.

We are now in the process of determining the molecular weight, subunit structure, and metal content of the purified enzyme. After this work is completed, we intend to investigate the types of reactions that the enzyme is capable of carrying out. Since the protein can interact with molecular oxygen, other oxidase activities of physiological significance to the chloroplast, such as superoxide dismutase, catalase and/or peroxidase activities, will be tested with the purified protein.

The activation process is particularly intriguing. We believe that there are two possibilities: 1) The enzyme catalyzes a completely different reaction in vivo (catalase, dismutase, peroxidase, etc.), but exposure to linolenic acid allows a completely different, nonphysiological reaction (oxidation of L-dopa) to become manifest, or 2) The activation by linolenic acid is the key to the function of this enzyme, which is to produce anaerobiosis upon the liberation of linolenic acid from the photosynthetic membrane.

In order to examine the latter idea more fully, we needed to investigate some of the physiological responses of the chloroplast to fatty acids. In particular, we were interested in determining what effect linolenic acid had on the photosynthetic electron transport chain, especially at concentrations where the polyphenoloxidase enzyme became active in oxygen uptake. This work is described in the following section; because of its only recent relevance to the polyphenol oxidase problem, it was not included in the original proposal.

B. Stress Biochemistry and the Photosynthetic Membrane

1. Mechanism of Linolenic Acid-Induced Inhibition of Photosynthetic Electron Transport - Evidence for a New Mode of Inhibition in Photosystem II

In this study, we were interested in the effect of 10-100 μM linolenic acid on isolated spinach chloroplasts. We already knew from previous work in our laboratory that linolenic acid inhibited photosystem II (Hill activity) at low concentrations ($< 100 \mu\text{M}$) and photosystem I at higher concentrations ($\sim 200 \mu\text{M}$). However, we had no explanation for the mechanism of this inhibition. What gave the work particular impetus was the fact other laboratories reported that the damage to the photosynthetic membrane observed during stress and aging correlated with the presence of free fatty acid.

Consequently, we investigated the effect of linolenic acid on isolated chloroplasts and were able to: 1) Characterize the sites of inhibition in the electron transport chain, and 2) Provide a satisfactory biochemical explanation for the effect at the various sites. A paper describing this work has been submitted to Plant Physiology, and a copy of the manuscript is enclosed with this report/proposal (Appendix B). Its abstract reads as follows:

The effect on linolenic acid on photosynthetic electron transport reactions in chloroplasts has been localized to a site on the donor side of photosystem I and to two functionally distinct sites in photosystem II.

In photosystem I, an increase in the electron transport rate occurs in the presence of 10 to 100 μM linolenic acid, followed by a decline in rate from 100 to 200 μM linolenic acid. The increase may be due to an alteration of membrane structure that allows greater reactivity of the artificial donors DIPIP or TMPD with plastocyanin. The decrease is most likely the result of the loss of plastocyanin from the membrane since the addition of purified plastocyanin to the washed chloroplasts leads to the reestablishment of photosystem I rates.

In photosystem II, reversible and irreversible sites of inhibition have been located. At the irreversible site, there is a time-dependent loss of the loosely-bound pool of Mn implicated in the water-splitting mechanism. At the reversible site, the photochemical charge separation is rapidly inhibited as evidenced by the high initial fluorescence yield (F_i) upon illumination and the inhibition of artificial donor reactions in NH_2OH -washed chloroplasts. When the chloroplasts are washed free of linolenic acid, F_i returns to its original low value and there is a resumption of artificial donor activity from DPC + DPIP. This reversible inhibition of the photoact is clearly a unique characteristic of linolenic acid and indicates evidence for an entirely new mode of inhibition of photosystem II.

2. Stress and the Biochemical Response of the Photosynthetic Membrane

The ability of linolenic acid to "turn off" photosystem II is a curious phenomenon; other photosystem II inhibitors act either on the reducing side of the photoact (DCMU) or on the oxidizing side (CCCP, NH_2OH). In contrast, linolenic acid suppresses charge separation within the photochemical trapping center.

We believe that this phenomenon, as well as the activation of polyphenol oxidase by linolenic acid, may be a physiological response of the photosynthetic membranes to stress. Consider the following scenario: stress induced by aging or heat usually results in the hydrolysis of lipids within the photosynthetic membrane. Half of those released are in the form of the triunsaturated fatty acid, linolenic acid. Unchecked, the free linolenic acid has widespread potential for harm: 1) It causes the release of manganese from the water-splitting mechanism. In the absence of a suitable electron donor, the oxidant created by photosystem II causes rapid bleaching of the photosynthetic pigments (chlorophyll, carotenoids); 2) The oxidation of linolenic acid to malonaldehyde is known to be a free-radical chain reaction. Once this process

begins, there is potential for widespread damage to the membrane, since the process is autocatalytic. The end product, malonaldehyde, is, in turn, a potent protein cross-linking agent which has the ability to inactivate membrane-bound proteins. Membranes which contain bound iron-sulfur proteins are particularly susceptible, since decomposition of these proteins in the presence of oxygen may generate radicals which would initiate lipid autooxidation.

Now, consider the effect of linolenic acid on the photoact and on polyphenol oxidase. In the first instance, linolenic acid suppresses charge separation. If no photochemical oxidant is created, pigment decomposition will not occur. In the second instance, linolenic acid activates the polyphenol oxidase enzyme, causing oxygen uptake in the presence of a suitable reductant. If the chloroplasts were to become anaerobic, the oxidation of linolenic acid to malonaldehyde and the decomposition of the bound iron-sulfur proteins could not occur.

After the stress has been relieved, repair mechanisms could replace manganese and the photoact would resume. Our results show that the effect of linolenic acid on the photoact is reversible, but we have not yet investigated the ability of the polyphenol oxidase enzyme to "turn off" after the removal of fatty acids. We do know that the concentration of linolenic acid necessary to remove the manganese (and suppress the photoact) is in the same range as that required to activate the polyphenol oxidase enzyme within the chloroplast; in fact, the complementarity between these two processes is striking.

PROPOSED WORK

We propose to build upon these ideas by initiating a series of studies devoted to stress biochemistry and the response of the photosynthetic membrane.

We propose to study this phenomenon by addressing the following questions:

- 1) Are the relative rates of bleaching of chlorophyll and carotenoids modulated by the ability of linolenic acid to "turn off" photosystem II? Bleaching studies in the presence of other photosystem II inhibitors have been performed by other groups; but our interest is to determine whether linolenic acid is effective at physiologically significant concentrations. Since we can remove the manganese with 0.8 M Tris or 5 mM NH_2OH without affecting the photoact, we can monitor the relative rates of bleaching in the presence and absence of linolenic acid. We will also monitor the relative rates of malonaldehyde formation under the same conditions as a measure of membrane breakdown.
- 2) What is the role of polyphenoloxidase under conditions of stress? We know that the enzyme can be inhibited with KCN or azide. We will assess the importance of polyphenoloxidase in preventing lipid autooxidation by measuring the rate of malonaldehyde formation in the presence and absence of the enzyme inhibitor. Stress will be applied by high intensity light, heat and/or age. Since the membrane breakdown is autocatalytic, the response should be rapid and easily measured.
- 3) Do the bound iron-sulfur proteins play an important role in initiating lipid hydrolysis? The bound iron-sulfur centers can be inactivated with 2 M urea - 5 mM ferricyanide. If destabilized iron-sulfur proteins or their decomposition products are potent catalysts or initiators of lipid autooxidation, there should be an increased rate of malonaldehyde formation following destabilization. Linolenic acid should protect against this process by activating polyphenol oxidase and removing molecular oxygen.

III. LIGHT HARVESTING AND ELECTRON TRANSPORT IN C₄ PLANTS

A paper from this laboratory entitled "Comparison of Photosynthetic Electron Transport Activities of Spinach Chloroplasts with Those of Corn Mesophyll and Corn Bundle Sheath Tissue" recently appeared in Plant Physiology (Hardt, H. and B. Kok, Plant Physiol. 62, 59-63, 1978). One of the major conclusions of this paper was that corn bundle sheath chloroplasts are capable of high rates of O₂ evolution. However, data presented in the same paper also showed that there was twice as much P₇₀₀ per chlorophyll in the bundle sheath as in the mesophyll or spinach preparations. Since all three tissues have the same photosystem I cross-sections, this would imply that all the pigment in the bundle sheath was devoted to system I, leaving no room for any photosystem II.

Subsequent work in this area has been devoted to understanding and rationalizing these apparently contradictory results. Probably the most surprising outcome was that, contrary to the results of Hardt and Kok, it was possible to get pure ($\geq 95\%$) bundle sheath preparations by both enzymatic and differential grinding techniques that had very low system II content, assayed by several different techniques (e.g., fluorescence and pH changes in the presence and absence of artificial donors, O₂ evolution, etc.).

There are several plausible reasons for the discrepancy between the earlier results of Hardt and Kok, and the later results (which, until the last few months, were also obtained under the supervision of Kok). A few, in no particular order are: 1) Differences in the ages of the leaves used; young leaves often have more photosystem II than mature leaves (Raghavendra, A. and V. Das, Plant Sci. Lett. 12, 355-360, 1978); 2) Contamination of the

bundle sheath preparation (by mesophyll) in the experiments of Hardt and Kok; their a/b ratios were often quite low. 3) Preparation procedures that yield pure bundle sheath chloroplasts may selectively inhibit photosystem II; this may be particularly significant with some enzyme techniques.

In an effort to determine and monitor the quality of our preparations we have used several techniques:

1) Scanning Electron Microscopy. The three preparations were fixed in glutaraldehyde before examination. The fixation techniques can be quite tricky, and we claim no special expertise. Still, we could observe striking differences between the bundle sheath and mesophyll preparations; both preparations contained complete (i.e., non-fragmented) chloroplasts and no discernable "particles".

2) Fluorescence Microscopy. Since the two types of corn chloroplasts have widely disparate fluorescence characteristics, this technique provided a ready means to taken an accurate census of the two preparations. Surprisingly, we observed a higher percentage of bundle sheath chloroplasts in the mesophyll preparation than vice versa.

3) Fluorescence Micrography. Using a Zeiss Universal Microscope, we were able to obtain high-quality phase-contrast and fluorescence micrographs of the same field. Both showed that the preparations contained complete, non-fragmented chloroplasts with different characteristic morphologies. Even more striking were the results obtained when the fluorescence micrographs were taken using infra-red sensitive film (Kodak Ektachrome IE 135-20). Under these conditions the fluorescence emitted by the three preparations appeared as three different colors!

It should be evident from the discussion above that this particular task has been in a state of uncertainty and flux. On the one hand, the early data obtained in our laboratory pointed to a high photosystem II content in bundle sheath tissue, a finding subsequently corroborated by Walker and Izawa (Plant Physiol. 63, 133-138, 1979). If true, this finding would have the effect of unifying the C₄ photosynthesis picture. (At present only NAIP-ME plants (such as corn) are reputed to have very low photosystem II activity.) However, subsequent work has presented a different picture, which is more in accord with the findings of Anderson, et al. (Biochim. Biophys. Acta 245, 253-258, 1971) and Edwards, et al. (in CO₂ Metabolism and Plant Productivity, R.H. Burris and C.C. Black, eds., University Park Press, Baltimore, 1976). We find that the amount of detectable photosystem II activity in a given bundle sheath preparation correlates well with the fraction of contaminating mesophyll chloroplasts.

Although our latest results suggest that bundle sheath chloroplasts contain a very low population of photosystem II centers, the number is probably not zero. We routinely observe 5-10% photosystem II activity in bundle sheath tissue (compared to mesophyll) even after correction for cross-contamination. Experiments which monitored the response of bundle sheath P₇₀₀ to strong illumination in the presence and absence of DCMU showed that a significant fraction of the P₇₀₀ resides in thylakoids that contain photosystem II centers. These findings were further corroborated by measurements in which we monitored a flash-induced pH change associated with either photosystem. (For photosystem I we measured proton uptake associated with the viologen-catalyzed reduction; for photosystem II we monitored proton evolution associated with the photooxidation of tetraphenylboron.)

In summary, on the basis of many different types of measurements, we estimate that corn bundle sheath chloroplasts contain only about 10% as much photosystem II as photosystem I. This enrichment of photosystem I occurs at the expense of photosystem II, so that the trap/chlorophyll ratio remains relatively constant. Our only substantial reservation concerning this estimated photosystem II/photosystem I ratio is the differential destructive effect that the various preparation procedures may have on the two photosystems. Experiments to date suggest that this ratio is the same in preparations prepared either by enzymatic digestion or differential grinding.

PROPOSED WORK

The third task of our earlier proposal was presented under three separate categories, and we will adhere to these same headings.

A. Preparative Procedures

As described above, the limiting aspect of this whole C₃-C₄ program seems to be the preparation of pure, fully active, and well-characterized preparations. Our laboratory has a considerable arsenal of analytical techniques; however, the application of these techniques is of limited value in the absence of reliable chloroplast preparations. During the past year we have made excellent progress in obtaining very pure bundle sheath and mesophyll preparations from corn, although we still retain nagging doubts concerning the possible destruction of some activities, particularly photosystem II, by our procedures.

In the second year of the program we propose to compare these preparative procedures with respect to their effects on various photosynthetic reactions. Particular emphasis will be given to the effects of enzymatic

digestion and differential grinding techniques on various photosystem II activities (O_2 evolution, fluorescence, tetraphenylboron oxidation, etc.). If the results of this comparison warrant, we will also explore the use of gradient techniques and preinfiltration techniques in an effort to obtain clean preparations that reflect the photosynthetic activities present in the whole plant.

B. Photosystem II Assays

Of the several photosystem II assays mentioned in our earlier proposal, the one based on measurement of the flash-induced pH-change associated with oxidation of tetraphenylboron (TPB) proved to be the most suitable. It is technically straightforward and measures photosystem II without interference from photosystem I.

We found that this assay works well for spinach and (corn) mesophyll chloroplasts, even if they are aged or tris-washed. At present we have little insight into the mechanism of TPB's interaction with photosystem II. For instance, we have observed that it dramatically affects the behavior of cytochrome b559, although we have no ready explanation for our observations. In the absence of such understanding, we obviously cannot have complete confidence that the method is foolproof. Our study of TPB's effects will continue.

Other measurements, such as the induction of absorption changes due to C550, X320, or the transmembrane electrical field, are better established as measures for photosystem II. However, these tests are subject to much larger interference by photosystem I photochemistry; and since all these tests require that, after dark adaptation, photosystem II can turn over at least once, they are presumably more susceptible to aging or some other unspecified

inhibitory effects (during the isolation procedure) than TPB oxidation. These other methods of assay will, therefore, only be used to obtain backup evidence in cases where we measure -- polarographically or by the TPB test -- a high photosystem II content.

The measurement of the turnover of P_{700} under repetitive flashing light as an assay for photosystem II is certainly only useable with undamaged chloroplasts. We find, as before, that intersystem electron transfer is easily impeded. This lesion is probably related to an apparent deficiency in (active) plastocyanin. It now seems that part of the chloroplast plastocyanin is nonfunctional. When we abolish compartmentation by the use of detergent (Triton X-100), the difference in plastocyanin content between spinach and mesophyll chloroplasts is small. However, the bundle sheath chloroplasts still contain considerably less (per P_{700}) than spinach. Of course, this might be due to part of the plastocyanin having been washed away during chloroplast isolation.

On the other hand, we presently believe (on the basis of evidence not discussed here) that each plastocyanin molecule serves more than one reaction center. If, in fact, this is so, there would be no reason to expect an equal plastocyanin content (per P_{700}) for chloroplasts from different tissues. We have to test this hypothesis by determining whether bundle sheath and mesophyll chloroplasts also have different plastocyanin contents in vivo.

C. Stoichiometry: Cyclic Operation of Photosystem I

The large enrichment in photosystem I compared to photosystem II characteristic for bundle sheath chloroplasts of corn leads inevitably to the conclusion that a major function of these organelles is to drive cyclic electron flow around photosystem I.

The circumstance that cyclic phosphorylation plays a major role in the photosynthesis of at least one major crop is reason, for us, to consider this process more worthy of study than we used to think it was. Presently, the mechanism of this process is ill-understood. It has been the subject of investigation by a few other laboratories for a number of years. However, we feel that some of their basic premises are erroneous.

The work of Hind and coworkers, and of Bohme in Germany, seems to show that the turnover of cytochrome b₆ is a reaction specific for the cyclic process. In contrast, our own recent work shows that the reported cytochrome b₆ kinetics can be observed under conditions of linear electron flow as well, and are typical for a highly reduced plastoquinone pool. It thus appears that cytochrome b₆ is a component that is common to the linear and cyclic chain.

One of the earliest and steadiest investigators of cyclic phosphorylation has been D.I. Arnon. His work and that of his coworkers points to a central role for ferredoxin in the cyclic chain. Another valuable concept introduced by Arnon has been that of regulation by "redox-poising". According to this concept, the "overoxidation" as well as the "overreduction" of the cyclic chain can inhibit its operation. In this way, one can understand the relief of inhibition by such diverse treatments as admission of oxygen and application of low concentrations of DCMU.

Recently, Arnon and coworkers showed that the primary photosystem II acceptor, C-550, can be reduced by ferredoxin, presumably via cytochrome b₆ and plastoquinone. This interpretation of this finding was again in terms of "poising". It was argued that the function of the C-550 reducing reaction would be to decrease electron pressure from photosystem II and thereby

prevent overreduction. To us, this seems an improbable explanation. The proposed mechanism essentially says that a plant attempts to prevent reduction of C-550 and plastoquinone by reducing Q and plastoquinone. This, of course, makes little sense.

We believe, and will try to establish in the next phase of this sub-task, that instead of a mechanism for 'poising', ferredoxin-mediated reduction of plastoquinone is the essential step in cyclic electron flow, and that it is the only reaction, occurring in place of photosystem II reduction of plastoquinone, in which the cyclic and non-cyclic processes differ. One of the things we will have to show is that the rate of reduction of plastoquinone by reduced ferredoxin is compatible with its supposed function in cyclic phosphorylation. We propose to study this interaction by spectrophotometrically monitoring the redox state of plastoquinone under conditions in which initially the ferredoxin is largely reduced.

APPENDIX A

ON THE LIGHT-DRIVEN UPTAKE OF O_2 , CO_2 , and HCO_3^-
BY THE GREEN ALGA SCENEDESMUS¹

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ABSTRACT

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 O_2 uptake was observed in a mutant lacking photosystem I. Thus, we conclude the carbon-cycle-independent reduction of O_2 occurs at the expense of photosystem I-generated reducing equivalents.

2) The commonly observed differences between CO_2 -grown and air-grown Scenedesmus with respect to CO_2 uptake and glycolate formation cannot be ascribed to differences in their capacity for light-driven O_2 uptake. We found no intrinsic differences in O_2 uptake capacity between the two physiological types under conditions in which CO_2 was saturating or CO_2 uptake was inhibited. It was only under CO_2 -limited conditions that pronounced differences between the two physiological types were observed. This fact suggests that differences in O_2 metabolism and sensitivity between the two types really reflect differences in their capacity to assimilate inorganic carbon; in this respect they are analogous to C_3 and C_4 plants.

3) We tested the hypothesis that air-grown Scenedesmus can assimilate HCO_3^- by directly monitoring the time course of dissolved CO_2 , O_2 uptake, and O_2 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 CO_2 concentration deviated from equilibrium (with the

other inorganic carbon species) during the course of illumination. The observed kinetics in air-grown and CO_2 -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 HCO_3^- at a high rate. Our data suggest that these algae preferentially assimilate CO_2 , and supply the balance of their needs by taking up HCO_3^- . Since (unlike C_4 plants) these algae have no special " CO_2 pump," and thus have a relatively low affinity for CO_2 , HCO_3^- assimilation will be the major carbon uptake process at alkaline pH even when the total CO_2 is present in millimolar concentrations.

INTRODUCTION

One of the major impediments to efficient photosynthetic energy conversion is that the organism must reduce CO_2 , present at very low concentrations (0.03%), in the presence of 21% O_2 . This problem can be even worse for aquatic plants; since the air-water equilibration and equilibration between different CO_2 species can be quite slow, aquatic organisms can readily encounter extremely limiting conditions of free CO_2 . Some algae can apparently circumvent this CO_2 limitation by taking up HCO_3^- , although the evidence to date is somewhat indirect (22).

Earlier, we reported that Scenedesmus can reduce O_2 at a high rate under conditions in which CO_2 fixation is limited. The rate of this O_2 uptake is quite sensitive to the instantaneous concentration of CO_2 , and seems to reflect a direct competition between O_2 and CO_2 for photosynthetically generated reducing power. This O_2 uptake takes place by a process that is independent of carbon cycle (18,19).

In the experiments described in this communication we examined several aspects of this CO_2 - O_2 competition. By monitoring the time course of O_2 uptake, O_2 evolution, and CO_2 uptake, we were also able to infer the uptake of HCO_3^- , and the degree to which this process supplemented CO_2 uptake.

MATERIALS AND METHODS

The mass spectrometer inlet system, data acquisition and processing procedures, and assay techniques used in these experiments were similar to those used in previous experiments (18,20).

Autotrophic cultures of Scenedesmus obliquus (Gaffron strain D₃) were maintained in Roux bottles containing 500 ml of inorganic medium (14) with urea as the nitrogen source. Cultures were bubbled either with filtered air ("air-grown") or 3% CO₂ in air ("CO₂-grown"). Heterotrophic cultures of the wild-type and Mutant #8 (2) were maintained in the dark in the same inorganic medium amended with 0.5% glucose and 0.5% yeast extract. Chlorophyll concentrations were determined by the methods described in (4).

Catalase and carbonic anhydrase were obtained from General Biochemicals and Worthington Biochemicals respectively. Diamox (5-acetamido-1,3,4-thiadiazole-2-sulfonamide) was obtained from Pfaltz and Bauer. Gaseous ¹⁸O₂ (99 atom %) was obtained from Bio-Rad Laboratories.

RESULTS AND DISCUSSION

Photosystem I is Required for Significant Rates of Light-Driven Oxygen Uptake In Vivo.

Light-driven O_2 uptake in whole algae can occur at high rates via a pathway that does not involve the photosynthetic carbon cycle (18). The most likely site of interaction is one of the strong reductants generated by photosystem I (e.g. reduced ferredoxin, NADPH, etc.), although this is by no means proven. One could posit that electron backpressure from photosystem I, due to limitations in CO_2 fixation, would result in the reduction of O_2 by photosystem II. The interaction of O_2 with the light-generated photosystem II reductant has been observed in chloroplasts (24) and algae (16), although this reaction usually occurs at a very low rate (17).

Table I shows the results obtained when O_2 uptake and evolution were monitored in Scenedesmus Mutant #8, which lacks photosystem I activity (2), and heterotrophically and autotrophically cultured wild-type Scenedesmus. As shown in the first three lines of the table, no discernable light-driven O_2 uptake was observed in the mutant either in the absence or presence of the carbon cycle inhibitors iodoacetamide or KCN. This is in sharp contrast to the O_2 uptake observed in both the heterotrophically and autotrophically grown wild-type algae, under both normal conditions and those in which CO_2 fixation was inhibited. Under non-inhibited CO_2 -sufficient conditions, the transient differences between the mutant and the wild type were even more marked than shown in the table. The data of Table I reflect the rates observed

several minutes after the induction lag; during the lag under CO_2 -sufficient conditions, O_2 uptake occurred at a rate comparable to that of O_2 evolution (cf. 18).

Although we observed some striking differences between the autotrophically grown and heterotrophically grown algae -- for example, a four-to-five-fold difference in chlorophyll content per cell volume -- there was little apparent difference in photosynthetic capacity on a chlorophyll basis. Thus the lack of light-driven O_2 uptake (and the near-zero O_2 evolution rate) by the mutant cannot be ascribed to the heterotrophic growth conditions. We conclude, therefore, that light-driven O_2 uptake in these algae requires an operational photosystem I, and that O_2 reduction occurs at the expense of photosystem I-generated reducing equivalents.

O₂ Uptake in Algae Adapted to Different Levels of CO₂

Algae grown under high CO₂ can have physiological characteristics that are quite different from those of algae grown under air. The ability to efficiently utilize CO₂ is correlated with the physiological status of the algae: cultures grown at high CO₂ concentrations have high CO₂-compensation points (similar to C₃ plants), whereas those grown at air-level-CO₂ have compensation points near zero (6). Low-CO₂ algae also have higher levels of carbonic anhydrase activity (8).

The pronounced differences in the capacity for CO₂ assimilation between these two physiological types might be related to their interaction with O₂. Contradictory reports have appeared in the literature concerning the presence (23) or absence (3) of a Warburg effect in *Chlorella*, and some attribute this discrepancy to differences in physiological status (1). Differences in glycolic acid metabolism have also been reported (13): CO₂-grown algae excrete glycolate during photosynthesis, while air-grown algae do not. In view of these differences in O₂ and glycolate metabolism, one could postulate that analogous differences in the capacity to reduce O₂ also occur. Consequently, we examined the O₂ uptake capacity in the two physiological types.

Figure 1 is a comparison of the time courses of O₂ evolution (E_O), O₂ uptake (U_O), and CO₂ uptake (U_C) by CO₂-grown and air-grown *Scenedesmus* with ample CO₂. Since the samples in the two experiments contained the same cell density (10 μ l packed cells/ml) with about the same chlorophyll content (19 and 20 μ g chl/ μ l packed cells for the CO₂-grown and air-grown respectively), they can be directly compared. In both cases the strong light induced a high rate of steady state photosynthesis -- 34 and

53 cell volume O_2 /hr for the CO_2 - and air-grown respectively². After the initial induction lag, O_2 uptake (U_o) decreased in both cases to a value <10% of the concomitant O_2 evolution. [The kinetics of U_o , U_c and E_o during the induction lag were described previously (18); the apparent lag at light-on evident in the first -- and possibly the second -- data point of E_o reflects the 3 sec time constant of the apparatus]. These results demonstrate that, given adequate CO_2 , there is no appreciable difference between the two physiological types with respect to light-driven O_2 uptake. In both cases U_o is a minor fraction of the total electron balance ($E_o - U_o - U_c$).

Figure 2 shows the time course of O_2 uptake and evolution by the two physiological types in the presence of iodacetamide, an inhibitor of CO_2 fixation. Note that the O_2 cycle turned over at a rate proportional to the CO_2 -supported rate (cf. Fig. 1), again indicating a similar capacity for O_2 reduction in the two physiological types.

Figure 3 shows the results obtained when the algae were illuminated under conditions in which CO_2 could become limiting. In this case we observed striking differences between the two physiological types. In the air-grown algae, the CO_2 was rapidly depleted to a near-zero level, at which time O_2 uptake increased to compensate. Throughout the experiment E_o was relatively invariant, and comparable to that observed under CO_2 -sufficient conditions (cf. Fig. 1). In contrast, CO_2 -grown algae only slowly depleted the CO_2 , and during the 5 min duration of the experiment (only 3 of which are shown in the figure) the CO_2 level never approached the near-zero level attained by

the air-grown algae in 2 min. (The higher CO_2 level at $t = 0$ was due to carryover from the CO_2 -fed algal culture.) Again, as in the air-grown algae, E_o occurred at a high rate and was relatively invariant during the ~5 min illumination. During the entire experiment O_2 uptake increased as CO_2 uptake decreased, so that the two processes were complementary, and the balance expression ($E_o - U_o - U_c$) remained relatively constant³.

These results indicate that the two physiological types have the same intrinsic capacity for O_2 uptake. Observed differences in O_2 sensitivity (Warburg effect) (23,3) are attributable to differences in the capacity of the two types to assimilate inorganic carbon (see below), and not to differences in the O_2 uptake pathway per se. In this respect the two types are closely analogous to C_3 and C_4 plants.

CO₂ and HCO₃⁻ Uptake by Air-Adapted and CO₂-Adapted Algae

As described above, the ability of Scenedesmus to efficiently utilize CO₂ is correlated with its physiological status. This efficient CO₂ utilization may reflect the ability of these algae to assimilate HCO₃⁻ (16), a process that may be quite prevalent in aquatic plants (e.g.21). Bicarbonate utilization is usually inferred from the relative rates of photosynthesis at two different CO₂/HCO₃⁻ ratios, set by two different pH values (22). However, evidence obtained in this manner must be considered equivocal (22;7) since the pH and CO₂/HCO₃⁻-ratio cannot be varied independently, and changes in pH are known to affect photosynthetic pathways in a manner unrelated to the resultant differences in CO₂ availability (15). Models based on calculations of diffusion and reaction rates also suggest that bicarbonate can serve as the primary source of inorganic carbon under some conditions (10).

In the experiments described in this section we used a different, possibly more direct, approach in which we monitored dissolved CO₂. Because the time response of our apparatus is fast compared to the non-enzymatic rate of equilibrium between the inorganic carbon species, we were able to determine the concentration of CO₂ -- as opposed to "total CO₂" (i.e. CO₂ + H₂CO₃ + HCO₃⁻ + CO₃⁼) -- and the deviation of the CO₂ concentration from equilibrium. We thus could, at least in principle, distinguish between a rapid efficient uptake of CO₂ (similar to that occurring in C₄ plants) and the assimilation of HCO₃⁻. For the case of rapid CO₂ uptake, one would expect to see, in strong light, a precipitous decrease of CO₂ to a low level, markedly out of equilibrium with the HCO₃⁻. If HCO₃⁻ were the primary specie assimilated at this pH, one would expect to see a much less striking CO₂ depletion to a level commensurate with: 1) the CO₂ uptake observed at this pH and CO₂ concentration with

non-adapted algae (there is no reason to suspect a priori that adapted algae lose the ability to assimilate CO_2 ; and 2) the CO_2 - HCO_3^- equilibration kinetics characteristic of this pH and total CO_2 concentration.

Figures 4 and 5 illustrate the time course of light-driven O_2 evolution, O_2 uptake, and CO_2 uptake induced by air-grown Scenedesmus at pH 8 in the presence and absence of carbonic anhydrase. The evolution and uptake of O_2 were virtually identical in the two cases, indicating that the rate of carbon assimilation was about the same in the two experiments. [The uptake of O_2 is a sensitive indicator of CO_2 availability (18,19)].

In the presence of carbonic anhydrase (Fig. 4) CO_2 uptake attained a constant rate after a distinct lag. Because of the rapid CO_2 - HCO_3^- equilibrium induced by the enzyme, the time course of CO_2 in this experiment accurately reflected that of the total CO_2 .

In the absence of added carbonic anhydrase (Fig. 5), we observed a transient uptake of CO_2 at the onset of illumination, followed by a slower decrease that paralleled that shown in Fig. 4. At light-off a rapid rise and distinct overshoot (in comparison with time zero) of CO_2 was observed.

We interpret the CO_2 uptake kinetics of Fig. 5 as follows. At the onset of illumination, CO_2 will be depleted to a value concordant with its uptake by the algae and its restitution via the dehydration and dehydroxylation of H_2CO_3 and HCO_3^- respectively. We can obtain a rough idea of the relative magnitude of these processes by assuming that the uptake of CO_2 is only a weak function of CO_2 concentration over these small changes in

substrate concentration, and that some sort of pseudo-steady state is attained.

Consider the following reactions and rate constants (9):

$$k_1 = .03 \text{ sec}^{-1}$$

$$d(\text{H}_2\text{CO}_3)/dt = k_3 (\text{H}_2\text{CO}_3)$$

$$k_3 = 20 \text{ sec}^{-1}$$

$$k_2 = 8500 \text{ sec}^{-1} \text{ M}^{-1}$$

$$d(\text{HCO}_3^-)/dt = k_4 (\text{HCO}_3^-)$$

$$k_4 = 2 \times 10^{-4} \text{ sec}^{-1}$$

$$3) \quad K = \frac{(\text{CO}_2)}{(\text{H}_2\text{CO}_3)} = \frac{k_3}{k_1} = 667$$

$$4) \quad K_a = \frac{(\text{H}^+) (\text{HCO}_3^-)}{(\text{H}_2\text{CO}_3)} = 2.5 \times 10^{-4}$$

$$5) \quad K_1 = \frac{(\text{H}^+) (\text{HCO}_3^-)}{(\text{CO}_2 + \text{H}_2\text{CO}_3)} = 4.45 \times 10^{-7}$$

The rate of CO_2 uptake will be given by the expression

$$6) \quad d(\text{CO}_2)/dt = -k_1(\text{CO}_2) - k_2(\text{OH}^-)(\text{CO}_2) \\ - k_p + k_3 (\text{H}_2\text{CO}_3) + k_4 (\text{HCO}_3^-)$$

At (pseudo) steady state the rate of photosynthetic CO_2 uptake (k_p) will be

$$7) \quad k_p = -k_1 (\text{CO}_2) - k_2 (\text{OH}^-) (\text{CO}_2) \\ + k_3 (\text{H}_2\text{CO}_3) + k_4 (\text{HCO}_3^-)$$

For the case shown in Fig. 5, total $\text{CO}_2 = 1 \text{ mM}$, $\text{pH} = 8.0$, and CO_2 was depleted from 22 to 19 μM ; we calculate that $k_p = 0.1 \mu\text{M sec}^{-1}$.

The dot-dashed line in Fig. 5 shows the results obtained when the rate equation (eq. 6) and the equivalent expressions for $d(\text{H}_2\text{CO}_3)/dt$ and $d(\text{HCO}_3^-)/dt$ were solved explicitly with the aid of a computer, using the value $k_p = 0.1 \mu\text{M sec}^{-1}$. The agreement is quite good in the initial transient phase of CO_2 uptake, lending credence to the rather rough approximations used above.

The very modest rate of carbon assimilation occurring during the initial lag at the onset of illumination is difficult to compare with the net evolution of O_2 ($E_0 - U_0$) or the uptake of total CO_2 in Fig. 4. Our data suggest, but do not prove, that only CO_2 is taken up at the onset of illumination, and in quantities sufficient to provide the entire required carbon during this phase of very low CO_2 assimilation.

A more accessible question addressed by these experiments is whether CO_2 uptake in these air-grown algae occurs at a high enough rate to provide the sole carbon source during periods of rapid photosynthesis, i.e., whether these algae have a CO_2 pump similar to C_4 plants. The dotted line in Fig. 5 shows the predicted time course of (CO_2) generated by explicitly solving the

differential equations for the three carbon species, assuming that CO_2 was assimilated at a rate inferred from the data on Fig. 4 and the slow second phase of CO_2 uptake in Fig. 5. The large disparity between this computed curve and the observed CO_2 time course suggests that the uptake of species other than CO_2 is occurring during the course of the experiment.

The dashed line of Fig. 5 shows the predicted time course of (CO_2) when CO_2 uptake (at a rate of $0.1 \mu\text{M sec}^{-1}$) was supplemented by sufficient HCO_3^- uptake to satisfy the total CO_2 demand inferred from the data of Figs. 4 and 5. The good agreement between the observed and computed time courses lends credence to this model.

Fig. 6 shows the results obtained when high- CO_2 -grown algae were illuminated under the same conditions as those of Fig. 5. In this case, we observed the same initial transient as in Fig. 5, but very little net photosynthesis, as evidenced by U_c or $(E_o - U_o)$. In fact, the CO_2 time course was almost identical to that predicted when only CO_2 was being taken up (cf. initial transient in Fig. 6 with the dot-dashed line of Fig. 5). This suggests that air-grown algae have an HCO_3^- assimilation process superimposed on the basic CO_2 assimilation process.

Our data also suggest that CO_2 uptake is not replaced by HCO_3^- uptake in air-grown algae. If this were to occur to an appreciable extent, one would expect to see a net increase in the CO_2 trace shown in Fig. 5. For example, using the same methods as those used to generate the theoretical traces shown in Fig. 5, we computed that the CO_2 concentration would rise ~30% from its pseudo-steady-state value if half of the CO_2 uptake were replaced by HCO_3^- uptake. Thus, in air-grown algae CO_2 uptake occurs at a

constant or increasing rate, and HCO_3^- uptake is a complementary rather than competing process.

A nagging reservation concerning the data interpretation presented above was the possibility that the uptake of HCO_3^- could be mimicked by an increased uptake of CO_2 (compared to high- CO_2 grown algae) coupled with a low level of (excreted) extracellular carbonic anhydrase. To test this possibility, we examined the effect of the carbonic anhydrase inhibitor Diamox on CO_2 uptake kinetics. The results of these experiments, shown in Table II, indicate that the increased rate of total CO_2 uptake is not attributable to extracellular carbonic anhydrase activity. An identical CO_2 transient was observed in both the presence and absence of sufficient Diamox to inhibit at least 100 units of carbonic anhydrase (items #3 and 4). These CO_2 transients were, in turn, identical to that observed in the absence of both the enzyme and the inhibitor (Item #1 and Fig. 5). Only in the presence of uninhibited added enzyme was the CO_2 transient suppressed (Item #2 and Fig. 4). Consequently, unless we postulate the excretion of a low-activity carbonic anhydrase enzyme that is not inhibited by Diamox, we must conclude that extracellular carbonic anhydrase does not play a role in the facilitated uptake of inorganic carbon by air-grown algae.

An interesting aspect of the data shown in Figures 4-6 is the behavior of the CO_2 and "total CO_2 " at light-off. Like higher plants, some algae rapidly release CO_2 for a short dark period immediately after illumination, which may reflect processes occurring in the prior light period (25). A comparison of Figs. 4 and 6 indicates that these processes are much more predominant in the CO_2 -grown algae, a finding in accord with

other reports (6). That the released specie is CO_2 (at least in the air-grown algae), and not another form of inorganic carbon, is shown in Fig. 5. The observed CO_2 transient at light-off in this experiment really represents the superposition of two components, the reequilibration of the carbon species -- comparable to the decrease observed at light-on -- and the efflux of CO_2 from the algae, which may arise (at least in part) from an internal pool of actively assimilated inorganic carbon (see 19). The long descending phase, only the first part of which is shown in the figure, reflects the slow hydration of the released CO_2 .

In summary, our data support the concept that air-adapted Scenedesmus are able to assimilate HCO_3^- at a sufficient rate to support photosynthesis under high-light conditions. It appears that these algae preferentially assimilate CO_2 ; when this is inadequate, they supply the balance of their needs by taking up HCO_3^- . Since the affinity for CO_2 does not increase during the air-adaption process, HCO_3^- assimilation will be a major process at alkaline pH even when the total CO_2 is present in millimolar concentrations, and at acid pH during the last stages of CO_2 depletion (cf. Fig. 3).

TABLE 1. Rates of Oxygen Evolution and Uptake by *Scenedesmus* Mutant #8 and the Autotrophically and Heterotrophically Cultured Wild-Type.

		O ₂ Uptake	O ₂ Evolution
Mutant #8:	+CO ₂	<1	18
	+IA ²	<1	6
	+KCN	<1	6
Heterotroph:	+CO ₂	5	132
	+IA ²	68	72
	+KCN	72	94
Autotroph:	+CO ₂	20	136
	+IA ²	111	118
	+KCN	90	101

The tabulated rates, in units of $\mu\text{moles O}_2 \text{ mg chl}^{-1} \text{ hr}^{-1}$, were obtained several minutes after the onset of illumination. An initial high rate of O₂ uptake was observed in the absence of inhibitors in both the heterotroph and the autotroph, but not in the mutant (c.f. ref. 18). Because of the high respiration rates in the heterotrophically grown wild-type and Mutant #8 algae, all tabulated rates were corrected for dark respiration. No discernible break in O₂ uptake was observed in the mutant at light-on or light-off.

The respective chlorophyll concentrations (in $\mu\text{g chl ml}^{-1}$) and packed-cell-volumes (in $\mu\text{l cell ml}^{-1}$) used in the assays were: Mutant #8, 89 and 13.5; heterotrophs, 97 and 17; autotrophs, 121 and 4.9. The reaction medium contained 100 mM phosphate buffer pH 6.0, 300 μM added NaHCO₃, 5 mM NH₄Cl, 100 units of carbonic anhydrase, and 200 units of catalase. Iodoacetamide (IA) and KCN concentrations were 5 mM; in the case of KCN, inhibition of CO₂ fixation was not 100%. The actinic light was about half-saturating. Initial O₂ concentrations ranged between 50 and 75%.

TABLE II. Effect of Carbonic Anhydrase and Diamox on the Initial CO₂ Uptake Transient in Air-Grown Algae.

	Carbonic Anhydrase (100 units)	Diamox (1mM)	Transient (c.f. Figs. 4 and 5)
1)	-	-	+
2)	+	-	-
3)	-	+	+
4)	+	+	+

Conditions were the same as those given in the legend of Fig. 4. The initial O₂ concentrations were all in the range of 60 to 68%.

The Diamox was added immediately before the experiment was run. No "deadaptation" from air-grown characteristics was observed, a phenomenon which often occurs after long-term incubation with the inhibitor (8). The efficacy of both the added enzyme and the inhibitor were verified by monitoring their effect on the isotope exchange reactions

FIGURE LEGENDS

Figure 1. Time course of gas exchange by CO_2 -grown (left) and air-grown (right) Scenedesmus in the presence of ample CO_2 (1 mM added NaHCO_3). Gas exchange was monitored at a rate of 14 cycles (70 m/e values)/min. The reaction was carried out in 100 mM potassium phosphate buffer, pH 6.0, 5 mM NH_4Cl , and 200 units of catalase. The cell concentration was 1% (v/v), and the actinic light was about half-saturating. The initial O_2 concentration was 37% in both experiments.

Figure 2. Time course of gas exchange by CO_2 -grown (left) and air-grown (right) Scenedesmus in the presence of 5 mM iodoacetamide. Initial O_2 concentrations were 53% (left) and 60% (right) respectively. For other conditions, see legend of Fig. 1.

Figure 3. Time course of gas exchange by CO_2 -grown (left) and air-grown (right) Scenedesmus under conditions of limiting CO_2 . Initial O_2 concentrations were 62% (left) and 66% (right) respectively. A small amount of NaHCO_3 (100 μM) was added at the beginning of the experiment; the additional CO_2 present at the onset of illumination was derived from respiration in the preceding (~5 min) dark period and carryover from the culture. For other conditions, see legend of Fig. 1.

Figure 4. Time course of gas exchange by air-grown Scenedesmus at pH 8.0 in the presence of carbonic anhydrase. The reaction mixture contained 100 mM potassium phosphate, 1 mM added NaHCO_3 , 5 mM NH_4Cl , 200 units of catalase, 100 units of carbonic anhydrase, and 0.5% cells (v/v). The actinic light was about half-saturating. Gas exchange was monitored

at a rate of 14.5 cycles (58 m/e values)/min. "Total CO_2 " was calibrated using standard HCO_3^- solutions in which the CO_2 signal was measured at pH 6.0, 7.0 and 8.0; the differences between the measured and calculated values at these pH values was <5%. The initial O_2 concentration was 68%.

Figure 5. Time course of gas exchange by air-grown Scenedesmus at pH 8.0 in the absence of carbonic anhydrase. The " CO_2 " trace reflects the amount of free CO_2 dissolved in the medium, and was not always proportional to the "total CO_2 ." The initial O_2 concentration was 75%. The theoretical time courses (dotted, dashed, and dot-dashed lines) were generated by solving the system of equations describing the various CO_2 species (see text) by the fourth-order Runge-Kutta method. For other conditions, see legend of Fig. 4.

Figure 6. Time course of gas exchange by CO_2 -grown Scenedesmus at pH 8.0 in the absence of carbonic anhydrase. The initial O_2 concentration was 68%. For other conditions and procedures, see legends of Figs. 4 and 5.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

FIG. 5

Fig. 6

FOOTNOTES

- 1 This work was supported by grants from the USDA/SEA Competitive Grants Office (5901-0410-8-0179-0) and the Department of Energy (EY-76-C-02-3326).
- 2 We routinely observe higher rates with air-grown than with CO₂-grown Scenedesmus, both on a packed-cell-volume and chlorophyll basis. This is in contrast to the report of Findenegg (5). However, our algae were grown under air for several days, while those of Findenegg were grown on CO₂ before being adapted to air. Thus the two instances may not be directly comparable.
- 3 The assimilatory quotient γ ($= \Delta\text{CO}_2/\Delta\text{O}_2$) observed in these experiments was usually about 0.7, which is somewhat lower than one would predict for long-term steady-state photosynthesis with urea as the nitrogen source (12). Such deviation from the theoretical γ value (≈ 0.9 in this case) has often been observed, even in relatively long-term experiments (11,12), and probably reflects a rather gradual adjustment to changes in conditions.

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MECHANISM OF LINOLENIC ACID-INDUCED INHIBITION OF
PHOTOSYNTHETIC ELECTRON TRANSPORT¹

Evidence for a New Mode of Inhibition in Photosystem II

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APPENDIX B

ABSTRACT

The effect of linolenic acid on photosynthetic electron transport reactions in chloroplasts has been localized to a site on the donor side of photosystem I and to two functionally distinct sites in photosystem II.

In photosystem I, an increase in the electron transport rate occurs in the presence of 10 to 100 μM linolenic acid, followed by a decline in rate from 100 to 200 μM linolenic acid. The increase may be due to an alteration of membrane structure that allows greater reactivity of the artificial donors DPIP or TMPD with plastocyanin. The decrease is most likely the result of the loss of plastocyanin from the membrane since the addition of purified plastocyanin to the washed chloroplasts leads to the reestablishment of photosystem I rates.

In photosystem II, reversible and irreversible sites of inhibition have been located. At the irreversible site, there is a time-dependent loss of the loosely bound pool of Mn implicated in the water-splitting mechanism. At the reversible site, the photochemical charge separation is rapidly inhibited as evidenced by the high initial fluorescence yield (F_i) upon illumination and the inhibition of artificial donor reactions in NH_2OH -washed chloroplasts. When the chloroplasts are washed free of linolenic acid, F_i returns to its original low value and there is a resumption of artificial donor activity from DPC $\rightarrow$ DPIP. This reversible inhibition of the photoact is clearly a unique characteristic of linolenic acid and indicates evidence for an entirely new mode of inhibition of photosystem II.

INTRODUCTION

Inhibition of electron transport reactions by unsaturated C_{18} fatty acids was first reported by Krogmann and Jagendorf in 1959 (19). Since then, the topic has received considerable attention because damage to the photosynthetic membrane observed during stress and aging has been correlated with the presence of free fatty acid. McCarty and Jagendorf (22) and Molotkovsky and Zheskova (23) showed that the addition of linolenic acid to freshly isolated chloroplasts caused damage resembling that which occurs in chloroplasts isolated at pH 6.0 or after inactivation by gentle heating. The latter two authors suggested that heat-activated lipases hydrolyze membrane lipids, resulting in the release of free fatty acids. Kato and San Pietro (19) reported an inhibition of $NADP^+$ reduction by linolenic acid, but noted also a stimulation of the photosystem I-mediated DPIP to $NADP^+$ reaction at intermediate concentrations of fatty acid. Since this reaction eventually was inhibited in chloroplasts without Hill activity, it suggested that high concentrations of linolenic acid inhibit photosystem I as well as photosystem II.

Cohen et al. (10) further resolved these concentration-dependent effects of linolenic acid into two major classes: a) At fatty acid/chlorophyll (FA/Chl) ratios of 2-3 the variable part of the fluorescence induction was inhibited 50%, but the photosystem I-dependent DPIP to $NADP^+$ pathway was stimulated. b) At FA/Chl ratios of 10, low-temperature fluorescence at 735 nm was reduced 50% and the DPIP to $NADP^+$ pathway was inhibited.

Other effects of linolenic acid on chloroplasts have been described which are relevant to this study: a) Photophosphorylation is uncoupled (22); b) The chloroplasts swell, and there are large changes in the appearance of the grana lamellae (10,24,25); c) The population of system II reaction centers appears to decline (8).

The reversibility of the photosystem II inhibition is controversial (10,25,26,33,34) and is one of the major topics of this paper.

Although the investigations cited in the literature describe the effects of linolenic acid, a satisfactory biochemical explanation has not yet been offered. In this study, we found that the irreversible inhibition of photosystem II is accompanied by the removal of the loosely bound pool of manganese, and that the inhibition of photosystem I after washing is due to the loss of plastocyanin from the membrane. The stimulation of photosystem I flow was reinvestigated, and we concluded that linolenic acid accelerates a rate-limiting donor reaction on the oxidizing side of P700. Finally, we found evidence for a new type of photosystem II inhibition, based primarily on fluorescence studies and artificial donor reactions in isolated chloroplasts. In particular, linolenic acid was found to block charge separation rapidly and reversibly within the photosystem II trap.

MATERIALS AND METHODS

Chloroplasts were isolated from greenhouse-grown spinach in a medium consisting of 0.4 M sucrose, 0.05 M Tris, and 0.01 M NaCl, pH 7.40 (STN) according to the method of Schwartz (31). Chlorophyll concentrations were determined in 80% acetone (3). Oxygen evolution and uptake rates were measured polarographically as described by Cheniae and Martin (9).

Fluorescence induction was measured at room temperature as described by Malkin and Kok (21). The chloroplasts were suspended in STN buffer containing 5 mM $MgCl_2$ to a chlorophyll concentration of 20 $\mu g/ml$. During studies of the kinetics of variable fluorescence yield, there was a 10-min dark equilibration period before addition of the inhibitor.

The effect of linolenic acid on electron transport rates was determined with chloroplasts at 14 μg or 20 μg chlorophyll/ml, depending on the experiment (see figure legends). Complete inhibition over a suitable linolenic acid concentration range could not be obtained at chlorophyll concentrations greater than 20 $\mu g/ml$. The incubations were carried out at room temperature because of the limited solubility of linolenic acid in aqueous solution. Some studies were made directly in the presence of linolenic acid, while others were made after washing the treated chloroplasts with STN buffer $\pm$ 2 mM EDTA at 0°C.

Kinetics of linolenic acid inhibition of electron transport activity were investigated directly in the polarograph. After a 2- to 3-minute dark equilibration, linolenic acid was added to the methylviologen reaction

mixture through the vessel cap with a syringe, and illumination was begun at specified times.

Experiments to study reversal of the inhibitory effect were conducted in a test tube. After a suitable incubation period with linolenic acid, Mn^{+2} or BSA was added directly to the reaction mixture to affect recovery of rates. The suspension was then immediately transferred to the polarograph for assay. Preliminary experiments showed that this method could be used because no further decline in oxygen rates was observed after these additions.

Manganese and copper were determined by atomic absorption spectroscopy (Perkin Elmer, Model 303). Chloroplast suspensions were wet-ashed prior to analysis with 0.25 ml conc. sulfuric and 5 ml conc. nitric acid. Standards were prepared from Fisher Certified Atomic Absorption Standards.

Plastocyanin was released from the membrane by sonication and was determined by the method of Plesnicar and Bendall (27). The supernatant, after centrifugation at 144,000 xg for 2 hrs, was used directly for analysis. Plastocyanin standards were prepared after purification from spinach leaves (18).

Mn-free chloroplasts were prepared by washing with 5 mM NH_4OH as described by Cheniae and Martin (9), or with 0.8 M Tris according to the method of Yamashita and Butler (37).

All reagents were of the highest commercial grade and were used without further purification. Bovine serum albumin-fraction V, α -linolenic acid (9,12,15 - octadecatrienoic acid), Trizma base, and EDTA-disodium salt were obtained from Sigma Chemical Co.. Hydroxylamine-HCl and manganous chloride were 'analyzed reagent grade' from J. T. Baker Chemical Co..

Baker was also the supplier for p-phenylenediamine dihydrochloride ('Baker' grade). Diphenylcarbazide and methylviologen (dimethyl dipyridyl chloride) were purchased from K & K Laboratories, Inc. and DPIP, sodium salt from Fisher Scientific Co..

RESULTS

In this study, our goal was to characterize the site(s) of linolenic acid-induced inhibition in the electron transport chain and to clarify the conditions for the reversal of inhibition. Fig. 1 shows the effect of increasing concentrations of α -linolenic acid on the Hill reaction ($H_2O + PD_{ox}$) and the photosystem I (DPIP $\rightarrow$ MV) partial reaction. Also included are the rates of electron transport after the chloroplasts had been washed free of the inhibitor (first with STN + 2 mM EDTA, then with STN). As previously shown (17,10,32), the Hill reaction (squares, Fig. 1) is more sensitive to linolenic acid than is the partial reaction involving only photosystem I (closed circles). In agreement with these studies, we found that with increasing concentrations of linolenic acid the photosystem I pathway from DPIP $\rightarrow$ MV became accelerated, reaching a rate nearly double the control at the same concentration of linolenic acid that completely inhibits oxygen evolution (100 μ M linolenic acid).

Photosystem II activity did not recover when the chloroplasts were washed free of linolenic acid following a 10-minute treatment (triangles, Fig. 1). In contrast, photosystem I activity was greatly affected by removal of linolenic acid (open circles). The control rate was recovered at (what was) 40- μ M linolenic acid, followed by more rapid loss in rate between 50 and 80 μ M. Although not shown in the figure, less than 10% of the photosystem I rate remained intact at concentrations of linolenic acid near its solubility limit.

Photosystem I

In Fig. 1, the decline in the rate of photosystem I electron flow (open circles) suggests that a component normally required in this pathway was removed upon washing the chloroplasts free of linolenic acid. Since plastocyanin is required for high rates of photosystem I electron flow and is readily released from the chloroplast membrane (28), we investigated the chloroplast plastocyanin content before and after treatment with linolenic acid.

Because recent studies report more than one plastocyanin molecule per electron transport chain (27,4,20,16), we first redetermined the copper and plastocyanin content of our EDTA-washed chloroplasts. Two separate cases were studied: normal, EDTA-washed chloroplasts and chloroplasts that had been passed 3 times through the Yeda press. The latter procedure was investigated because preliminary experiments in our laboratory showed that this gentle procedure liberates plastocyanin from the photosynthetic membrane without destroying the water-splitting mechanism. Table I shows that the EDTA-washed chloroplasts contain 5-6 Mn, 3-4 Cu and nearly 2 plastocyanins-per-electron transport chain (assuming 400 Chl/trap). Breakage in the Yeda press resulted in only a slight loss of Mn, but in nearly a complete (avg. 90%) loss of plastocyanin from the lamellae. The difference in the total copper content of whole and Yeda-pressed chloroplasts was within 15% of the plastocyanin content of whole chloroplasts (except in Expt. #2, where one third of the copper in the Yeda press fraction is unaccounted for). Since plastocyanin contains one atom of Cu/molecule (11)

and since very little was liberated from the Yeda-pressed fragments following sonication (Table I), we concluded that passage through the Yeda press causes the quantitative removal of plastocyanin from the thylakoid membrane. Table I also indicates that the oxygen-evolving mechanism survived the passage through the Yeda press, but only 15-20% of the flow from H_2O to MV remained intact.

Figure 2 is a plot of the photosystem I rate (DPIP→MV) after treatment with increasing concentrations of linolenic acid against lamellar copper content. Following treatment with linolenic acid for 10 min, the chloroplasts were centrifuged, resuspended in STN buffer containing 2 mM EDTA and washed a second time in STN before assay. The data show that a) Two copper atoms are eventually lost at high concentrations of linolenic acid. b) There is a distinct relationship between the photosystem I rate and the remaining lamellar copper content. We conclude from these results that the decline in the photosystem I rate after washing is due to the loss of plastocyanin from the linolenic acid-treated chloroplasts.

We confirmed this conclusion with reconstitution experiments using purified plastocyanin. Chloroplasts were treated with 200- μ M linolenic acid for 10 min and washed according to the protocol described in the legend of Fig. 1. Following this procedure, the treated, washed chloroplasts retained only 6% of the DPIP→MV rate of normal chloroplasts (Table II). Although the addition of plastocyanin to untreated chloroplasts caused only a slight (~5%) increase in rate, its addition to the linolenic acid-treated chloroplasts led to the reestablishment of system I rates commensurate with the amount added. A double reciprocal plot (not

shown) indicated that at limiting concentrations of plastocyanin, the photosystem I rate becomes $\sim 620 \mu\text{moles O}_2/\text{mg Chl}\cdot\text{hr}$, or twice the rate measured in control chloroplasts. This result implies that an independent competing process is responsible for the decline in the photosystem I rate (Fig. 1) after exposure to higher concentrations of linolenic acid.

The ability of the washed lamellae to reestablish photosystem I rates after addition of purified plastocyanin indicated that the decline in rate from 100-200 μM linolenic acid was most likely due to the loss of plastocyanin rather than to a destruction of the system I photochemical apparatus. We deem it significant that plastocyanin was effective in restoring rates of linolenic acid-treated chloroplasts without the need for detergents or sonic oscillation. Linolenic acid apparently causes lesions in the photosynthetic membrane large enough for plastocyanin to enter and function as an efficient electron donor to P700.

The identity of the remaining copper in the linolenic acid-washed and Yeda-pressed chloroplasts is open to question (Table I, Fig. 2). Although there are indications that a "tightly bound" pool of plastocyanin is deeply imbedded in the chloroplast membrane, Malkin and Bearden (20) have shown with ESR techniques that less than 10% of the total plastocyanin remains bound to the membrane following sonication. It is more likely that the remaining copper protein is at least partially accounted for by the polyphenol oxidase enzyme (O-Diphenol: oxygen oxidoreductase, EC 1.10.3.1) that has been reported to exist in the chloroplast (35).

The stimulation of system I activity with increasing concentrations of linolenate is more difficult to explain. Since the increase in the

photosystem I rate occurred already in the presence of methylamine (Fig. 1) or gramicidin (data not shown), an uncoupling of photophosphorylation by linolenic acid, which has been suggested as a cause of similar stimulation of overall electron flow (22), is not relevant here. This conclusion is further strengthened by comparing DPIP and TMPD-mediated rates. Even if DPIP supported a short proton cycle across the membrane, TMPD, a non-protonated donor, would show none of these effects if the stimulation by linolenic acid were due solely to uncoupling. As shown in Table III, TMPD-mediated rates were stimulated to nearly the same degree as are DPIP rates, albeit with a slight offset in the concentration of linolenic acid necessary to produce the maximum effect. Therefore, the affected step appears to be one that nominally limits electron flow from DPIP to MV in photosystem I other than proton translocation.

Another possible explanation for the doubling of the photosystem I rate (as measured by oxygen uptake) is that linolenic acid inactivates an endogenous superoxide dismutase (SOD) that normally decomposes the $O_2^{\cdot -}$ (produced by $MV^{\cdot -}$) to $1/2 O_2$ and $1/2 H_2O_2$. If the resulting, non-enzymatic dismutation of $O_2^{\cdot -}$ were relatively slow, the ascorbate might reduce $O_2^{\cdot -}$ directly to H_2O_2 in a reaction sequence described by Epel and Neumann (13) and Allen and Hall (1). In the absence of catalase activity the result would be a doubling of the rate of oxygen uptake. However, if the stimulation were due solely to the inhibition of SOD and if the inhibition of SOD were completely reversed by washing, the rate of O_2 uptake should be restored to control level rather than to less than 25% of the control rate (Fig. 1). Furthermore, because the majority of the superoxide dismutase in

spinach chloroplasts is CN^- sensitive (2), the enzyme would most likely be inhibited in the KCN-containing reaction mix.

Following exposure to linolenic acid the chloroplast thylakoids become "leaky" and lose plastocyanin (Fig. 2). Digitonin also disrupts membrane structure (16). Therefore, we inquired whether the effects of digitonin on photosystem I were similar to those observed with linolenic acid. As shown in Table IV, the DPIP to MV partial reaction was accelerated to 164% of the control rate in the presence of low concentrations of the detergent. Higher concentrations were inhibitory, as with linolenic acid. Since digitonin partially solubilizes membrane structures and, at high concentrations, causes the release of up to 70% of the plastocyanin from the membrane (28), the acceleration in rate in both instances may be due to an alteration of a restrictive membrane structure that places a limitation on the reactivity of plastocyanin with DPIP or TMPD³.

Photosystem II-Inhibition

The failure of the linolenic acid-treated chloroplasts to recover photosystem II activity after washing led us to examine the possibility that the loosely-bound pool of manganese (9) was affected by exposure to linolenic acid. The experimental procedure was similar to that shown in Fig. 1: chloroplasts at 20 $\mu\text{g/ml}$ chlorophyll were incubated with linolenic acid for 10 minutes and washed by centrifugation (first with STN + 2mM EDTA, then with STN). A metal analysis of the chloroplasts showed that with increasing concentrations of linolenic acid, a greater loss of manganese occurred from the photosynthetic membrane. In Fig. 2, the remaining photo-

system II Hill rates are plotted against manganese content; the linear relationship with an intercept at 2 Mn/400 chlorophylls suggests that the removal of the loosely-bound pool of manganese (~ 3 Mn/400 Chl) is responsible for the irreversible loss of the photosystem II Hill reaction. In this regard, linolenic acid resembles the effect of 0.8 M Tris (38) or 5 mM NH_2OH (9) on isolated chloroplasts.

Figure 3 shows the effect of 200- μM -linolenic acid (added in the dark) on the fluorescence rise curve of dark adapted chloroplasts following a 30-second incubation period. This concentration of linolenic acid was chosen because it completely inhibits oxygen evolution (Fig. 1). We found not only that the variable fluorescence (F_{var}) was missing, but also that the initial level of fluorescence (F_i) had risen to match the maximum level of fluorescence (F_{max}). All subsequent illuminations showed the same behavior. Significantly, the presence of 0.5% BSA prior to the addition of linolenic acid protected the fluorescence characteristics against linolenic acid-induced damage.

In Figure 4, the fluorescence changes are shown as a function of time of incubation with 80- μM linolenic acid. The time-course shows that the effect of linolenic acid on the fluorescence yield was very rapid and may well have been within the mixing time of the reagents. The complete loss of F_{var} and the increase in F_i to match F_{max} was achieved in less than 20 seconds of exposure under the specified conditions. Following the initial, rapid increase in F_i , there was a slower decline in the fluorescence yield. The meaning of the rise in F_i and F_{max} beyond the

$F_{\max}$ of the control chloroplasts and the significance of the slower decline in F_i are still unclear.

The author wishes to emphasize that the effect on the fluorescence yield is a unique characteristic of linolenic acid. This result clearly differs from that observed in Tris or NH_2OH -washed chloroplasts where F_{var} is missing but F_i remains low. Only under certain circumstances can a high F_i be observed after NH_2OH treatment (6). In the presence of NH_2OH and DCMU, chloroplasts show a normal DCMU-rise curve on the first illumination provided NH_2OH is added to equilibrated chloroplasts in complete darkness. Upon a second illumination, separated from the first by a suitable dark period, the fluorescence rise appears similar to that of the linolenic acid-treated chloroplasts; that is, F_i is high, equaling $F_{\max}$. Under these conditions, Q remains reduced following the first illumination since DCMU prevents electron flow to the plastoquinone pool, and the back-reaction of Q^- is slow in the absence of the S_2 state.

In the linolenic acid-treated chloroplasts, the dark reduction of Q cannot be the cause of the high F_i ; rather it appears that the photochemical charge separation may be inhibited. If the charge separation were prevented, the fluorescence would remain unquenched, behaving as if Q^- were present prior to illumination. It appears, therefore, that linolenic acid functions either by inhibiting the transfer of energy from the antennae chlorophyll to the trap or by inhibiting the charge separation within the system II trap.

In order to examine further our newly proposed site of inhibition, we examined artificial donor activities in photosystem II as a function of

linolenic acid concentration. The chloroplasts used in this experiment were NH_2OH -washed prior to assay with DPC in the presence of linolenic acid to avoid mixed H_2O -to-DPIP and DPC-to-DPIP rates. We found that the inhibition of the specific photosystem II donor reaction $\text{DPC} \rightarrow \text{DPIIC}$ depended on linolenic acid concentration to nearly the same degree as the Hill reaction with PD_{ox} using normal chloroplasts. (The data not shown, but see Fig. 8 for a comparable experiment.)

We observed nearly the same effect of linolenic acid on artificial donor activities in a purified photosystem II particle. A TSF-2 particle was prepared from spinach chloroplasts by the method of Vernon and Shaw (36) and incubated with linolenic acid for 2 minutes prior to measurement. The chlorophyll concentration was adjusted to 14 $\mu\text{g}/\text{ml}$, somewhat below the chlorophyll content in the chloroplasts. As shown in Fig. 5, concentrations of linolenic acid similar to those effective with chloroplasts inhibited the DPC to DPIP photoreduction in the isolated photosystem II particle.

We conclude from these results that linolenic acid affects two sites in system II: 1) It slowly (see later section) induces the loss of the loosely-bound pool of manganese from the water-splitting site. 2) It rapidly (<20 sec) inhibits the photochemical charge separation in photosystem II.

Photosystem II - Prevention and Reversal of Inhibition

Several reports that inhibition of the Hill reaction by linolenic acid may be partially reversible (25,26) led us to investigate the conditions

under which BSA and inorganic metal ions prevent or reverse damage to either photosystem II inhibition site. In agreement with other studies (22,10,25), we found that adding 0.5 to 2% BSA in the chloroplast reaction mixture prior to addition of linolenic acid prevented linolenic acid-induced damage to the Hill reaction and to the fluorescence characteristics (Fig. 3). Similarly, the inclusion of 10 mM Mn^{++} , Ni^{++} , or Ca^{++} in the reaction mix prior to addition of 80 μ M linolenic acid preserved 78% (Mn), 75% (Ni), and 93% (Ca) of the pathway from H_2O to MV; without the ions, the rate after 2 minutes was nearly zero. Curiously, 10 mM Mg^{++} offered little or no protection against linolenic acid-induced damage. Monovalent cations, even at high concentrations (0.2 M), were ineffective at preventing inhibition.

An important but controversial consideration is whether BSA or Mn^{++} is effective in reversing the inhibition to either photosystem II site once inhibition has already occurred. We first attempted to reestablish the pathway from H_2O to MV by adding BSA to the reaction mix after incubating chloroplasts for various time intervals with 80- μ M linolenic acid. The lower curve of Fig. 6a shows the rates of MV-mediated O_2 uptake (H_2O as donor) as a function of incubation time in the presence of 80- μ M linolenic acid. After an initial rapid decline to only 30% of the control rate within 3 to 5 seconds, the rate less rapidly reached zero at 2 minutes. The upper curve shows the recovery of electron transport rates at specified times following the addition of 0.5% BSA. It is clear that a) Addition of BSA to the linolenic acid-treated chloroplasts reverses the inhibition of the electron transport chain, and b) The degree of recovery depends strongly

on exposure time to the inhibitor. We observed nearly identical results with PD_{ox} as acceptor.

As seen in Fig. 6b, the addition of Mn^{++} to the reaction mix also reversed the inhibition of the $H_2O \rightarrow MV$ pathway, and, as with BSA, recovery was highly dependent on the exposure time to the fatty acid⁵. Regardless of the extent of loss, the recovery rate after addition of Mn^{++} remained stable with time. In the presence of 40- μM linolenic acid, the rate of decay followed first-order kinetics ($t/2 = 147$ seconds) and, with 80- μM , the rate appeared first-order kinetics ($t/2 = 48$ seconds) only after an initial faster decay. The slow, time-dependent nature of this inhibition, which is in contrast to the fast effect upon the fluorescence yield (Fig. 4), strongly supports the suggestion that two independent inhibitory processes occur in system II: a rapid, but reversible inhibition of the photoact followed by a slower, irreversible decay of the water-splitting mechanism. According to this model, Mn^{+2} added within seconds after the linolenic acid affords the recovery of oxygen evolution because the inhibition of the charge separation is reversed. The onset of the slower, irreversible loss of Mn from the water-splitting mechanism has yet to occur in these short time ranges.

The fluorescence characteristics of the BSA-treated samples are shown in Fig. 7. In the presence of 80- μM linolenic acid, F_i is within 90% of F_{max} and only a small portion of F_{var} remains. The "reversal" experiment, performed by adding 2% BSA to the chloroplasts after 20 seconds of incubation in linolenic acid shows two important characteristics:

- the recovery of the variable portion of fluorescence, F_{var} , and
- b) the

return to a "low" F_i . We found, however, that the recovery of F_{var} was accompanied by an extended rise curve which we attributed to the disintegration of the manganese-containing donor complex in photosystem II and to the acceleration in photosystem I rates. Since the rise curve is a balance between the rate of photoreduction of Q and A by system II and the effect of system I (which tends to drain the A pool), any effect which diminishes photosystem II and/or accelerates photosystem I would extend the time required to photoreduce the plastoquinone pool. Although the data are not shown, Mn^{+2} also reversed the effect of linolenic acid on F_i and had an effect similar to BSA on the recovery of F_{var} .

The observation of a reversible site of inhibition at the photoact was supported by the recovery of artificial donor reactions ($DPC \rightarrow DPIP$) after the chloroplasts were washed with BSA. In this experiment, spinach chloroplasts were pre-washed with 5 mM NH_2OH for 15 minutes to remove the loosely-bound pool of manganese; the Hill activity after this treatment was negligible. Figure 8 shows rates of DPC-mediated DPIP reduction, measured after 6 minutes of exposure to linolenic acid and again following treatment with 0.5% BSA and washing the chloroplasts by centrifugation (1x with STN + 0.5% BSA, 1x with STN alone). Even though ~90% of artificial donor rates through photosystem II were inhibited in the presence of 80- μM linolenic acid, washing with BSA allows recovery of all but 10% of the activity. At concentrations of linolenic acid greater than 100- μM , either irreversible damage to the photosynthetic lamellae occurred or the inhibitor was not completely removed.

DISCUSSION

The results of these experiments show that the effect of linolenic acid on photosynthetic electron transport is localized at a site on the donor side of photosystem I and at two functionally separate sites in photosystem II.

In photosystem I, the most striking event was the increase in DPIP + MV rates up to 100- μ M linolenic acid and the sharp decline from 100 to 200- μ M linolenic acid. Qualitatively, it appears that two competing processes are caused by linolenic acid: one which causes an acceleration in the DPIP + MV pathway and another which causes its decline. The stimulation may be related to the structure of the lipid membrane which restricts the interaction of components on the oxidizing side of photosystem I. Because the oxidation kinetics of plastocyanin and the reduction kinetics of P700 show electron transfer with a half-time less than 20 μ s (15), the rate-limiting step probably occurs prior to this reaction. Therefore, the increase in rate may be due to the greater reactivity of DPIP or TMPD with plastocyanin in a membrane that has been made permeable by linolenic acid.

Okamoto et al. (25) has studied in detail the effects of linolenic acid on the ultrastructure of chloroplasts. At 15- μ M linolenic acid, chloroplasts at 20 μ g/ml chlorophyll were reported to retain gross membrane structure although intergrana and end grana thylakoids appeared swollen. Beginning at 40- μ M linolenic acid, many grana thylakoids were destacked and unfolded into extended membranes, although not all were equally affected. At 100 μ M, grana structure disappeared completely and vesicles

of various sizes appeared many of which were enclosed in a balloon-like outer membrane. These findings are consistent with the above suggestion that there may be large changes in membrane permeability induced by exposure to 40 - 100- μ M linolenic acid.

The decline in rate at 100 to 200- μ M linolenic acid may be caused either by the direct loss of plastocyanin from the permeable, balloon-shaped vesicles or by the larger volume that plastocyanin would occupy if it were present within the intrathylakoid space. In support of the latter proposal, Bendall and Wood (5) found that by increasing the osmolarity of the medium ten-fold with either sucrose or KCl, a 2-3x stimulation in TMPD to MV rates was obtained. One explanation of this is that plastocyanin is in equilibrium with the binding site on the thylakoid membrane and the intrathylakoid volume. Accordingly, any increase in the intrathylakoid volume would result in a shift away from binding and thus cause a decline in photosystem I-mediated rate. It is, therefore, probable that the decreased rate at high concentrations of linolenic acid is due to a change in the reactivity of plastocyanin, either because it is lost from the extended vesicles or because it is distributed within a much larger volume in the thylakoid.

In photosystem II, two inhibitory sites can be distinguished. We found that, within a few seconds after chloroplasts were exposed to 100- μ M linolenic acid, the Hill reaction became inhibited, and a high initial level of fluorescence occurred. The inhibition to the Hill reaction was almost completely reversible when the inhibitor was removed from the chloroplasts within seconds of its introduction. After its removal, F_i

approached its original value and F_{var} returned, albeit with a slightly extended rise time. An irreversible inhibition of the Hill reaction occurred when chloroplasts were incubated for longer periods of time.

This slow, irreversible inhibition of photosystem II was shown to be due to the loss of manganese from the water-splitting mechanism. Accordingly, the time course of water-splitting recovery shown in Fig. 6 represents the kinetics of removal of the loosely-bound pool of the Mn from its binding site(s).

In our experiments, the reversibility of the fluorescence characteristics after washing the chloroplasts with BSA strongly suggested that the major site of inhibition of the Hill reaction was the suppression of the photochemical charge separation. These data imply that the fast, reversible inhibition site occurs at the photoact itself. It may involve a mechanism that either blocks the charge separation or prevents energy from funneling from the antennae chlorophyll into the trap. This site of inhibition was completely reversible provided the chloroplasts had been exposed to moderate concentrations ($<100 \mu\text{M}$) of linolenic acid. Artificial donor studies confirmed this conclusion by showing that electron flow from DPC to DPIP was restored under the same conditions that allowed F_i to recover its low, original value. Although the recovery of F_i and of artificial donor rates were incomplete at higher concentrations of linolenic acid, this may be understood in light of studies with ^{14}C -linolenic acid, which show that removal of fatty acid from the membrane is incomplete even after several washings (26). Perhaps even lower values of F_i and greater

recovery of artificial donor rates could be obtained by exhaustive washing with BSA or Mn^{++} .

Okamoto et al. (25) reported that the linolenic acid-induced structural changes to the chloroplasts are completely reversible. Even though 100 μM linolenic acid transformed the photosynthetic membrane into assemblies of open vesicles, addition of 2 mg/ml BSA restored the original grana stacks and intragranal thylakoids. Similar refolding of the grana stacks was observed on washing the chloroplasts with a large quantity of preparation medium. They concluded that the reversibility of structural changes as well as Hill activity correlate with reversibility of fatty acid binding.

Our results indicated a low specificity of divalent cations for prevention or reversal of the linolenic acid effects. Ni^{++} and Ca^{++} , but not Mg^{++} , were as effective as Mn^{++} . Co^{++} has effects similar to Mn^{++} and Ca^{++4} . It seems likely that these effects are a consequence of formation of insoluble salts of linolenic acid with the various cations.

The mechanism of the inhibition of the photoact may ultimately lie in the ability of linolenic acid to penetrate its hydrophobic tail into the lipid membrane and change the orientation of the electron donor and acceptor complexes relative to one another. Alternatively, the hydrophobic tail might interact with the antenna chlorophyll bed and inhibit transfer of energy to the photoactive trap. Either mechanism would explain the high initial fluorescence yield.

A potentially more damaging effect of linolenic acid would be its

direct interaction with membrane proteins. Fragata (14) suggests that thylakoid transformations during aging results from conformational changes in the membrane proteins because of their interaction with free fatty acids. Since lipids are important in the three-dimensional structure of lipoproteins (29,30), and since photosystem II and its accessory pigments are most likely bound in a membrane-protein complex, it is reasonable to suppose that alteration of the membrane structure would induce organizational changes in the associated peptides. These altered structures might be incapable of facilitating energy transfer to the trap or of initiating a charge separation between the electron donor and acceptor complexes.

We are aware of only one other treatment that inhibits photosystem II and causes a high initial fluorescence yield upon illumination. Berry et al. (7) indicated an increase in F_0 in a study of thermal damage to Atriplex sabulosa after heat treatment of whole leaves. In the presence of DCMU, F_0/F_{max} increased from 40 to 50% for undamaged tissue to 70 to 90% for severely damaged chloroplasts. In contrast to the changes in F_0 , no changes in the magnitude of F_{max} occurred. Based partly on this evidence, Berry et al. (7) suggested that the anomalous fluorescence characteristics reflect heat damage to the reaction center itself. In light of the linolenic acid-induced effects on chloroplasts discussed in this paper, we suspect that the effect of heat on A. sabulosa is due to linolenic acid acting on the reaction center of system II following its liberation from the photosynthetic membrane.

TABLE I

The Effect of the Yeda Press on Mn, Cu, Plastocyanin
Content and Photosystem II Rates in Spinach Chloroplasts

Experiment	Conditions	H ₂ O→PD _{ox}	H ₂ O→MV	Mn/400 Chl	Cu/400 Chl	PC/400 Chl
1	Control	292	417	4.83	3.14	1.59
	Yeda Press	291	63	4.43	1.24	.15
2	Control	298	360	5.93	4.65	1.98
	Yeda Press	288	67	5.20	1.68	.17
3	Control	260		5.43	2.67	1.43
	Yeda Press	257		5.20	1.00	.13

To release plastocyanin from the lamellae, spinach chloroplasts were suspended in Tricine buffer (0.025 M, pH 7.8) containing 0.01 M KCl and 1 mM EDTA, and passed 3x through the Yeda press at 112 kg/cm². The fragments were spun at 106,000 xg for 2 hr and the pellets were resuspended in Tricine buffer (0.025 M pH 7.8) containing 0.01 M KCl. The plastocyanin content was determined by the method of Plesnicar and Bendall (27); the chloroplasts and the washed Yeda press fragments were sonicated for 1 min (six 10-second intervals) using the highest setting of a Bronson sonifier and pelleted by centrifugation at 180,000 xg for 3 hr. Since sonication releases plastocyanin quantitatively from the membrane (28,20), the supernatant was used directly for the analysis. System II rates were determined as described in Fig. 1. Uncoupled rates from H₂O to MV were determined in STN buffer, 0.1 mM MV, 0.1 mM KCN and 0.03 M methylamine.

TABLE II

The Effect of Plastocyanin on Photosystem I Rates in Control
and Linolenic Acid-Treated Chloroplasts

Conditions	nmol PC	$\mu\text{mol O}_2/\text{mg Chl}\cdot\text{hr}$
Control	0	307
	4.85	323
Treated (200 μM Linolenic Acid)	0	19.7
	1.21	36.2
	2.42	71.5
	4.85	124.5
	12.12	214.5
	30.30	357.1

Chloroplasts at 20 $\mu\text{g}/\text{ml}$ chlorophyll were treated with 200 μM linolenic acid for 10 min in STN buffer and centrifuged at 25,000 $\times g$ for 5 minutes. The pellet was washed in STN buffer and 2 mM EDTA for 5 minutes, centrifuged again and washed with STN buffer alone. The 2 ml assay mixture included 10^{-4} M MV, .03 M methylamine, 10^{-4} M TMPD, 10^{-4} M KCN, 10^{-5} M DCMU, 5×10^{-3} M ascorbate and chloroplasts at 20 $\mu\text{g}/\text{ml}$ chlorophyll.

TABLE III

The Effect of Linolenic Acid on DPIP and TMPD-Mediated
Photosystem I Rates

Linolenic acid μM	O_2 Uptake (DPIP) $\mu\text{mol/mg Chl}\cdot\text{hr}$	O_2 Uptake (TMPD) $\mu\text{mol/mg Chl}\cdot\text{hr}$
0	363	368
40	420	389
60	471	434
100	577	530
110	630	551
120	250	583
140	201	508
160	-	254
200	106	138

Conditions: Photosystem I rates were determined with chloroplasts at 20 $\mu\text{g/ml}$ chlorophyll in STN buffer, pH 7.4, containing 10^{-4} M MV, 10^{-4} M KCN, .03 M methylamine, 2 μM DCMU, 10^{-4} M DPIP or TMPD, 5 mM ascorbate and linolenic acid as specified. The same batch of chloroplasts was used for both studies.

TABLE IV

The Effect of Low Concentrations of Digitonin on
Methylviologen-Mediated Oxygen Uptake

<u>Digitonin</u>	<u>O₂ Uptake</u> (% Control)
0%	100%
0.01	110
0.05	164
0.10	163
0.25	86

Chloroplasts at 400 $\mu\text{g}/\text{ml}$ chlorophyll were incubated with digitonin in STN buffer for 6 hrs and assayed for photosystem I rates (MV-mediated O₂ uptake) after dilution to 20 $\mu\text{g}/\text{ml}$ chlorophyll as described in Fig. 1.

LEGENDS FOR FIGURES

Fig. 1. Electron Transport Rates in Chloroplasts Incubated with α -Linolenic Acid

In the first series of experiments, spinach chloroplasts at 20 $\mu\text{g/ml}$ chlorophyll were incubated with α -linolenic acid for 10 minutes and assayed for photosystem I rates (closed circles) in: STN buffer containing 10^{-4} M MV, 10^{-4} M KCN, 0.03 M methyamine, 5 mM ascorbate, 50 mM DPIP, 10 μM DCMU and for oxygen evolution (squares) in: STN buffer containing 0.6 mM PD_{ox} and 3 mM $\text{K}_3\text{Fe}(\text{CN})_6$. In the second series of experiments, the chloroplasts were treated similarly but after 10 minutes of incubation, were washed by centrifugation, resuspended at 0°C in STN buffer containing 2 mM EDTA, and washed a second time in STN before assay. Photosystem I and II rates after washing are indicated by open circles and triangles, respectively. Photosystem I rates are expressed as (MV-mediated) oxygen uptake and photosystem II rates as oxygen evolution.

Fig. 2. Photosystem I and Photosystem II as a Function of Copper and Manganese Content.

Spinach chloroplasts at 20 $\mu\text{g/ml}$ were incubated in various concentrations of linolenic acid at room temperature for 10 minutes and washed free of the inhibitor as described in Fig. 1. The copper and manganese remaining in the membrane after washing are plotted against photosystem I (DPIP to MV) and photosystem II (H_2O to PD_{ox}) rates, respectively.

Fig. 3. Fluorescence Characteristics of Spinach Chloroplasts in the Presence of Linolenic Acid.

Top: Spinach chloroplasts at 20 $\mu\text{g/ml}$ chlorophyll and 200 μM linolenic acid in STN buffer containing 5 mM MgCl_2 . The linolenic acid was added in complete darkness to 10-minute dark-adapted chloroplasts. F_{max} was determined after addition of DCMU to the assay mixture.

Middle: Control chloroplasts

Bottom: Effect of 2% BSA added to the assay mixture prior to the addition of 200 μM linolenic acid.

Fig. 4. Effect of Time of Incubation of Spinach Chloroplasts with Linolenic Acid

Chloroplasts at 20 $\mu\text{g/ml}$ chlorophyll in STN buffer (pH 7.5) containing 5 mM MgCl_2 were incubated in the dark at room temperature for 10 minutes. Linolenic acid was then added to 80 μM , and the suspension was illuminated at the specified times. The initial (F_i) and maximum (F_{max}) levels of fluorescence were determined as indicated in Fig. 3 and plotted against time of incubation. A fresh sample was used for each determination. ΔF corresponds to F_{var} , the difference in magnitude between F_{max} and F_i .

Fig. 5. Artificial Donor Activity in an Enriched System II Particle as a Function of Linolenic Acid Concentration.

TSF-2 particles, at 14 $\mu\text{g/ml}$ chlorophyll, were incubated for 2 minutes with linolenic acid and measured spectrophotometrically for DPIF reduction at 590 nm in a reaction mix containing: sucrose.

(0.25 M), phosphate (0.03 M, pH 6.4), DPIP (0.064 mM) and DPC (.05 mM). An identical reaction mix containing 16.7 DCMU was prepared; the DCMU rate was subtracted from the former to obtain the DCMU-dependent photosystem II rate.

Fig. 6. Time Course of BSA and Manganese-Induced Reversal of Inhibition of the Hill Reaction.

Spinach chloroplasts at 20 $\mu\text{g/ml}$ chlorophyll were incubated with 40 and 80 μM linolenic acid in STN buffer, pH 7.5 containing 10^{-4} M MV, 10^{-4} M KCN, and 30 mM methylamine. After a period of incubation, BSA (a) or Mn^{++} (b) was added to a final concentration of .5% and 10 mM respectively, and an aliquot was removed for assay. The assay mix contained chloroplasts at 20 $\mu\text{g/ml}$ in STN buffer with 0.1 mM MV, 0.1 mM KCN and 0.03 M methylamine.

Fig. 7. Fluorescence Characteristics Following Addition of BSA to Linolenic Acid-Inhibited Chloroplasts.

Top: Chloroplasts at 20 $\mu\text{g/ml}$ chlorophyll and 80 μM linolenic acid in STN buffer containing 5 mM MgCl_2 . F_{max} was determined after addition of DCMU to the assay mixture.

Middle: Control chloroplasts

Bottom: Effect of 2% BSA added to the mixture 2 minutes after the addition of 80 μM linolenic acid. Both BSA and linolenic acid were added to the chloroplasts in complete darkness.

Fig. 8. The Effect of Washing on Artificial Donor Activity in Linolenic Acid-Treated Chloroplasts.

Spinach chloroplasts at 200 $\mu\text{g/ml}$ chlorophyll were extracted with 5 mM NH_2OH in STN buffer (pH 7.8) for 15 minutes at 0°C , washed by centrifugation, and resuspended in buffer containing 0.25 M sucrose and 0.03 M PO_4^{3-} (pH 6.4). Aliquots of 20 mM stock linolenic acid (in 95% ethanol) were added to a chloroplast suspension at 20 $\mu\text{g/ml}$ chlorophyll. After 6 minutes of incubation at room temperature 2 ml was removed for the DPC-to-DPIP assay and the remainder was centrifuged at 27,000xg for 3 minutes. The pellet was washed with STN buffer containing 0.5% BSA and again with STN buffer alone. The final precipitate was resuspended in buffer containing 0.25 M sucrose and 0.03 M PO_4^{3-} (pH 6.4) for assay.

Fig. 5

Fig. 6

Fig. 7

FOOTNOTES

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² Abbreviations: DPIP, 2,6-dichlorophenolindophenol; TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine; MV, methylviologen; PD_{ox}, oxidized form of phenylenediamine; DPC, diphenylcarbazide; PC, plastocyanin.

³ A second possibility exists: only one of the two plastocyanins functions on the linear pathway from Q to P700. The other may be involved in another segment of the electron transport chain but the effect of linolenic acid on the membrane structure permits it to couple to P700+ reduction. This could act to double the rate of electron flow through photosystem I and yet place the rate limitation on the oxidizing side of P700.

⁴ Personal communication, W. Cohen.

⁵ The addition of 10 mM Mn⁺⁺ to normal chloroplasts resulted in a doubling of the control rate of oxygen uptake in the presence of MV. However, as proposed by Epel and Neumann (13), Mn⁺⁺ reduces O₂⁻ to H₂O₂ directly, thereby preventing its disproportionation to 1/2 O₂ and 1/2 H₂O₂. With water as donor, the uptake should triple in the presence of Mn⁺⁺; we usually found a two-fold increase.

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