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PHOTOSYSTEM II REACTIONS IN THYLAKOID MEMBRANES

BY

BARBARA ANN ZILINSKAS

B.A., Massachusetts State College (Framingham), 1969
M.S., University of Illinois, 1970

THESIS

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Biology
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Govindjee
Chairman

William F. Ogren

Edwin J. Feit

Constantin A. Rebeiz

† Required for doctor's degree but not for master's

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LIST OF ABBREVIATIONS

AS	12-(9-anthroyl) - stearic acid
ATP	adenoisine triphosphate
Chl	chlorophyll
Chl *a*	chlorophyll *a*
Cyt f	cytochrome f
DABS	diaminobenzene sulfonic acid
DCMU	3-(3′, 4′-dichlorophenyl)-1, 1-dimethylurea
DCPIP	2,6-dichlorophenol indophenol
$DCPIPH_2$	2,6-dichlorophenol indophenol: ascorbate (300:1)
DLE	delayed light emission
DNA	deoxyribonucleic acid
DPC	diphenylcarbazide
EPR	electron paramagnetic resonance
Fd	ferredoxin
FeCy	ferricyanide
F_o	minimum (or "constant") fluorescence yield
F_∞	maximum fluorescence yield
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
OEF	oxygen-evolution factor
OEI	oxygen-evolving intermediate

P or P_{680}	photosystem II reaction center
P_{700}	photosystem I reaction center
PC	plastocyanin
PMS	phenazine methosulfate
PPNR	photosynthetic pyridine nucleotide reductase
PQ	plastoquinone
PS I	photosystem I
PS II	photosystem II
Q	primary electron acceptor of photosystem II
Reductase	ferredoxin - NADP - reductase
RNA	ribonucleic acid
SiMo	silicomolybdate
SiTu	silicotungstate
X	primary electron acceptor of photosystem I
Z	electron donor of photosystem II

I. INTRODUCTION

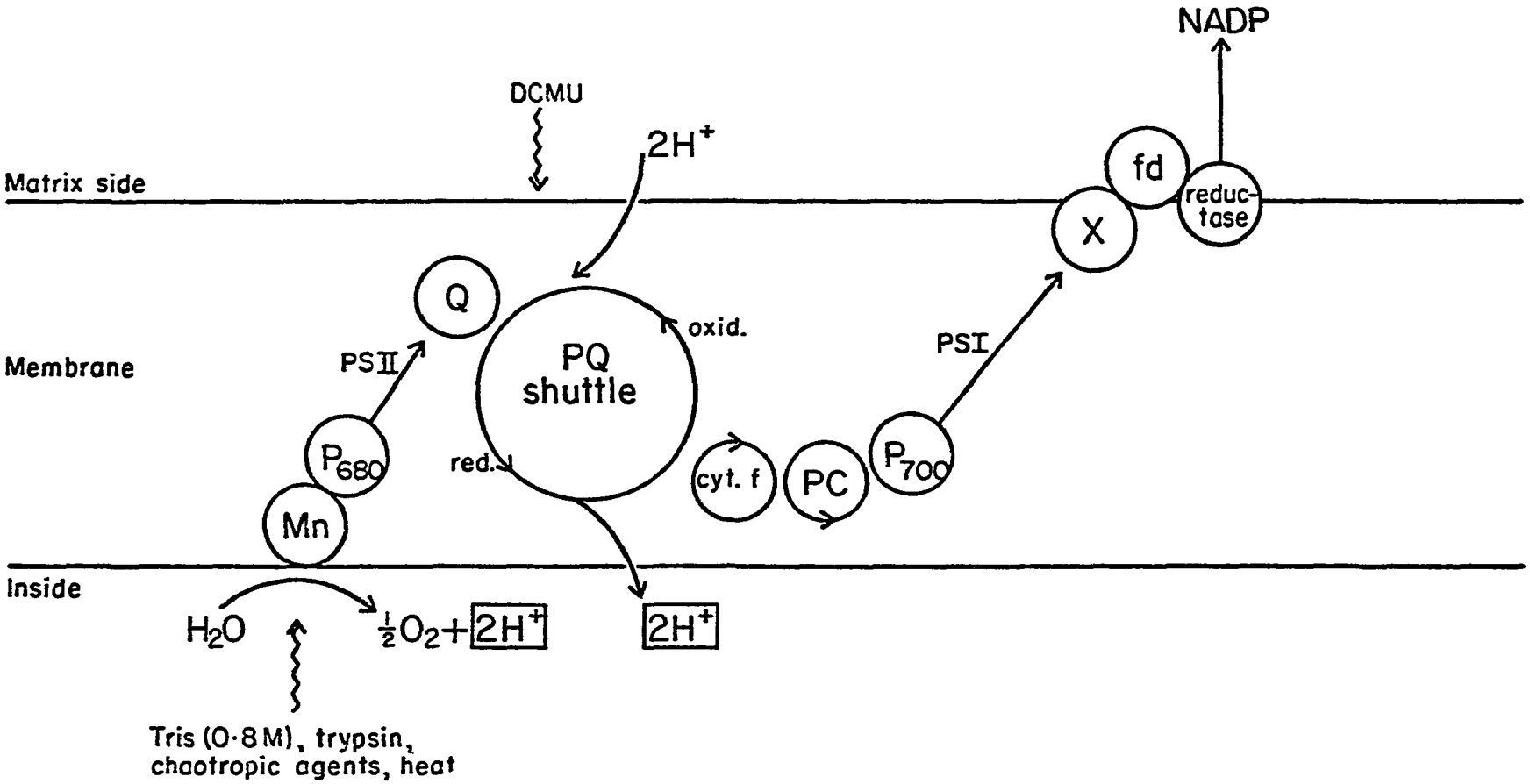
The light reactions of green plant photosynthesis lead to the production of oxygen, reducing power in the form of reduced nicotinamide adenine dinucleotide phosphate (NADPH), and adenosine triphosphate (ATP). The latter two products are utilized in fixation of CO_2 in dark enzymatic reactions into an organic carbon source which is the support of life in the plant that produces it as well as ultimately all heterotrophs. The light reactions, generally accepted to be two, are sensitized by two "photosystems" embedded in a lamellar membrane, each system with its own light harvesting pigment molecules, specialized chlorophyll a (Chl a) reaction center molecule which undergoes the photochemical primary reaction, and associated electron transport carriers. The Calvin cycle enzymes, on the other hand, are located in the stroma of the chloroplast, bounded by the outer chloroplast membrane and the lamellar, "thylakoid" membranes.

Since the introduction of the original chemiosmotic hypothesis by Mitchell (1) as a mechanism for phosphorylation in biological membranes, interest has been sparked in the study of the location of electron transport components in the thylakoid. Basically, the model requires an asymmetric distribution of hydrogen atom carriers and electron acceptors in the

membrane, such that one side of the membrane will accumulate hydrogen ions; this production of a pH gradient and accompanying membrane potential, then, in terms of the chemiosmotic hypothesis, is the driving force for phosphorylation. Research done in the laboratory of Witt (see references 2 and 3) was the first to suggest an establishment of a separation of charge and creation of an electric field across the membrane as a result of both of the primary photoacts, with the positive charges toward the inside. To accommodate this data, as well as evidence derived from the use of antibodies against specific components of the photosynthetic chain, impermeable protein modifiers, hydrophilic and hydrophobic electron donors and acceptors, and structural and biochemical analysis of detergent fractionated chloroplast membranes, Trebst (4) has proposed a tentative model for the organization of electron transport components in the membrane as shown in Figure 1.

Basically, the asymmetric distribution of the two pigment systems in the membrane as proposed by Arntzen et al. (5) and Briantais (6) is more or less conserved in this model with photosystem I (PS I) pigments and associated components being exteriorly localized (close to the stroma) and photosystem II (PS II) being oriented more toward the locular (inner) surface of the thylakoids. Mitochondrial membranes have been very well characterized as to their sidedness and localization of electron carriers, because, unlike thylakoid membranes, they can be turned

Fig. 1.--Photosynthetic electron transport from water to $NADP^+$, showing tentative localization of intermediates and proton release within the membrane. Mn, the oxygen-evolving intermediate; P_{680}, the reaction center of photosystem II; PS II, photosystem II pigments; Q, the primary electron acceptor of PS II; PQ, plastoquinone; cyt f, cytochrome f; PC, plastocyanin; P_{700}, reaction center of photosystem I; PS I, photosystem I pigments; X, primary electron acceptor of PS I; fd, ferredoxin; reductase, ferredoxin-NADP-reductase; $NADP^+$, nicotinamide adenine dinucleotide phosphate.



Adapted from Trebst, Ann. Rev. Plant Physiol. (1974)

inside out by sonication, and thus both sides can be specifically labelled. It has not yet been possible to do the same with thylakoid membranes, and there are conflicting reports in the literature with regard to the organization of its constituents [see Trebst (4)].

The localization of many of the photosystem I components is, however, well established to be toward the outside of the membrane. The immunological data is the most convincing in this regard, with antibodies against photosystem I endogenous acceptors severely inhibiting electron transport to $NADP^+$ and dramatically agglutinating chloroplasts either directly or indirectly (7-9, see below for further discussion). Also supportive are the accessibility to hydrophilic artificial acceptors, the greater labeling by the impermeable protein modifiers diamobenzene sulfonic acid (DABS) (10, 11) and lactoperoxidase catalyzed iodination (12), and detergent fractionation studies that show photosystem I being stripped off the membrane first (5, 6).

The localization of P_{700}, the reaction center of PS I, is less clear. Racker et al.(13) have suggested a localization on the outside of the chloroplast thylakoid based on data obtained with an antibody, prepared against a P_{700} enriched pigment-protein complex, which inhibits methylviologen reduction mediated by ascorbate and directly agglutinates chloroplasts; this antibody might equally be directed against X, the

primary electron acceptor of PS I. Based on the creation of an electric field where each photosystem was shown to contribute to one-half of the field formed (3), P_{700} was thought to be localized inside the membrane.

In contrast to the conclusions drawn from immunological studies on PS I acceptors, the secondary electron donors to PS I, plastocyanin and chytochrome f, are buried in the hydrophobic region of the membrane. Antibodies to plastocyanin (13, 14) and cytochrome f (14) do not inhibit electron transport unless the site is exposed by sonication in the presence of the antıbodies. Also, lipophilic but not hydrophilic coupounds can donate to these components (15).

The arrangement of PS II components in the membrane is still largely subject for debate. In all likelihood, Q, the primary electron acceptor is more surface localized than not, although there are suggestions that it is still in a liphophilic region of the membrane, as would be expected of a quinone (if Q is actually a quinone) (see Trebst, 4). Suggestions have been made (16, 17) that silicomolybdate and silicotungstate alter membrane structure in such a way as to allow the hydrophilic electron acceptor, ferricyanide, to accept electrons directly from Q. We have examined this problem and provide evidence (Chapter IV) indicating that this is probably not the case; the silico-compounds themselves are shown to be the electron acceptors. However, as it is thought

that Q is not exposed to the hydrophilic region of the membrane although being close to the thylakoid exterior (18, 19), and these very large inorganic molecules could not be expected to permeate the membrane, there is probably some membrane alteration by simple addition of these compounds to the membrane, making them more accessible to Q; this availability is increased by additional light-induced conformational changes in the membrane.

It has been suggested by work with antibodies to chlorophyll (20) and enzymatic iodination of surface-localized components (21) that the PS II reaction center, P_{680}, is exteriorly located on the membrane. If this is the case and if the water oxidation components are localized on the inner surface of the membrane (see below), then there must be some means to allow for very fast tunneling of electrons from the secondary electron donor to P_{680} to allow for a separation of charges and a creation of an electric field across the membrane within a very short time range.

Our main concern has been with the localization and nature of the intermediate(s) involved in the oxidation of water by PS II (see Chapters II and III). The work of Fowler and Kok (22) is especially convincing with regard to placement of the oxygen evolution component close to the interior of the membrane, as they have shown that proton deposition, which follows almost the same kinetics of oxygen evolution, occurs inside

the thylakoid. The charge-accumulating species associated with oxygen evolution, situated on the inside of the membrane, and the primary electron acceptor of system II, located on the opposing membrane face, could allow for the charge separation which can, in turn, account for a creation of membrane potential and provide a driving force for phosphorylation as well as delayed light emission (1, 2, 23).

Contrary to the above finding, work done until recently was usually taken to suggest that the water oxidation component was localized closer toward the outside face of the thylakoid than the inside (4). This notion was supported by inhibition of oxygen evolution by the impermeable membrane modifier, DABS (24, 25), inhibition of oxygen evolution by several different antisera (26 - 29), and the action of trypsin specifically on oxygen evolution (30, 31). As these are all impermeable molecules, their ability to inhibit oxygen evolution was taken by some to indicate that the water oxidation intermediates are more surface localized than not. However, it is very important to note that the inhibition by DABS of PS II is strictly light-potentiated (24, 25), the antisera inhibit by only 15 to 20 percent (26 - 28), and an antiserum to lutein can inhibit oxygen evolution to 65 percent, provided the chloroplasts are extremely well coupled to phosphorylation (29). These data suggest that the site being inhibited by these agents is not readily accessible, but perhaps some conformational change in the membrane is better exposing the

intermediate or some component complexed to it that is able to transfer its alteration to the oxygen-evolving intermediate.

It might be appropriate to think in terms of the fluid mosaic model of membrane structure (32) where amphipathic proteins, having highly polar and non-polar ends, can migrate in the plane of the membrane (32, 33) and may, in fact, transverse much of the distance of the membrane (34). Nicolson (35) has shown gross movement of certain components, probably glycoproteins, in mouse fibroblasts with very mild proteolysis; perhaps, an analogy can be drawn with trypsin treated chloroplasts. With this model in mind, we have examined the action of several impermeable inhibitors of oxygen evolution, as well as permeable or physical inhibitors, to determine more clearly the localization of the oxygen-evolving intermediate in the membrane, as well as the mode of action of the various inhibitors of water oxidation (Chapter III).

As far as water oxidation is concerned, much is known of the kinetics of the reaction (36, 37), but we are yet ignorant of chemical nature of the intermediate(s) involved other than the fact that somehow manganese (38), chloride (38), and bicarbonate ions (39, 40) are implicated. The stumbling block appears to be the extreme lability of the component (41) as well as a lack of a suitable assay procedure. Our original research plans were focused around the use of the immunochemical techniques to avail us in the isolation of the oxygen-

evolving intermediate. Although we were successful in obtaining antisera which partially inhibited oxygen evolution reactions specifically against two of the five antigens used in immunization, we were unable to fulfill the original intentions for reasons described below. Nevertheless, the antisera provided us with information on the localization of the intermediate (Chapters II and III).

CHAPTER II

ANTISERA AGAINST A COMPONENT ON THE OXYGEN-EVOLVING SIDE OF PHOTOSYSTEM II

A. Introduction

The chemical intermediates of the dark reactions of photosystem II (PS II) associated with evolution of oxygen from water are basically unknown. Work with chemical inhibition by Tris (42), chaotropic agents (41, 43), proteases (44, 45), UV light inactivation (46), aging (47), and mild heating effects (48) seem to indicate that at least one chemical component, probably proteinaceous in nature, is involved, the isolation and characterization of which have not been possible due to its extreme lability (41) and lack of suitable assay procedure. Manganese (38), chloride (38), and bicarbonate (39, 40) ions have been implicated in oxygen evolution; in fact, a model system has been evolved (39) to illustrate how manganese ions may be directly participating in the charge accumulating mechanism of oxygen evolution which has been proposed to explain detailed kinetic data (36, 37.)

Very recently, Tel-Or and Avron (50) isolated a manganese-containing, low molecular weight component from a blue-green alga, Phormidium luridum, which is able to restore

oxygen evolution to mechanically ruptured membrane fragments of this alga. On the surface, this appears to be the greatest progress made on this baffling problem. However, there are a few obvious questions raised that require further experimentation before any universal conclusions can be drawn. First, the component is released and readded to blue-green algae fragments with remarkable readiness, quite unlike comparative attempts with higher plant chloroplasts; this raises interesting possibilities for an alternate arrangement of oxygen evolution component(s) in cyanophycean thylakoid membranes. Also curious is the fact this factor and its associated manganese have been shown to be extremely heat stabile, while oxygen evolution has been shown to be severely inhibited by heat treatment, accompanied by a loss of chloroplast bound manganese (41, 51). Taking these as precautionary examples, as well as other equally exciting reports in the literature dealing with the isolation of a critical factor involved with oxygen evolution that later proved disappointing (52, 53), we must await further data before believing the oxygen evolution intermediate problem has begun to be solved.

Our approach to the problem has been an immunochemical one, originally with the intention of being able to identify a component specifically involved with oxygen evolution by employing the specificity of the antibody-antigen interaction. However, several suggestions have now been made to

the effect that this component might exist in a transmembrane complex which bridges the oxygen-evolving intermediate with the reaction center and primary electron acceptor of PS II, and as this oxygen-evolving component is easily disturbed by changes in its environment, one does not know if antibodies inhibiting oxygen evolution are directed at the oxygen-evolving intermediate itself or at some other component of the complex. Obviously, antibodies against a structural component would not be very helpful in the isolation and characterization of the oxygen-evolving intermediate, and, therefore, the naiveté of the originally proposed plan becomes evident. Moreover, there are technical difficulties inherent in the isolation of a "pure component" by using the immunochemical approach, for rather circular reasons. As the antigen injected contains several immunogenic substances, the antibody titer is likely to be lower than if a pure antigen is used; also, affinity of the antibody for the extracted (and probably altered) antigen may be greater than for the native component. These problems, coupled with the apparent limited accessibility of the antigen in the chloroplast for the added antibody, result in a low level of inhibition of oxygen evolution which makes further studies on the isolation of the oxygen-evolving intermediate by this method difficult, if not impossible.

Nevertheless, this immunological approach was applied to chloroplast systems with an alternate purpose, i.e., the organization of components in the thylakoid. The immunological technique has provided valuable information on the localization of photosynthetic components in the membrane, as discussed in Chapter I. Roughly approximating, one might say that PS II (unlike PS I) is not surface exposed, as antisera produced against chloroplasts inhibit only PS I activity (54, 55). However, later immunochemical experiments have suggested a partial accessibility of reaction center II (20) and the oxygen-evolving intermediate (26-29) to their respective antisera. [The work to be presented by this author in this chapter has already been published (26, 27)]. The low levels of inhibition of PS II activity, an indication of the limited availability of the antibodies for the corresponding antigens, is in agreement with the agglutination studies of Briantais and Picaud (56); they show with antisera against PS I and PS II particles that PS I is more exteriorly localized on the membrane.

We shall show that immunization of rabbits with PS II particles from spinach and a solubilized extract from repeatedly frozen and thawed spinach chloroplasts produces antisera that specifically (but only partially) inhibit oxygen evolution at a site prior to diphenylcarbazide (DPC) donation (57), that is, probably against the same intermediate inactivated by Tris-washing (42) and a series of other oxygen evolution inhibitors (see Chapter III). As the nature of this problem

does not allow one to deal with purified antigens, it is impossible to make definite conclusions on the basis of typical immunological tests, e.g., agglutination reactions, with regard to localization of components in the chloroplast membrane. For the same reasons, an inhibition of oxygen evolution does not necessarily indicate that the active antibody in the respective antisera is actually specific for the oxygen-evolving component; there is also the likelihood, considering the proposed existence of this component as being part of a transmembrane complex and being very sensitive to perturbations of the membrane (see Chapter III), that the antiserum is against a component intimately associated with the actual oxygen-evolving site.

B. Materials and Methods

1. Chloroplast preparation

Chloroplasts were prepared by homogenizing in a Waring blender for 10 seconds depetiolated spinach leaves in 50 mM Tris-Cl buffer, pH 7.2, containing 0.4 M sucrose, 10 mM NaCl and 1 mM $MgCl_2$. The homogenate was filtered through eight layers of cheesecloth, centrifuged at 200 X g for two minutes, and then at 1000 X g for 10 minutes. The pellet was suspended in 50 mM Tris-Cl buffer and centrifuged at 1000 X g for 10 minutes; this final pellet was resuspended in a small amount of the homogenizing medium. In most experiments reported here, these chloroplasts were quickly frozen in a dry

ice and acetone slurry and used for several weeks thereafter without significant loss in photochemical activity.

2. Antigen preparation

a. Photosystem II particles

Photosystem II particles were isolated from market spinach according to Huzisige et al. (58) or Briantais (6) with minor modifications. The chlorophyll a to chlorophyll b ratio of these preparations varied from 1.9 to 2.4; the "Huzisige-type" particles were stored at -15°C in 50 mM Tris-Cl buffer, pH 7.2, containing 0.4 M sucrose, while the "Briantais-type" particles were stored in 100 mM Tricine-maleate buffer, pH 6.6, containing 0.4 M sucrose.

b. Extract from frozen and thawed chloroplasts

The extract from frozen and thawed chloroplasts was prepared as follows: First, chloroplast fragments were isolated from market spinach as described above. The final suspension of chloroplasts (∿500 μg Chl/ml) was lightly sonicated (sonication at 0°C on position four with sonifier cell disruptor, model W1850 with 1/2 inch tip, Heat Systems-Ultrasonics, Inc., Plainview, N. J.) for 30 seconds, and then centrifuged at 5000 X g for 20 minutes. (In such preparations, stroma proteins are lost.) The pellet was resuspended in a small volume of 50 mM Tris-Cl buffer, pH 7.2, containing 10 mM NaCl and 1 mM $MgCl_2$ and frozen quickly with dry ice and

acetone. These fragments were then thawed at room temperature in the dark, followed by slow refreezing at -15°C. After the thawing and freezing cycle was repeated five times [for a comparable method, see Black (53)], the chloroplast fragments were then centrifuged at 10000 X g for 45 minutes, the pellet discarded, and the supernatant stored at -15°C. The supernatant contains the antigen(s) referred to as "soluble extract."

c. Other antigens

Other antigens were prepared, based on suggestions in the literature that certain chloroplast treatments might result in release of a factor involved in oxygen evolution, as follows.

The "oxygen evolution factor" (OEF) was prepared according to the method of Huzisige et al. (52). Rabbits were also immunized with the supernatant from Tris-washed chloroplasts. The Tris-washing was done according to the method of Yamashita and Butler (42); however, following the incubation in Tris for 20 minutes, the chloroplasts were centrifuged at 1000 X g for 10 minutes, the pellet discarded, and then the supernatant centrifuged at 17000 X g for 30 minutes. The supernatant was concentrated several fold with lyphogel, no attempt was made to dialyze away the Tris for fear of losing small, low molecular weight molecules by dialysis, and the supernatant (referred to as Tris-wash supernatant) was stored at -15°C. Cheniae and Martin (41) reported that

it was possible to isolate a manganese containing protein in micellar form with Triton, although no method other than concentration of the detergent was given. We homogenized spinach leaves in 0.017 M Tris-Cl buffer, pH 7.2, centrifuged the homogenate, filtered through eight layers of cheesecloth, at 200xg for two minutes and then centrifuged the supernatant at 5000xg for 10 minutes. The chloroplasts were resuspended in 0.017 M Tris-Cl, pH 7.2, to ∿500 μg Chl/ml, and were then incubated in two percent Triton-X-100 for 30 minutes. This material was then centrifuged at 20000 X g for one hour and the supernatant used immediately thereafter for immunization.

3. Antigen analysis

In the case of two of the antigens used, PS II particles and the extract from repeatedly frozen and thawed chloroplasts, some investigation was done on the characteristics and/or purity. In the case of the PS II particles, ratios of chlorophyll a to b were determined using the equations of MacKinney (59); in addition, fluorescence spectra at liquid nitrogen temperature were made according to Cho et al. (60), using the spectrofluorometer described elsewhere (61).

The absorption spectrum of the extract was measured with a Cary-14 spectrophotometer. Protein concentration was estimated by Warburg and Christian's method (62) as well as

by a modified Folin-Ciocalteu test (63), arbitrarily using bovine serum albumin as a standard. As there was considerable absorbance at 260 nm, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) content was measured, applying the diphenylamine reaction (64) with calf thymus DNA as standard and the orcinol test (65) with ribose as standard, respectively. Manganese content was measured colorimetrically after oxidation by periodate (66), as well as by electron paramagnetic resonance (EPR) spectroscopy, measured according to the protocol of Blankenship and Sauer (67). In addition, a crude separation of the extract components was done by ammonium sulfate fractionation (25 percent, 50 percent, 75 percent and 100 percent cuts). The 100 percent $(NH_4)_2SO_4$ fraction was concentrated after dialysis by lyophilization. SDS acrylamide gels according to the method of Hoober (68) were run on those fractions as well as the original extract.

4. Immunization procedure

The various chloroplast preparations were injected into New Zealand white rabbits as soon as possible after preparation. The immunization course began with injection of antigens containing ∿10mg protein (or ∿2mg chlorophyll, in the case of PS II particles or the Triton-X-100 treated chloroplasts, discussed above) emulsified with an equivalent volume of complete Freund's adjuvant into the hind-foot pads

and intramuscularly into the back. After five weeks, the rabbit was boostered in the back muscles with antigen (5-7mg protein or 1-1.5mg Chl) emulsified with incomplete Freund's adjuvant; this was repeated at two-month intervals for three more injections. Bleedings were taken from the marginal ear vein weekly after the third injection, as well as intermittently earlier in the immunization course; sera were stored at -15°C. Control sera were collected prior to the first injection as well as weekly from two non-immunized rabbits to compare with immune sera of equal age. The immunoglobulin fraction was isolated from the antisera, essentially according to the method used by Hauska et al. (14), with the isolated globulins finally being suspended in 10 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl, according to Tel-Or and Avron (69).

5. Immunochemical tests

Agglutination of chloroplasts and system II particles was done essentially according to the method used by Briantais and Picaud (56) with modifications as described in the legends of the Tables. Indirect agglutination was done using goat anti-rabbit immunoglobulin (IgA+IgG+IgM), provided by Cappel Laboratories, Inc. (Lot 58221). After the antigen and antibodies were incubated for 30 minutes at 37°C, they were washed with physiological saline (0.8 percent), followed by centrifugation at 1000 X g for 10 minutes for chloroplast antigens

and 10000 X g for 30 minutes for PS II particles. The pellet was resuspended in the original volume and type buffer, and an equal volume of goat anti-rabbit immunoglobulins was added. Immunodiffusion tests were done in a gel containing 1 percent agar, 0.1 percent sodium azide and 0.15 M NaCl; precipitation, using the double diffusion technique, was done at 5°C, and 72 hours were allowed for full development of precipitin lines.

6. Hill reaction measurements

Chloroplasts used in the various assays, to check antiserum inhibitory activity, were prepared from market spinach as described above. Twenty µl of (anti) serum were preincubated at 0°C in the dark for 15 minutes with these broken chloroplasts ([Chl], 8µg) to allow for the antibody-antigen interaction.

Other chloroplast preparations for testing the inhibitory properties of the antisera included Tris-washing of chloroplasts, done according to the method of Yamashita and Butler (42) with incubation in the 0.8 M Tris-Cl extended to 30 minutes and done in dim light. Photosynthetic pyridine nucleotide reductase (PPNR) was prepared according to San Pietro and Lang (70). Chlorophyll estimation was done following MacKinney's method (59).

Hill activity was measured with a Cary-14 spectrophotometer equipped with a side attachment that provided actinic illumination at right angles to the measuring beam.

To monitor initial rates of dichlorophenol indophenol (DCPIP) reduction (at 597 nm), the sample was illuminated simultaneously with saturating red light [G.E. 120V, 650W, DYV tungsten lamp; Corning glass filter, C.S. 2-59 (3mm, plus a "heat" filter)]. The photomultiplier was protected by a 597 nm Farrand interference filter (half-band width, 10 nm). The intensity of the incident actinic light, measured by a Yellow Springs Instrument Radiometer, was $1.5 \cdot 10^5$ μwatts cm^{-2}.

Steady state rather than initial rates of DCPIP photoreduction were measured in most of the early experiments (see Table 2 and 5, A) with the PS II antiserum. The chloroplasts were illuminated for 45 seconds in saturating white light according to Mohanty et al. (71), and then measured for absorbance decrease at 603 nm.

$NADP^+$ reduction was measured at 340 nm, the photomultiplier protected by two C.S. 7-60 filters, with the sample simultaneously illuminated with saturating red light (Schott glass filter, RG 645). The intensity of the incident light was 10^5 μwatts cm^{-2}.

Ferricyanide reduction was measured at 420 nm, the photomultiplier protected by a 424 nm Farrand interference filter (half-band width, 12.5 nm), with the sample simultaneously illuminated with saturating red light (as for DCPIP reduction).

Mn^{2+} donation experiments were done following the experimental protocol of Ben-Hayyim and Avron (72), measuring oxygen uptake by methylviologen with a Yellow Springs Clark (concentration) electrode.

C. Results

1. Effects of antisera against OEF, triton-X-100 treated chloroplasts, and Tris-washed chloroplast supernatant

Of the five antigens injected into rabbits, only the antisera produced against PS II particles and an extract from repeatedly frozen and thawed chloroplasts were effective in specifically inhibiting oxygen evolution, as will be discussed below. Antisera to two of the other antigens did not alter rates of DCPIP photoreduction with respect to rates with the control sera; these sera were taken from rabbits immunized with the "oxygen-evolution factor" of Huzisige et al. (52) and the Triton-X-100 treated chloroplast extract (41), as discussed in Materials and Methods. However, antibodies were elicited against these injected materials, as precipitation reactions in double diffusion gels were seen, but these antibodies had no effect on photosynthetic electron transport rates as measured with $NADP^+$ and DCPIP as electron acceptors.

The remaining antigen, the supernatant from Tris-washed chloroplasts, was effective in inhibiting a system I reaction, DCPIP-ascorbate to $NADP^+$; it also inhibited the water to DCPIP and water to $NADP^+$ reactions, but this inhibition was not restorable with DPC (Table 1). A significantly lesser degree of inhibition was observed in isolated PS II particles. This antiserum caused a remarkable amount of direct agglutination of broken chloroplasts but not PS II particles to be discussed below. As there was no significant difference in

TABLE I

EFFECT OF ANTISERUM AGAINST TRIS-WASHED CHLOROPLAST SUPERNATANT ON CHLOROPLAST REACTIONS

Material	Assay	% Inhibition of Control Serum
Broken Chloroplasts*	$H_2O \rightarrow DCPIP$	28.5
Broken Chloroplasts	$H_2O \rightarrow NADP^+$	32
Broken Chloroplasts	$DCPIPH_2 \rightarrow NADP^+$	35
Tris-washed Chloroplasts	$DPC \rightarrow DCPIP$	31.1
Photsystem II Particles	$H_2O \rightarrow DCPIP$	6.6

Conditions as described in Tables 2, 5 and 7.

*Here and elsewhere, unless indicated otherwise, broken chloroplasts are used.

the rates of photosynthetic electron transport with water or diphenylcarbizide as electron donors, this antiserum, unlike the two we exploited, was not against the oxygen-evolving side, and, therefore, we did not pursue it further.

2. Effects of antisera against photosystem II particles and soluble extract

The antisera produced in response to immunization of rabbits with photosystem II particles or the extract from repeatedly frozen and thawed chloroplasts appear to inhibit photosynthesis in exactly the same manner on the oxidizing side of photosystem II. Tables 2(A) and 3(A) indicate that the addition of the antisera against system II particles and the "soluble extract" to broken chloroplasts results in approximately a 15 percent inhibition of DCPIP photoreduction. This

inhibition represents an average of 15 to 20 measurements of Hill reaction rates in the presence of the antiserum compared with rates in the presence of the control serum. The degree of inhibition varies somewhat with the activity of the chloroplasts before serum addition.

TABLE 2

INHIBITION OF HILL REACTION BY ANTIBODIES MADE AGAINST PHOTOSYSTEM II PARTICLES

Additions		μmoles $DCPIPH_2$ (A) or NADPH (B) $(mgChl \cdot hr)^{-1}$	% Inhibition of Control Serum
(A)	None	290±5	
	0.04 ml Control Serum	241±5	
	0.04 ml Antiserum	205±4	14.9±2
(B)	None	48.2±0.8	
	0.16 ml Control Serum	44.2±0.9	
	0.16 ml Antiserum	38.8±0.8	12.2±2
	DCMU, $DCPIPH_2$	15.7±0.1	
	DCMU, $DCPIPH_2$ plus 0.16 ml Control Serum	14.4±0.1	
	DCMU, $DCPIPH_2$ plus 0.16 ml Antiserum	14.4±0.0	0.0±0

(A) Three ml sample, containing 15 μg Chl in 50 mM Tris-HCl buffer, pH 6.8, 10 mM NaCl, and 45 μM DCPIP, was illuminated for 45 sec in saturating white light filtered with 4.5 inch water filter and 1 inch clear plastic. Change in absorbance of DCPIP was measured at 603 nm. These values represent an average of 20 measurements.

(B) Three ml sample, containing 60 μg Chl in 0.05 M phosphate buffer, pH 7.2, 70 μmoles NaCl, 10 μmoles NaCl, 10 μmoles $MgCl_2$, 1 μmole $NADP^+$ and excess amounts of PPNR; where indicated, 0.03 μmoles DCMU, 0.02 μmoles DCPIP, and 6.0 μmoles Na ascorbate were added. $NADP^+$ photoreduction was measured at 340 nm after 2 min illumination. These values represent an average of 5 measurements.

TABLE 3

INHIBITION OF HILL REACTIONS BY ANTISERA AGAINST A SOLUBLE CHLOROPLAST FACTOR

Additions	μmoles $DCPIPH_2$ (A) of NADPH (B) $(mg\ Chl \cdot hr)^{-1}$	% Inhibition of Control Serum
(A) None	270±5	
0.02 ml Control Serum	232±4	
0.02 ml Antiserum	195±5	16
(B) None	54.0±0.9	
0.06 ml Control Serum	50.2±0.8	
0.06 ml Antiserum	43.1±0.8	14
DCMU, $DCPIPH_2$	22.3±0.1	
DCMU, $DCPIPH_2$ plus 0.06 ml Control Serum	20.9±0.1	
DCMU, $DCPIPH_2$ plus 0.06 ml Antiserum	20.7±0.1	1

(A) One ml of sample, containing 8μg Chl in 50 mM phosphate buffer, pH 6.8, 10 mM NaCl, and 45μM DCPIP, was simultaneously illuminated and measured for change in DCPIP asorbance at 597 nm as discussed in Methods. These values represent an average of 15 measurements.

(B) One ml sample, containing 24μg Chl in 50 mM phosphate buffer, Ph 7.2, 25 μmoles NaCl, 3 μmoles $MgCl_2$, 0.5 μmoles $NADP^+$ and excess amounts of PPNR; where indicated 0.01 μmoles DCMU, 0.01 μmoles DCPIP, and 2.0 μmoles Na ascorbate were added. (Note that plastocyanin was not added, but may be present in PPNR.) $NADP^+$ reduction was measured at 340 nm simultaneously with illumination as described in Methods. These values represent an average of 5 measurements.

Comparison is made here and elsewhere with control serum data. The inhibition of electron transport in the chloroplasts by the control serum appears to be non-specific, e.g., affecting system I and II reactions approximately equally, in contrast to the specific antiserum reported in this thesis. Aging of sera seems to increase the non-specific inhibitory activity.

Table 4 shows that heating the control serum at 65°C for 30 minutes removes the inhibitory activity of the normal serum; heating to 56°C for 30 minutes does not change the inhibition, so it is not a question of complement action, i.e., a destructive effect often following an antibody-antigen interaction inactivated by heating to 56°C for 30 minutes (see reference 73). Also, the inhibition does not appear to be caused by the immunoglobulin fraction of the serum, as isolation of immunoglobulins and subsequent incubation with chloroplasts does not inhibit electron transport rates; similarly, calf fetal serum, which does not contain any immunoglobulins, still inhibits DCPIP reduction. Therefore, it is unlikely that the inhibition seen in chloroplast Hill reaction by control sera is due to a natural immunoglobulin (74), but it appears to be due to some heat labile serum component, perhaps a protein.

With all probability, this inhibition by the antisera is real in view of the small errors and of negative results under other conditions. This low level of inhibition may be due to: (1) the limited accessibility of the antigen to the antibodies, (2) the weak affinity of the antibody-antigen interactions (assuming that the antigen has been somewhat altered from its native state during extraction, and, therefore, the antibody elicited against it will not strongly bind the native chloroplast component) or (3) the lack of significant conformational changes in these chloroplasts (see Chapter III and reference 29).

TABLE 4

INHIBITION OF HILL REACTION BY NORMAL SERUM

Additions	μmoles $DCPIPH_2$ $(mgChl \cdot hr)^{-1}$	% Inhibition of Control
None	189	
Control Serum	120	36
Control Serum, Heated 30 min., 56°C	138	27
Control Serum, Heated 30 min., 65°C	178	6
Immunoglobulins of Control Serum	189	0
Immunoglobulins of Control Serum, Heated 30 min., 65°C	186	2
Calf Fetal Serum	133	30

Conditions for measurement of Hill reaction activity as specified in Table 3 (A). Twenty μl of control serum, calf fetal serum and immunoglobulins were added to each 8 μg chlorophyll, where indicated, and allowed to incubate for the usual 15 minutes. The protein concentration of the immunoglobulin fraction was one-fifth of that of the whole serum. In whole serum, immunoglobulins constitute 20 percent of the total serum protein, so approximately equal amounts of immunoglobulin protein were present in all cases.

Radunz and Schmid (29) have shown that the typical 15-20 percent inhibition of oxygen-evolving capacity as seen by an antibody against lutein can be increased to approximately 65 percent if the chloroplasts are extremely well coupled, suggestive of a conformational change that better exposes the antigenic site. For several experiments, we took painstaking care to see that the chloroplasts as they were incubated with the antisera were well coupled to phosphorylation; in addition, ferricyanide was used as electron acceptor to avoid complications of possible uncoupling with DCPIP (75, 76). There were no significant differences in the percentage inhibition by the two antisera under these conditions as compared to the usual less-coupled chloroplasts.

To eliminate the possibility that the inhibition seen is due to an antibody against a PS I component detectable because DCPIP might also accept electrons from photosystem I, as suggested by several workers (30, 77), we measured $NADP^+$ photoreduction in the presence of the antiserum both with water and $DCPIPH_2$ as electron donors (Tables 2, B and 3, B). No decrease in reaction rates was observed with the antiserum when the artificial electron donor $DCPIPH_2$ (with DCMU) was used in place of water, indicating that the antibody inhibition must be before the site of $DCPIPH_2$ donation, and thus not in photosystem I.

To determine whether the site of inhibition is on the oxidizing or reducing side of photosystem II, chloroplasts were washed with 0.8 M Tris, pH 8.0, to inactivate oxygen evolution. DCPIP reduction was measured in the presence of the antiserum and control serum using DPC as electron donor. Table 5 shows that with this system, there is no longer any inhibition by either of the two antisera. As DPC has been shown to donate electrons between the Tris-block and reaction center II (57), the PS II antibody and the antibody against the extract from repeatedly frozen and thawed chloroplasts must attack (directly or indirectly) either at the site (or close to the site) of Tris-inactivation or at some unknown component between the Tris-block and water.

We then measured DCPIP reduction of chloroplasts preincubated with serum in the presence and absence of manganese chloride to further localize the site of action of the antiserum. According to Ben-Hayyim and Avron (72), and confirmed as shown here in Figure 2 with identical chloroplasts as used in the inhibition experiments, Mn^{2+} competes with water as an electron donor to PS II in normal chloroplasts, donating electrons between water and the Tris-block. The degree of inhibition of Hill reaction by the antiserum against the "soluble extract" (as well as the photosystem II antiserum, data not presented) was shown to be approximately the same in the presence or absence of $MnCl_2$, as shown in Table 6.

TABLE 5

EFFECT OF ANTISERA ON DCPIP PHOTOREDUCTION BY TRIS-WASHED CHLOROPLASTS

Chloroplast Treatment	Additions	μmoles $DCPIPH_2$ $(mgChl \cdot hr)^{-1}$	% Inhibition of Control Serum
(A) Experiments with Antisera to Photosystem II Particles:			
Normal	None	198.0	
Tris-washed	None	8.7	
Tris-washed	DPC	87.5	
Tris-washed	DPC+0.04 ml Control Serum	60.2±1.9	
Tris-washed	DPC+0.04 ml Antiserum	59.3±1.8	1.5±3
(B) Experiments with Antisera to Extract from Frozen and Thawed Chloroplasts:			
Normal	None	225	
Tris-washed	None	7.5	
Tris-washed	DPC	78.5	
Tris-washed	DPC+0.02 ml Control Serum	65.7±2.0	
Tris-washed	DPC+0.02 ml Antiserum	64.2±1.5	2.8±2

(A) Three ml of sample, containing 18 μg Chl. Reaction mixture and measurement are as in Table 2 (A); where indicated, 1.5 μmoles of DPC were added. These values represent an average of 15 measurements.

(B) One ml of sample, contained 8 μg Chl. Reaction mixture and measurement are as indicated in Table 3 (A); where indicated, 0.5 μmoles of DPC were added. These values represent an average of 10 measurements.

Fig. 2.--Effect of Mn^{2+} on oxygen exchange in chloroplasts. Left trace, effect of Mn^{2+} on O_2 uptake in the presence of methylviologen. Reaction mixture contained: Tricine (pH 8.0), 45 µmoles; NaCl, 60 µmoles; methylviologen, 0.03 µmoles; NaN_3, 3 µmoles and chloroplasts containing 60 µg chlorophyll in a total volume of 3.0 ml. Numbers in parentheses represent rates of O_2 uptake expressed in µmoles per mg Chl per hour.

Right trace, effect of Mn^{2+} on O_2 evolution with electron transport to $NADP^+$. Reaction mixture contained: Tricine (pH 8.0), 45 µmoles; NaCl, 60 µmoles; $NADP^+$, 0.5 µmoles; saturating amounts of PPNR and chloroplasts containing 60 µg Chl in 3.0 ml volume. Numbers in parentheses are initial rates of O_2 evolution expressed in O_2 per mg Chl per hour.

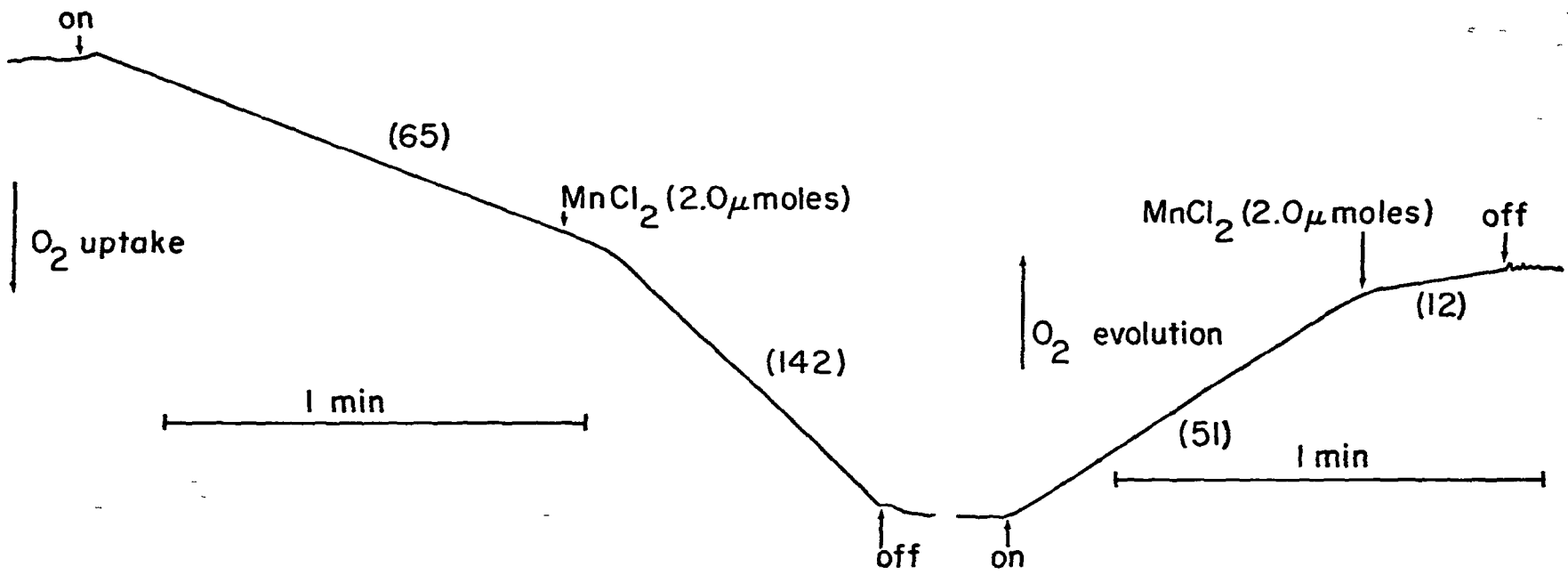

on
(65)
$MnCl_2$ (2.0μmoles)
O_2 uptake
1 min
(142)
off
on
$MnCl_2$ (2.0μmoles)
off
O_2 evolution
(12)
(51)
1 min

TABLE 6

INHIBITION OF DCPIP REDUCTION BY THE "SOLUBLE EXTRACT" ANTISERUM IN THE PRESENCE AND ABSENCE OF $MnCl_2$

Additions	μmoles $DCPIPH_2$ $(mg\ Chl \cdot hr)^{-1}$	% Inhibition of Control Serum
None	278±5	
0.02 ml Control Serum	238±5	
0.02 ml Antiserum	198±4	16.8±2
0.02 ml Control Serum plus $MnCl_2$	224±5	
0.02 ml Antiserum plus $MnCl_2$	185±4	17.4±2

One ml of sample contained 8 μg Chl in 50 mM Tris-Cl buffer, pH 7.2, 10 mM NaCl, and 45 μM DCPIP; where indicated, 0.3 μmoles of $MnCl_2$ are added. These results represent an average of 5 measurements.

We concluded (26, 27) from these experiments that the antisera must attack at a site between Mn^{2+} and DPC donation sites. As $MnCl_2$ was shown not to restore electron transport to $NADP^+$ in Tris-washed chloroplasts (72) and we found similarly no increase in DCPIP reduction with $MnCl_2$ addition to Tris-washed chloroplasts, it was concluded that it donates electrons before the Tris-block in the control chloroplasts. However, the conclusions presented in these original papers (26, 27), as well as the papers of Ben-Hayyim and Avron (72, 78,79), may be in error due to the ignorance of the superoxide dismutase reaction which was later recognized (80) with methylviologen mediated oxygen uptake. Epel and Neumann (80) showed that PS I

mediated production of radical superoxide, in the presence of manganese, becomes reduced to H_2O_2 and this increases apparent oxygen uptake. Similarly, the absence of oxygen evolution in chloroplasts effectively reducing $NADP^+$ in the presence of manganese is not to be understood as a competition between manganese and water (72,78,79), but rather the superoxide radicle reaction with Mn^{2+} (80).

That we could not see Mn^{2+} donation to Tris-washed chloroplasts may be also understood simply by a simple cyclic reaction, involving immediate reoxidation of reduced DCPIP by Mn^{4+}. [The ability of Mn^{2+} to restore electron flow to DCPIP in heat treated (81) and high salt treated (82) chloroplasts is not understood.] That the same cyclic reaction did not occur with control chloroplasts, where the water oxidation system is intact, simply confirms the fact that $MnCl_2$ is not competing with water in the normal chloroplast system. Therefore, the interpretations with the use of $MnCl_2$ no longer are valid, and it can only be said, on the basis of the lack of inhibition by antisera in the Tris-washed chloroplasts fed with DPC, that the site of inhibition is somewhere between water and the site of DPC donation.

Operating on the principle that the levels of inhibition (approximately 15 percent) seen were low because of the limited accessibility of the antigenic site(s) in the chloroplast membrane to the antibody(ies), we then looked at DCPIP reduction (water as donor) of PS II particles incubated in the

presence of the two types of antisera (as well as the control sera). As indicated in Table 7, the percent inhibition increased almost two-fold (approximately 30 percent inhibition), perhaps because the antigenic site(s) is more readily exposed in the particles than in the broken chloroplasts. On the same line of thinking, the lack of total inhibition may be explained by surmising that the site is yet only partially accessible in the particles and that the antibodies are against somewhat denatured components, and, thus, do not have a strong affinity for the native intermediate.

TABLE 7

EFFECT OF ANTISERA ON DCPIP REDUCTION BY PS II PARTICLES

Additions	μmoles $DCPIPH_2$ $(mgChl\ hr)^{-1}$	% Inhibition of Control Serum
None	92.0	
0.02 ml Control Serum	80.3±2.0	
0.02 ml PS II Antiserum	60.7±1.5	24
0.02 ml Extract Antiserum	58.5±1.5	27

One ml of sample contained 8 μg Chl. Reaction mixture and measurement as indicated in Table 3(A). These values represent an average of 10 measurements.

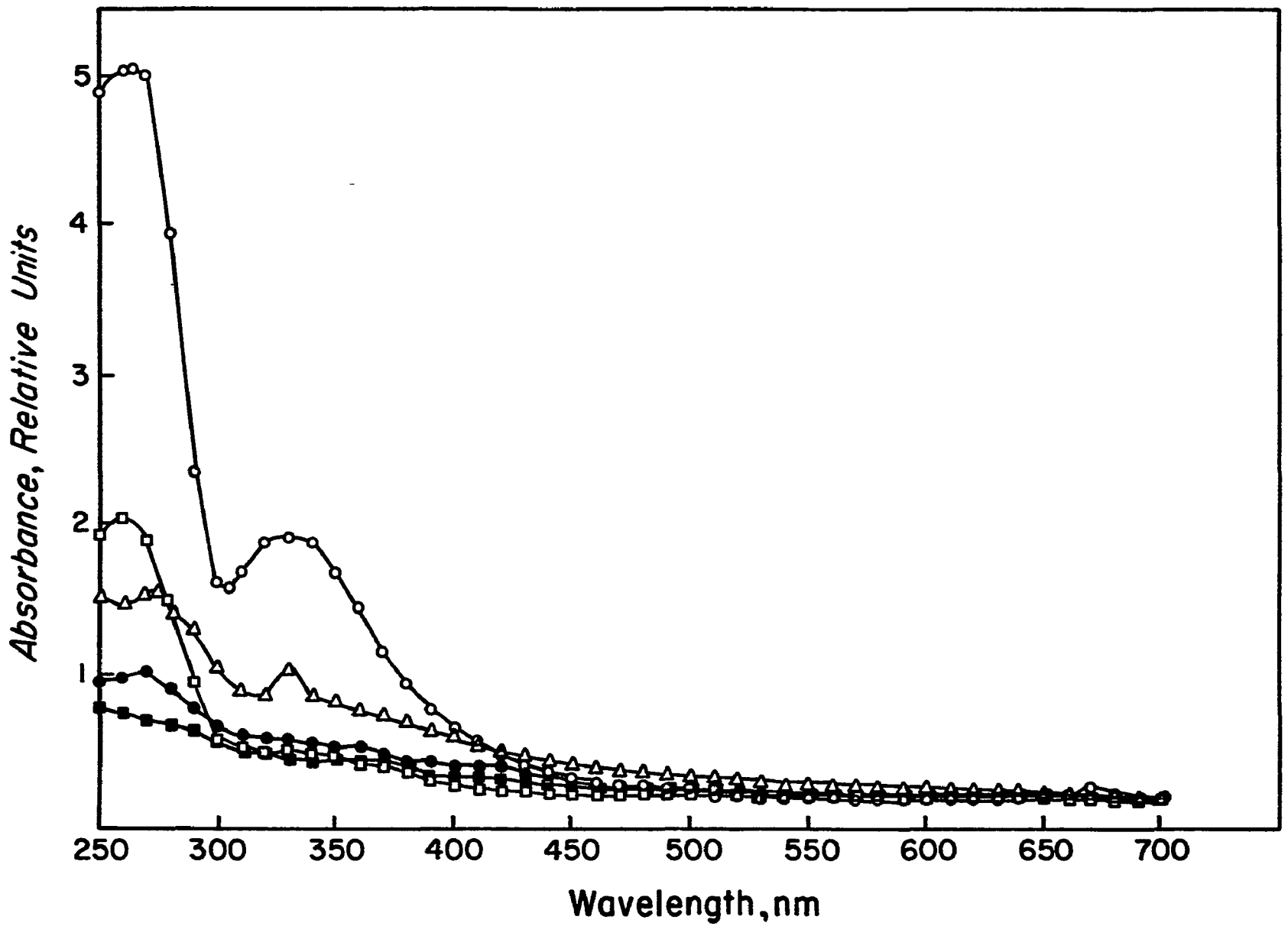
3. Characterization of soluble extract

As it would seem logical that the antigen prepared from frozen and thawed chloroplasts would be less complex

than the membrane bound PS II particles, we decided to do some simple characterization of this antigen. It can be stated with near certainty that the antibodies elicited against this extract were not made in response to some contaminating chlorophyll containing membrane fragment present in the extract. Careful absorption spectra measurements have shown a very slight absorbance detectable at 680 nm in most preparations. A typical extract preparation contained 0.5μg Chl/ml (based on an extinction coefficient of 100 $mM^{-1}cm^{-1}$ for chlorophyll a) for every mg of protein, i.e., 0.05 percent chlorophyll. Comparing this chlorophyll content with that of the chloroplast membrane itself (Chl/protein = 20 percent), one can see that the chlorophyll concentration of the extract is very small, i.e., the Chl/protein ratio of the extract being less than 0.5 percent of the Chl/protein ratio of the membrane. With some reservations, one can nearly eliminate the possibility of a Chl-protein membrane fragment contaminant as being the antigenic source. It is quite possible that if anything, the amount of total contaminant present in the immunization course might elicit a low dose tolerance in the rabbit rather than an immune response (74).

Figure 3 shows the absorption spectra of the crude extract as well as spectra of crude purifications of the

Fig. 3.--Absorption spectra of extract and $(NH_4)_2SO_4$ fractionations of the extract. All spectra normalized at 700nm. o——o, crude extract; ■——■ , 25 percent $(NH_4)_2SO_4$ fraction; ●——●, 50 percent $(NH_4)_2SO_4$ fraction; Δ——Δ, 75 percent $(NH_4)_2SO_4$ fraction; □——□ , 100 percent $(NH_4)_2SO_4$ fraction.


Absorbance, Relative Units
5
4
3
2
1
250
300
350
400
450
500
550
600
650
700
Wavelength, nm

extract with $(NH_4)_2SO_4$ fractionations at 25 percent, 50 percent, 75 percent and 100 percent $(NH_4)_2SO_4$. In the extract, the absorption maximum is at 265nm, and there is an additional absorption peak (although in some preparations, it is seen only as a shoulder) at 330nm. Absorption spectra of $(NH_4)_2SO_4$ fractions show a shift in the absorption maximum in all but the 100 percent $(NH_4)_2SO_4$ fraction to 275nm; the 330nm band is most obvious in the 75 percent fraction, and the chlorophyll has been concentrated in the 25 percent cut and is missing from all others. Rabbits were immunized with these fractions, but due to a large nonspecific inhibition seen in all the control sera, it was very difficult to see significant additional changes in the chlorplasts treated with antisera.

To see if the reason for absorbance at 260nm (rather than 280nm, which is more typical of protein absorption maxima) was due to a significant amount of nucleic acids (which absorb maximally at 260nm), DNA and RNA content were measured by the Dische (64) and orcinol (65) colorimetric assays. For the extract studied in this manner, the colorimetric assays showed DNA and RNA content to be 28 μg and 3 μg, respectively, per mg of protein, based on standards of calf thymus DNA and ribose, and the usual estimations for pyrimidine content in the orcinol test. The estimated total nucleic acid content, simply using absorbance of these compounds in the ultraviolet (62), is

slightly higher at 57 µg nucleic acid per mg protein in the extract. The difference may be accounted for by a greater accuracy of the colorimetric assays (or conversely, the assumptions that one makes in using arbitrary standards in the colorimetric tests). Only the 100 percent $(NH_4)_2SO_4$ fraction showed an absorbance maximum at 260 nm, and this might be explained by the fact that nucleic acids are not salted out with $(NH_4)_2SO_4$. (It is interesting to note that the new manganese-containing factor of Tel-Or and Avron (50) also has absorption characteristics not typical of a protein, with a maximum at 254 nm.)

The extract was analyzed for possible manganese content. First, a colorimetric test for manganese was done, measuring MnO_4^- after oxidation by periodate (66). This test is fairly sensitive, with the lower limit being 1 µg Mn^{2+}/ml, using 0-0.1 O.D. slidewire on a Cary-14 spectrophotometer. With a five-fold dilution of the extract, no manganese could be measured with this technique; it was not feasible to use the undiluted extract, because a large volume is needed.

We then measured manganese content by electron paramagnetic resonance techniques. Figure 4 shows the typical EPR signal from divalent, unbound Mn which results from the hyperfine interaction of the unpaired electrons of Mn with the 5/2 spin of the Mn nucleus (83). Figure 5 is an EPR

Fig. 4.--Room temperature EPR spectrum of a 3.6×10^{-5}M solution of $MnSO_4$. Instrumental conditions: microwave power, 20 mW; modulation amplitude, 16G; time constant, 1 second; scan range, 250 G/minute; receiver gain, 6.3×10^3.

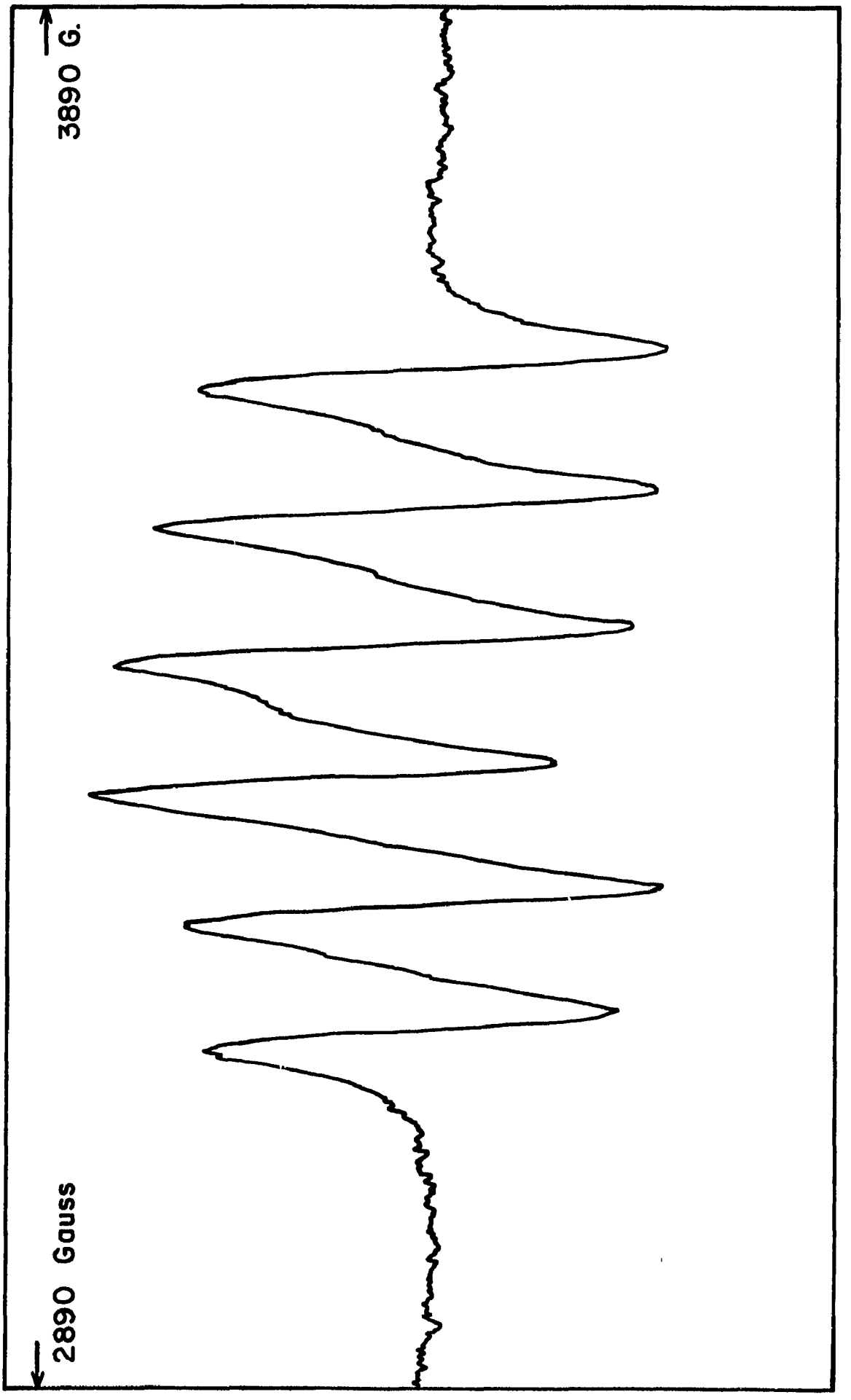
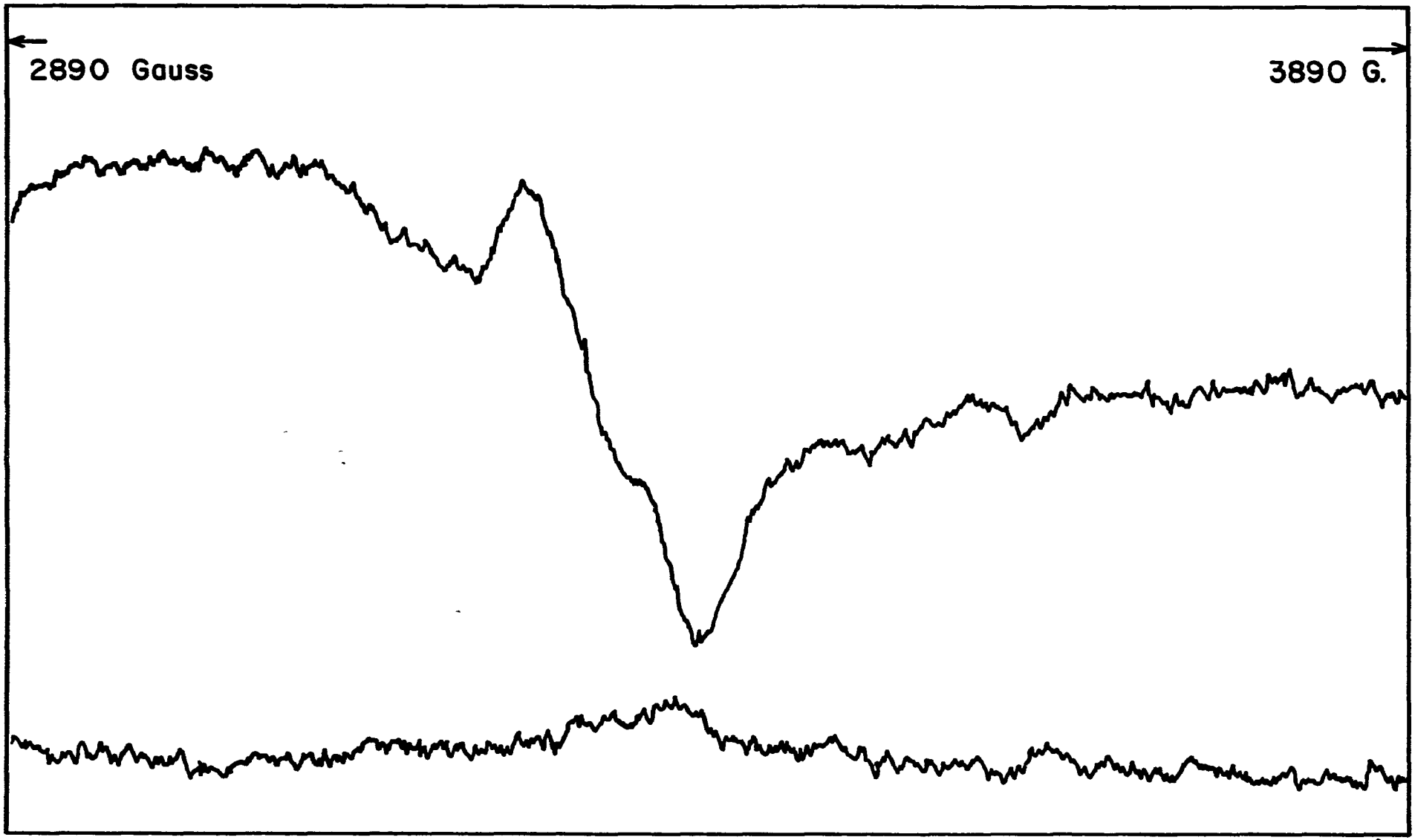

3890 G.
2890 Gauss

Fig. 5.--Room temperature EPR spectrum of undiluted extract from repeatedly frozen and thawed chloroplasts. Instrumental conditions: microwave power, 50 mW; modulation amplitude, 25G; time constant, 3 seconds; scan range, 125 G/minute; receiver gain, 8×10^3. Bottom trace is of buffer alone.


2890 Gauss
3890 G.

spectrum of the extract. The symmetric six-line EPR spectrum characteristic of unbound Mn^{2+}, is replaced by a very distorted spectrum that closely resembles reported spectra for manganese bound to some complex, e.g., the spectra of Reed et al. (84) for Mn EGTA and Mn EDTA. Whether this signal is from the long sought manganese containing oxygen-evolving component or from some insignificant material in the extract is not known; however, the concentration of the material in the extract must be quite low as the sensitivity of the spectrometer was increased about five-fold over that used for measuring the free Mn^{2+}, where the concentration was $3.6 \cdot 10^{-5}$M $MnSO_4$. Curiously enough, the amount of signal seen from the extract is fairly close to what might be expected if the loosely bound manganese were released with our repeated freeze-thawing. The chlorophyll concentration of the chloroplasts that were freeze-thawed was ∿1000 µg Chl/ml, i.e., 1 mM chlorophyll. Ignoring the distortion of the spectrum, one might assume manganese content to be ∿10 µM; this 100-fold difference closely approximates the reported figure for bound manganese in chloroplasts (see Cheniaé, reference 38).

Lastly, it should be mentioned only briefly that preliminary measurements of separation of polypeptides of the extract and the four fractions on polyacrylamide gel electrophoresis showed that the extract is composed of at least ten polypeptides. The 50 percent and 75 percent $(NH_4)_2SO_4$ fractions both showed fairly distinct separation into several

comparatively narrow bands (in each), seemingly with no overlaps. The 25 percent fraction showed one broad band and one narrow one; the 100 percent fraction had two narrow bands. As this work has not been carried out to the extent that the data can be neatly quantitated, no more can be said other than the suggestion that we are dealing with a fairly complex antigen that contains manganese and proteins.

4. Agglutination studies

Although the antigens used in this study contain a number of immunogenic components [as shown by multiple precipitin bands in Ouchterlony (85) double diffusion gels (data not presented)], we nevertheless looked at agglutination of chloroplasts and system II particles by the antisera to see if any suggestions could be made for the localization of these antigenic components in the thylakoid membrane (Tables 8 and 9). Not much weight can be placed on agglutination studies because of the complex nature of the materials used in immunization. However, some generalizations can be made in summary: (1) the antibodies causing direct agglutination of PS II particles (in the PS II antiserum) are not common to the extract antiserum, and are, therefore, probably not involved in the inhibitory action of oxygen evolution; also, these antibodies do not agglutinate broken chloroplasts, and, thus, this PS II site is not exposed in intact thylakoids, and (2) a common antigenic

TABLE 8

ANTIBODY-ANTIGEN INTERACTIONS

Serum	Antigen	Agglutination (without anti-rabbit IgG, IgM, IgA)	Agglutination (with anti-rabbit IgG, IgM, IgA)
Anti-PSII	Chloroplasts	++	++++
Anti-extract	Chloroplasts	+++	++++
Anti-Tris-washed supernatant	Chloroplasts	++++	++++
Control Serum	Chloroplasts	0	0
Anti-PS II	PS II Particles	+++	++++
Anti-extract	PS II Particles	0	++
Anti-Tris-washed supernatant	PS II Particles	0	0
Control Serum	PS II Particles	0	0

Chloroplasts, as prepared for photochemical studies, were incubated with antisera and control sera in the proportion of 2 µl serum/µg chlorophyll at 37°C for 30 minutes. The final chlorophyll concentration was approximately 200 µg Chl/ml. The same was done with PS II particle agglutination studies with the particles prepared by the method of Briantais (6) and finally resuspended in 10 mM phosphate buffer, pH 7.2, containing 10 mM NaCl to a final concentration of approximately 200 µg Chl/ml. Agglutination was scored qualitatively by size of clumps under a light microscope.

TABLE 9

ANTIBODY-ANTIGEN INTERACTIONS

Serum	Antigen Used in Preincubation	Antigen Used in Agglutination	Agglutination
Anti-PS II	Chloroplasts	Chloroplasts	0
Anti-PS II	PS II Particles	Chloroplasts	0
Anti-PS II	Chloroplasts	PS II Particles	+++
Anti-PS II	PS II Particles	PS II Particles	0
Anti-extract	Chloroplasts	Chloroplasts	0
Anti-extract	PS II Particles	Chloroplasts	0
Anti-extract	Chloroplasts	PS II Particles	0
Anti-extract	PS II Particles	PS II Particles	0
Anti-Tris-washed supernatant	Chloroplasts	Chloroplasts	0
Anti-Tris-washed supernatant	PS II Particles	Chloroplasts	++++
Anti-Tris-washed supernatant	Chloroplasts	PS II Particles	0
Anti-Tris-washed supernatant	PS II Particles	PS II Particles	0

Chloroplasts or photosystem II particles were preincubated with the three antisera as in Table 8 and were then centrifuged for one hour at 110,000 x g. The supernatants were incubated (same volume as in Table 8) with new samples of antigens, and, following incubation, were scored for agglutination.

site for both PS II particles and the extract antiserum, seen as a direct agglutination of chloroplasts by relatively high proportions of antiserum to chloroplasts and an indirect agglutination of photosystem II particles, may be that component whose alteration inhibits oxygen evolution.

It should be noted that addition of goat anti-rabbit immunoglobulins to chloroplasts increases the degree of agglutination with both PS II particle and soluble extract antisera (Table 9). It is feasible that this additional agglutination is the result of yet another common site on the thylakoid membrane that is in a crevice and only agglutinated by the help of a "go-between" anti-immunoglobulin which can link the two antibodies and effectively agglutinate the chloroplasts. This would parallel nicely the crevice seen in PS II particles, although in the case of particles, it is not really proper to assume, with fairly drastic fractionation procedures, that the structural organization of the isolated particle is necessarily like that in the intact chloroplast.

The fact that the percent inhibition of the water oxidation reactions by the antibodies is increased in isolated PS II particles suggests a greater accessibility of the antibodies to the site. This increased availability does not necessarily imply a greater surface orientation of the attached site, but may be equally well explained by being due in part to the

absence of a barrier to antibodies provided by grana stacking in intact chloroplasts. As the size of the immunoglobulin molecule is quite large, it is unlikely that antibodies can reach the partition regions where the thylakoids are closely appressed. This is in accord with the interpretations forwarded by Koenig et al. (28) for the low levels of inhibition seen with their antisera that inhibit water oxidation reactions. Similarly, Arntzen (personal communication) has shown that unstacking of chloroplasts allows a much larger inhibition by lactoperoxidase catalyzed iodination of the reaction center of PS II.

The antiserum against the Tris-washed chloroplast supernatant is unlike either of the other two sera. There is only a direct agglutination of chloroplasts, and it is a remarkably strong agglutination. Preincubation of this serum with system II particles does not change the serum's ability to agglutinate chloroplasts.

D. Discussion

Of several chloroplast treatments or extractions, only two, isolated photosystem II particles and an extract from repeatedly frozen and thawed chloroplasts, were effective immunogens in eliciting antibodies that specifically inhibit oxygen evolution. Through a series of experiments employing different donor and acceptor systems (see Tables 2, 3 and 5), we have shown that the inhibitory site is probably the same as inactivated by

0.8 M Tris, pH 8.0. It cannot be determined whether the effective antigen is the oxygen-evolving site itself or some component perhaps complexed with it (whose conformational alteration by the antiserum, in turn, effects inhibition of oxygen evolution), as it has been suggested that the oxygen-evolving intermediate might be a part of a transmembrane complex.

Analysis of the extract has shown that it is far from being a simple, purified antigen, but it is yet much less complex than the membranous PS II antigen. Obviously, the immunochemical technique used here is only an indirect means of looking at the unknown intermediate (or components complexed to it) involved in oxygen evolution. However, the commonly employed, more direct biochemical handles used in isolation and characterization of intermediates have not been successful in this case. We first started with the relatively crude PS II antigen, and then found it was feasible to obtain similar antisera (i.e., inhibiting oxygen evolution) by the use of simpler antigens, such as extracts from frozen and thawed chloroplasts. Crude antigens, including those as complex as whole chloroplasts (55), have been very successfully used in past studies in preparation of antibodies against certain photosynthetic components; later work with purified components showed that the same antibodies produced with immunization with chloroplasts were also made against these single, pure antigen systems. However, it appears that the oxygen-evolving component unlike

all other photosynthetic intermediates, is most susceptible to damage by alteration of the membrane or (in this case) of components complexed to it (Chapter III), and, therefore, we must not offer hasty conclusions.

Analysis of the extract does not negate the possibility of the oxygen-evolving component being included in the mixture of proteins (and small amounts of nucleic acids). The distorted EPR signal of the extract may be indicative of some component with bound manganese, present in concentrations roughly approximating what might be expected to be released from the chloroplasts. However, due to the relative ease of manganese dissociation from the oxygen-evolving intermediate by a number of fairly mild treatments (38), it is not too likely that the EPR signal seen is attributable to the long sought after intermediate. It would be interesting to look for an EPR signal from unbound manganese in the chloroplast pellet after the repeated freezing and thawing; the possibility exists that this treatment is similar to Tris-washing (67), and manganese is released to the interior space of the thylakoids.

CHAPTER III

STABILIZATION BY GLUTARALDEHYDE FIXATION OF CHLOROPLAST MEMBRANES AGAINST INHIBITORS OF OXYGEN EVOLUTION

A. Introduction

The several existing models for chloroplast membrane structure are themselves an issue of controversy. One of the models for chloroplast membrane organization is that of an asymmetric distribution of subunits and components on a binary membrane; the picture is an external localization of photosystem I and an internal positioning of photosystem II. Initially, this model was proposed from data derived from electron micrographs of freeze etched chloroplasts and photochemical partial reactions of thylakoid membranes fractionated with detergents (5, 6).

The recent findings on the localization of PS II intermediates do not concur with the above model, however. The reaction center chlorophyll of photosystem II has been suggested from immunological data (20) to be localized close to the exterior of the thylakoid. Similar conclusions have been recently arrived at by Arntzen et al. (12, 21) from data on enzymatic iodination of chloroplast membranes.

Experiments with large and/or non-permeant molecules [e.g., trypsin (30, 31), diazonium benzene sulfonic acid (DABS) (24, 25) and antisera prepared against several chloroplast preparations or extracts (26 - 29)], that partially inhibit oxygen evolution, were originally interpreted to suggest that the oxygen-evolving intermediate is at least partially accessible to the outside of the membrane. It was found that inhibition of oxygen evolution by DABS is dependent upon some sort of conformational change resultant from uncoupler-insensitive electron flow through photosystem II or supported by acid-base transition [but not H^+ ion accumulation or phosphorylation mediated by photosystem I (25)]. Antibodies to lutein exert a four-fold greater inhibition of oxygen evolution in tightly coupled as opposed to less coupled chloroplasts, a situation favorable to light-induced conformational changes (29). These data suggest that the site attacked by these impermeable inhibitors is exposed after some conformational change in the membrane, and is, therefore, not surface localized, but more likely closer to the hydrophobic interior of the membrane.

Mitchell (1), Witt (see reference 2) and Crofts et al. (23) have presented both theoretical predictions and experimental data which suggest a separation of charges across the chloroplast membrane which can account for the creation of membrane potential and provide a driving force for phosphorylation as well as delayed light emission. This separation of

charges would require a positive charge accumulating species (i.e., the oxygen-evolving intermediate) to be situated on one side of the membrane and the primary electron acceptor, Q, on the other side. The work of Fowler and Kok (22) is especially convincing with regard to a placement of the water oxidation component close to the interior of the membrane, as they have shown that proton deposition, following almost the same kinetics of oxygen evolution, occurs inside the thylakoid.

If one is to accommodate these findings as well as the suggestion that P_{680} is surface localized, then a transmembrane complex is easily visualized, perhaps connecting these photosynthetic components (as well as other yet unknown intermediates existing between water and reaction center II) in the membrane to facilitate transfer of electrons. It has been suggested that DABS (25) and antisera inhibiting oxygen evolution (27) may not, in fact, alter the active, oxygen-evolving site but rather some protein (perhaps existing in complex with the oxygen-evolving component) which is able to transmit its alteration to the active site and thereby inhibit water oxidation. Oxygen evolution is very sensitive to membrane disturbance as witnessed by the fact that various treatments of the chloroplasts including mild heating (48), ultraviolet light (46), aging (47), chaotropic agents (41, 43), and proteases (44, 45) effectively inhibit oxygen evolution before affecting activity of any other photosynthetic components. Considering the nature of all these destructive

treatments, we decided to see if the inhibition of oxygen evolution might be secondary to membrane disturbance, which may be especially likely if we must invoke a high degree of structural integrity to support a transmembrane electron flow. For this study, we used two general classes of oxygen evolution inhibitors, those that are impermeable and those that are either freely permeable or are merely physical treatments. We measured the oxygen-evolving capacity via dichlorophenol indophenol (DCPIP) reduction of control chloroplasts and chloroplasts fixed with glutaraldehyde, both treated in various ways to specifically impair oxygen evolution. Murakami and Packer (86) had already shown that glutaraldehyde fixed chloroplasts cannot undergo any configurational or osmotic, structural changes; however, small scale, microconformational changes can be induced by cations but not by strong actinic light in these fixed membranes, as measured by 90° light scattering changes. In this thesis we present a comparison of effects of oxygen evolution inhibitors on fixed and unfixed chloroplasts which has provided information regarding the involvement of a rigid structural requirement to maintain oxygen evolution as well as the localization of the oxygen-evolving intermediate in the membrane, as shall be discussed.

B. Materials and Methods

Broken, Class II, chloroplasts were isolated from

market spinach as described earlier (26) with modifications to better approximate the conditions used in the oxygen-evolution inhibition systems described below. In many cases, phosphate and Tris-Cl buffers were used interchangeably in the isolation procedures, and $MgCl_2$ was omitted from the homogenizing medium with no difference in the effects monitored.

To fix the chloroplasts, the samples, prepared as above, were washed twice in the same homogenization buffer without sucrose and then suspended to 150 μg chlorophyll per ml of the same washing buffer. Purified glutaraldehyde (Ladd Research Industries, Inc.) was diluted to a concentration of 10 percent and added to these chloroplasts to a final concentration of one percent. The suspension was constantly stirred at 0°C in the dark for five minutes and was subsequently centrifuged at 1500 X g for 10 minutes. The pellet was washed twice with the same volume of washing buffer and finally resuspended in a small volume of homogenizing medium. Formaldehyde was prepared from paraformaldehyde according to Park (87), although higher percent solutions were made and 10 mM instead of 30 mM phosphate buffer was used to dissolve the formaldehyde. Incubation of the chloroplasts with the formaldehyde and subsequent washings were done as with glutaraldehyde, although the time of incubation of the chloroplasts with the glutaraldehyde and formaldehyde in these comparative experiments was extended to 10 minutes.

Acetylation of the chloroplasts was done by incubating the chloroplasts (as prepared for glutaraldehyde fixation), suspended to 100 μg chlorophyll per ml of 10 mM Tris (or Tricine) buffer, pH 8.0, containing 10 mM NaCl, with 40 mM acetic anhydride in the dark at 0°C for 15 minutes (Dr. Elizabeth Gross, personal communication). This treatment was followed with washings as done with glutaraldehyde fixation.

Tris-washing of control and glutaraldehyde fixed chloroplasts was done according to Yamashita and Butler (42) with the time of incubation in Tris extended to 30 minutes. Fixed and unfixed chloroplasts were heat treated at 50°C for five minutes, as described by Yamashita and Butler (48). Inhibition of oxygen evolution by cadmium nitrate was accomplished by following the method of Bazzaz and Govindjee (88); in this case, it was essential that chloroplasts are isolated and fixed in HEPES buffer, as specified in their paper.

The method of Lozier et al. (28) was followed for treatment of chloroplasts with chaotropic agents; fixed and unfixed chloroplasts were incubated for 20 minutes at 0°C in 50 mM tricine buffer, pH 8.3, containing 0.8 M $NaClO_4$, 0.8 M guanidine or 1.0 M urea, followed by washing and resuspension in the chloroplast homogenzation medium.

Trypsin treatment was done according to the method of Selman and Bannister (30); fixation of the chloroplast membranes for trypsin treatment was done after the chloroplasts

had equilibrated in the 10 mM Tris-Cl buffer containing 10 mM NaCl, the same condition used when normal chloroplasts are trypsin treated. DABS treatment was essentially as that of Giaquinta et al. (25) although the chloroplasts were suspended in 50 mM phosphate buffer, pH 7.2, containing 10 mM NaCl, during incubation (and were washed and resuspended in the same plus 0.4M sucrose). The incubation time was shortened to 30 seconds, the actinic light was passed through a Corning filter, C.S. 2-59, and the reaction was stopped by a 40-fold dilution with buffer.

Treatment of chloroplasts with antisera to PS II particles and an extract from frozen and thawed chloroplasts was done as described earlier (26, 27). Chloroplasts were fixed with glutaraldehyde in the same buffering conditions in which unfixed chloroplasts are suspended during the incubation with antisera. Experiments reported in Tables 2 A, 3 A and 5 of Chapter II were repeated here to ascertain that we were dealing with the same antisera specifically inhibiting the oxygen evolution site.

DCPIP reduction was measured as described earlier (27) with a Cary-14 spectrophotometer. Chlorophyll a fluorescence was measured with a spectrofluorometer described elsewhere (61). Slow changes in chlorophyll a fluorescence were measured according to Papageorgiou and Govindjee (89). For measurements of emission spectra at liquid nitrogen temperature, the method of Cho et al. (60) was followed; the

spectra were corrected for the spectral variation of the monochromator and the photomultiplier (EMI 9558B): 12-(9-Anthroyl)- stearic acid (AS) fluorescence changes were measured with a spectrofluorometer described elsewhere (90), according to VanderMeulen and Govindjee (91).

Cation- and light-induced 90° scattering changes in fixed and unfixed chloroplasts were measured with the same instrument, according to VanderMeulen and Govindjee (90) and Murakami and Packer (86). The method of Packer et al. (92) was modified and then used as a measure of fixation of membranes, using a Cary-14 spectrophotometer. The method of Dilley (93) was used to measure proton pumping of control and fixed chloroplasts. Chlorophyll estimation was made according to MacKinney (59).

C. Results

1. Tris-washing, heating and cadmium nitrate treatments

Table 10 shows rates of DCPIP photoreduction (primarily a photosystem II reaction under the conditions used) of fixed and unfixed chloroplasts that had been treated with 0.8 M Tris-Cl, pH 8.0, and 0.5 mM cadmium nitrate or heated to 50°C for five minutes. In the case of control chloroplasts, as has been originally shown (42, 48, 88), each of the treatments severely depresses oxygen evolution capacity; diphenylcarbazide (DPC), an artificial electron donor, which promotes a 3-(3', 4'-dichlorophenyl-1, 1-dimethylurea (DCMU) sensitive

TABLE 10

EFFECT OF TRIS-WASHING, HEATING AND CADMIUM NITRATE ON ELECTRON FLOW IN FIXED AND UNFIXED CHLOROPLASTS

Chloroplast Treatment	Addition	μmoles $DCPIPH_2$ $(mg\ Chl \cdot hr)^{-1}$	% Inhibition of Control
Unfixed	None	86.3	--
Unfixed	DPC	131.2	--
Unfixed, Tris-washed	None	0	100
Unfixed, Tris-washed	DPC	126.0	4
Unfixed, 50°C, 5 min.	None	6.1	93
Unfixed, 50°C, 5 min.	DPC	102.5	14
Unfixed, 0.5 mM $Cd(NO_3)_2$	None	0	100
Fixed	None	19.6	--
Fixed	DPC	33.0	--
Fixed, Tris-washed	None	10.4	47
Fixed, Tris-washed	DPC	34.7	0
Fixed, 50°C, 5 min.	None	12.8	34
Fixed, 50°C, 5 min.	DPC	33.4	0
Fixed, 0.5 mM $Cd(NO_3)_2$	None	8.8	55

Two ml of sample, containing ∿15 μg Chl in 50 mM phosphate buffer, pH 6.8, 10 mM NaCl and 45 μM DCPIP, was simultaneously illuminated and measured for change in DCPIP absorbance at 597 nm. In the $Cd(NO_3)_2$ treatments, 50 mM HEPES buffer replaced the phosphate buffer, and as the rate of DCPIP photoreduction was slightly less in this buffer, the above rates for $Cd(NO_3)_2$ have been normalized to the control rates with phosphate buffer for simplicity. Details of treatment and measurements are given in Methods.

electron flow through photosystem II and bypasses the site of inhibition imposed by Tris, heating or $Cd(NO_3)_2$ (57), almost completely restores electron flow in these inhibited chloroplasts. Glutaraldehyde fixation permitted only a maximum of about 50 percent of the inhibition seen in the control chloroplasts treated with these agents; this inhibition in the fixed chloroplasts was likewise restorable with DPC. (Glutaraldehyde provides protection of about 50 percent against treatments with Tris, heat and $Cd(NO_3)_2$.)

2. Characterization of fixed membranes

The first question raised by the data presented in Table 10 was the possibility that some of the chloroplast membranes remained unfixed after the glutaraldehyde treatment. Therefore, it was necessary to verify by several independent means that the chloroplasts were completely fixed. To test whether the glutaraldehyde treatment was totally effective, we first observed the chloroplasts under the light microscope. Suspension of the unfixed chloroplasts in distilled water results in an osmotic shock and breakage of the chloroplast vesicles, while the fixed chloroplasts remain unchanged, as observed. A more quantitative method of looking at this lack of osmotic and structural changes in fixed chloroplasts involves measuring the optical density of chloroplasts at 680 nm and 546 nm (under conditions when scattered light is included in optical density measurements).

The ratio of optical density at 680 nm to the optical density at 546 nm will change if unfixed chloroplasts are suspended in distilled water or in a salt-containing medium, i.e., 50 mM phosphate buffer. The absorbance at 680 nm remains fixed, while the absorbance at 546 nm will change, reflecting the scattering difference as chloroplasts are suspended in salt or salt-free solutions. As fixed chloroplasts cannot undergo any osmotic changes, there will be no difference in the 680 nm/546 nm ratio. As shown in Figure 6, 0.5 percent glutaraldehyde treatment of chloroplasts (suspended to ∿150 μg Chl/ml) is sufficient to stop these osmotic, configurational changes.

Another convenient method to monitor fixation of membranes is the light-induced quenching of chlorophyll *a* fluorescence in the presence of phenazine methosulfate (PMS) which reflects structural modification of the thylakoid membranes (other than macroscopic volume changes) and has been shown to be absent in glutaraldehyde fixed chloroplasts (94). Figure 7 shows a concentration curve for the PMS quenching of Chl *a* fluorescence. As observed previously by Mohanty *et al*. (94), the light-induced PMS quenching is eliminated with fixation of the membranes; again 0.5 percent glutaraldehyde is sufficient to fix chloroplasts. However, as was not shown previously, there is an increase

Fig. 6.--Percentage change in the ratio of optical density at 680 nm and 546 nm with chloroplasts suspended in distilled water or in 50 mM phosphate buffer, pH 7.0 (designated as R) of chloroplasts incubated in various concentrations of glutaraldehyde. Chloroplasts containing ∿30 µg chlorophyll were placed in a 3 ml volume of the buffer or distilled water, and optical density was read in a Cary-14 spectrophotometer without transmission scattering attachment or an integrating sphere.

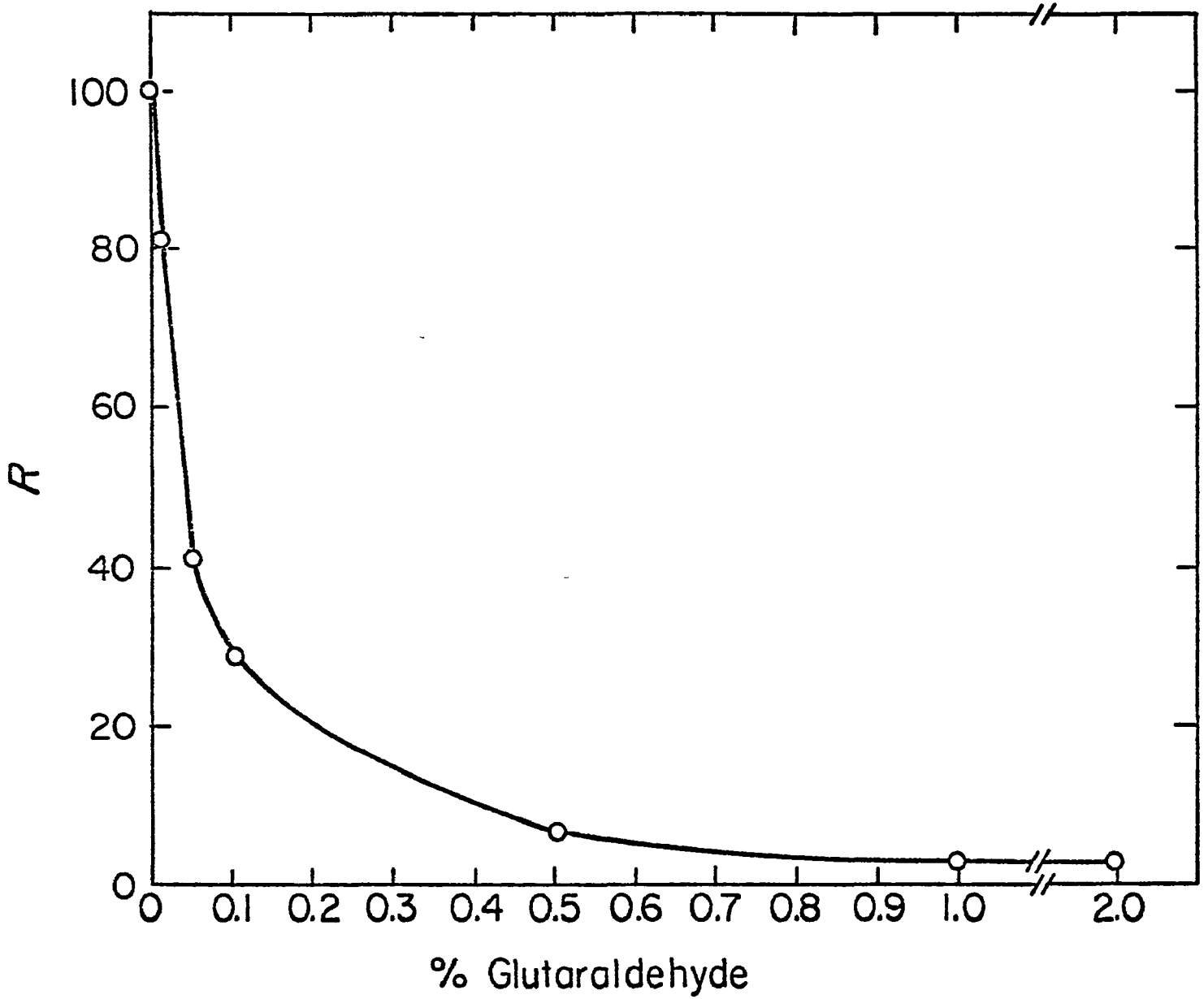
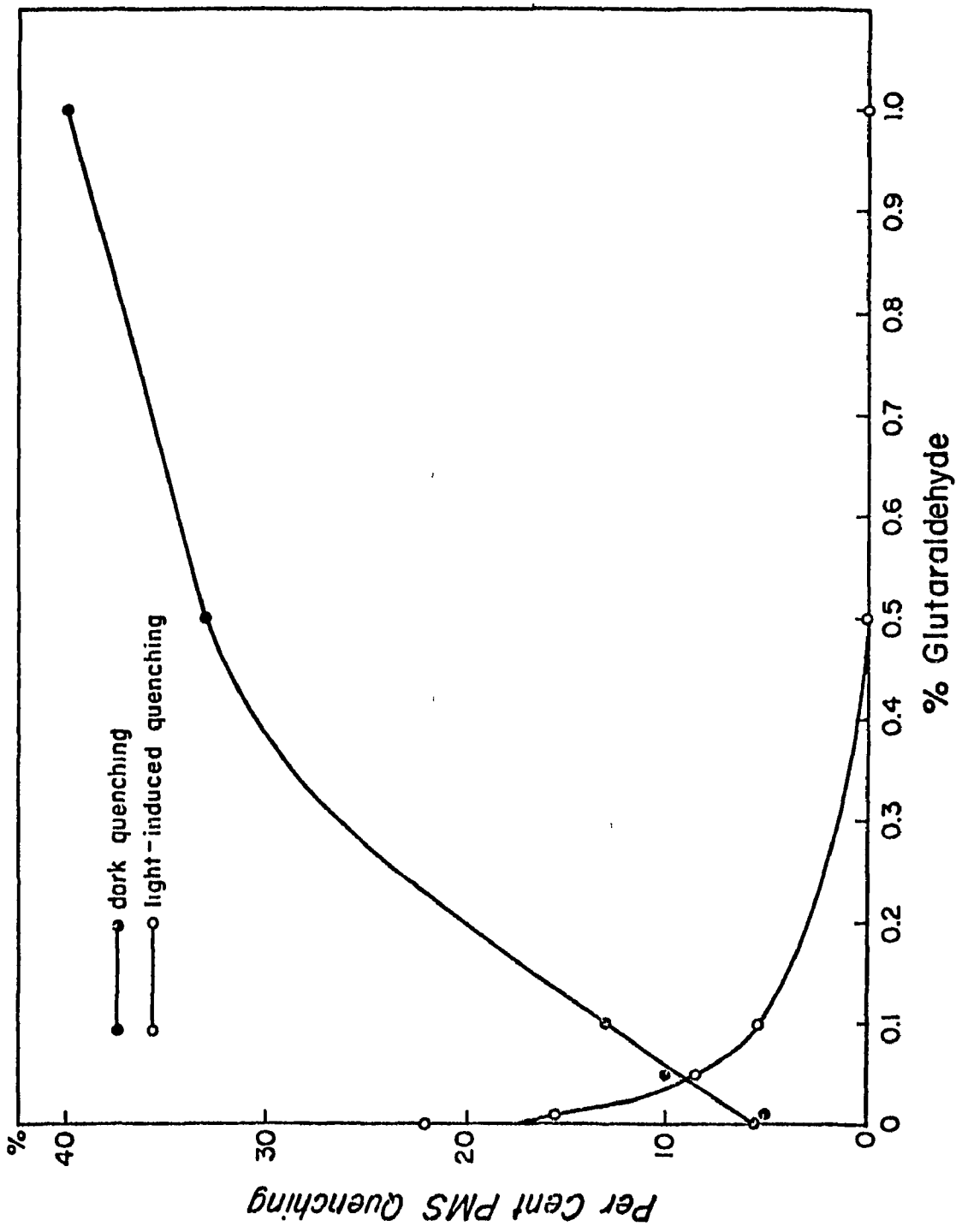

100
80
60
40
20
0
R
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
2.0
% Glutaraldehyde

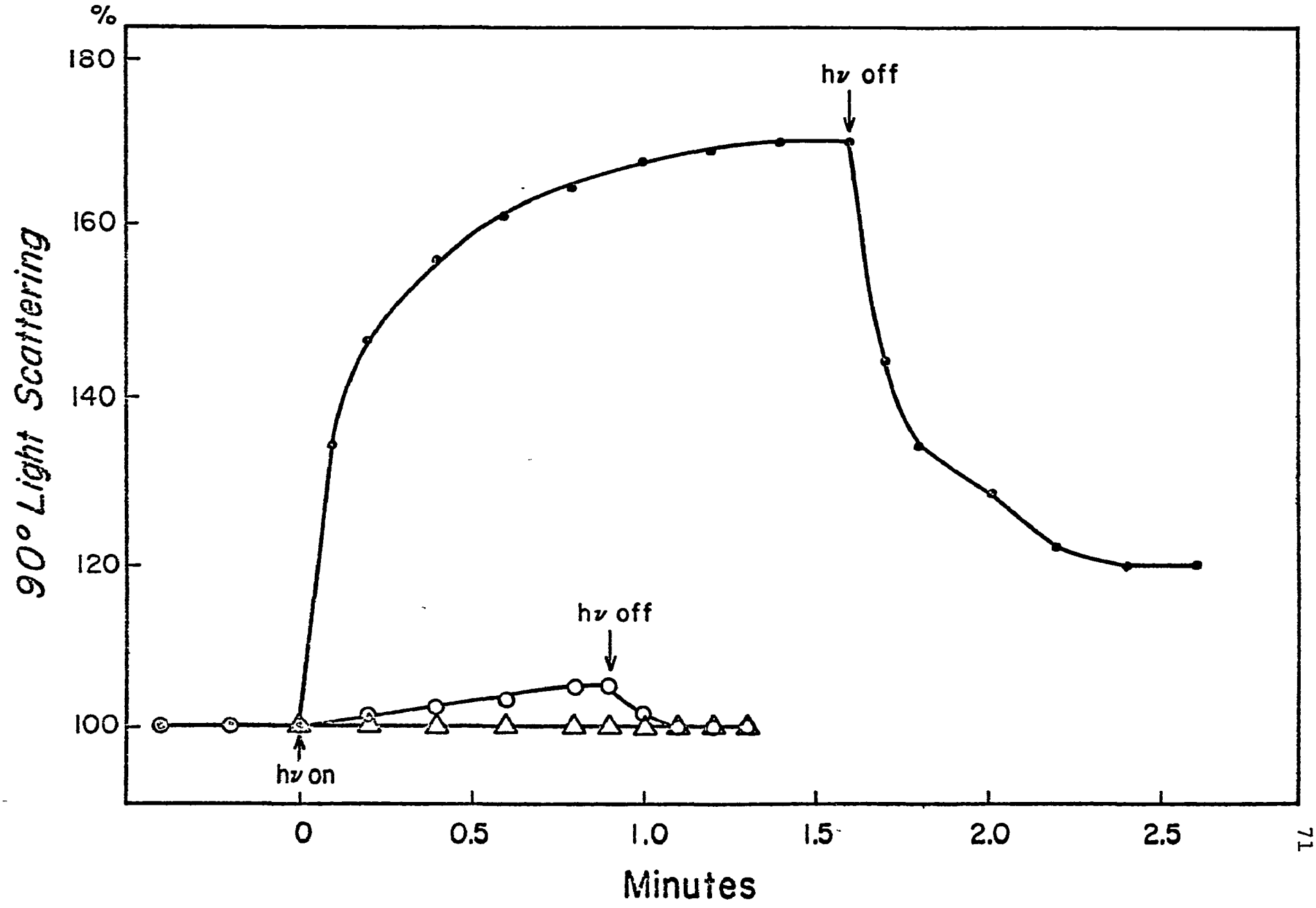
Fig. 7.--Percentage of PMS-induced quenching in the dark and light of chlorophyll a fluorescence yield in spinach chloroplasts in the presence of DCMU as a function of glutaraldehyde concentration during chloroplast fixation procedure. First, 5 μM DCMU was added to chloroplast suspension and relative fluorescence yield was measured. Then 30 μM PMS was added and after a 3 minute dark time to allow equilibration with the PMS, light-induced and dark PMS quenching was measured. Three ml samples contained approximately 15 μg chlorophyll/ml in 50 mM phosphate buffer, pH 7.8. Fluorescence was excited by broad-band blue light (C.S. 4-96 and C.S. 3-73) and was measured at 685 nm (half-band width, 6.6 nm). Corning C.S. 2-61 filter was placed before the observation monochromator. Glutaraldehyde fixation was done as described in Methods.


Per Cent PMS Quenching
%
40
30
20
10
0
dark quenching
light-induced quenching
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
% Glutaraldehyde

in PMS-induced quenching of Chl a fluorescence in the dark with glutaraldehyde fixation. Papageorgiou (95) has shown that the reduced form of PMS is a better direct quencher of chlorophyll a fluorescence than is oxidized PMS and has proposed that this is due to higher permeability and therefore greater accessibility of the reduced PMS to the chlorophyll bed. It is known that glutaraldehyde fixation increases permeability of the membrane (96), and, thus, the increased dark quenching in glutaraldehyde fixed chloroplasts may be attributed to greater accessibility of the (oxidized) PMS to the chlorophyll in the membrane.

A final check on the fixation of membranes was done by monitoring light-induced 90° light scattering. Murakami and Packer (86) have shown that glutaraldehyde fixed chloroplasts do not undergo light-induced 90° light scattering, although cation-induced 90° light scattering changes persist in the fixed chloroplasts. They have interpreted this data by suggesting that the light-induced scattering change is the result of osmotically controlled, macroconformational or configurational changes in membrane structure. As shown in Figure 8, 0.5 percent and one percent glutaraldehyde fixation stop the light-induced change, while some light-induced scattering difference is apparent at 0.25 percent glutaraldehyde.

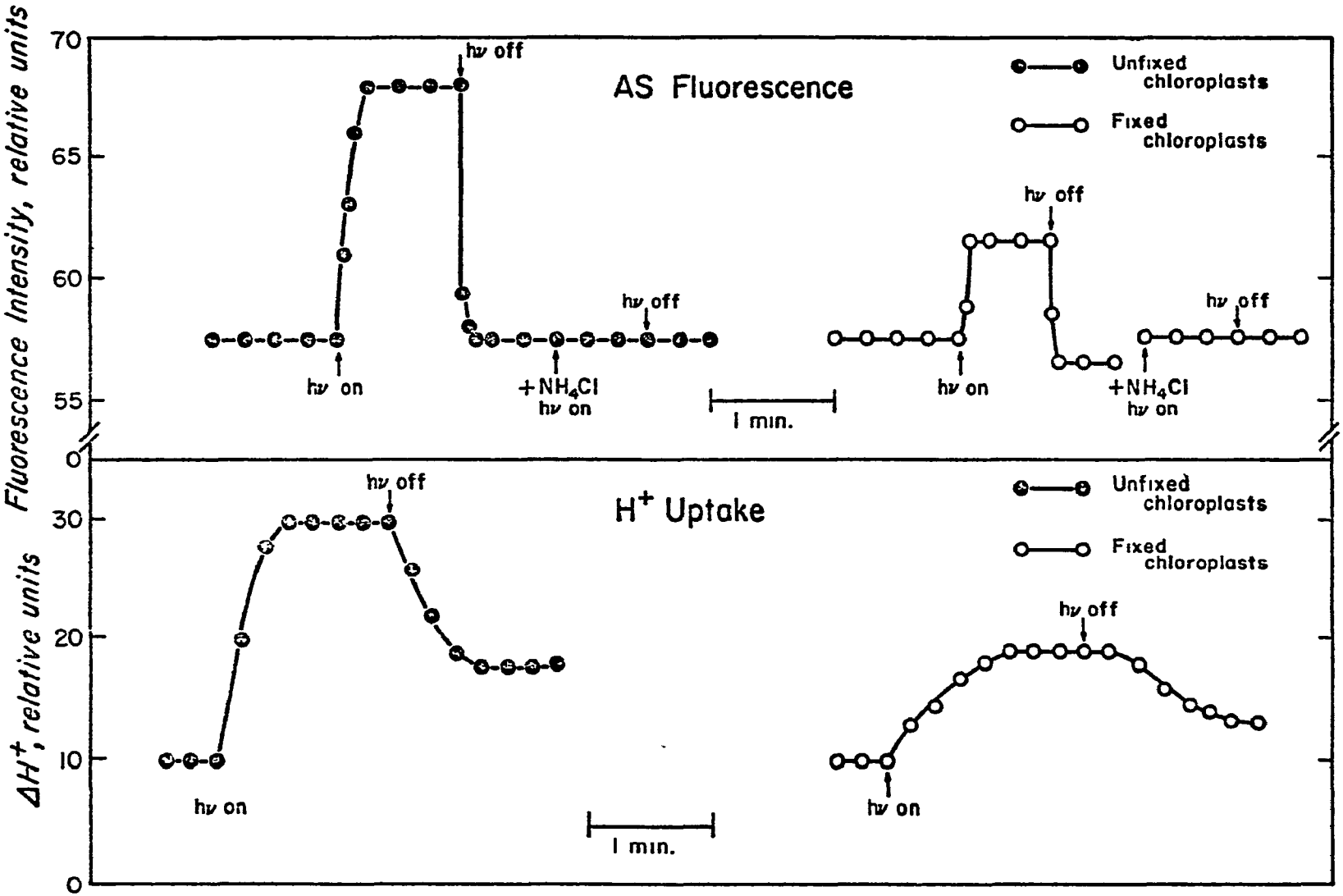
Fig. 8.--Kinetics of light-induced 90° light scattering of isolated spinach chloroplasts incubated in various concentrations of glutaraldehyde, accompanying photo-shrinkage in sodium acetate medium. Three ml of sample, containing 10 µg chlorophyll per ml of medium (200 mM Na acetate, pH 6.7, 20 µM PMS), was illuminated with saturating red light (C.S. 2-59 and C.S. 3-73) to see light-induced changes in scattering. Ninety degree light scattering was measured at 546 nm (half-band width of measuring light, 3.2 nm). A C.S. 4-96 filter was placed before the photomultiplier. Glutaraldehyde concentrations: 0% (●——●), 0.25% (o——o), and 0.5% or 1% (Δ——Δ).


%
180
160
140
120
100
90° Light Scattering
hν off
hν off
hν on
0
0.5
1.0
1.5
2.0
2.5
Minutes

Although incubation of chloroplasts with 0.5 percent glutaraldehyde for five minutes (150 μg Chl/ml) appears sufficient to completely fix the thylakoid membranes, we used one percent glutaraldehyde to be certain, and in earlier measurements (which have been averaged in with later ones), glutaraldehyde concentration was two percent [as used by Hallier and Park (97)]; the higher the concentration of glutaraldehyde, the lower the electron transport rates. It is disconcerting to note that Hill reaction rates are significantly decreased with fixation procedures, and it tends to lessen the dramatic protective effects offered by glutaraldehyde fixation against the oxygen evolution inhibitors. However, in individual experiments in which 0.5 percent glutaraldehyde was used as the fixative, electron transport rates were reduced only by approximately one-half, and the effectiveness of the oxygen evolution inhibitors was the same as reported here with the chloroplasts fixed with higher concentrations of glutaraldehyde. It is certain that under all the fixation conditions used that the chloroplasts are completely fixed, as in all of the preparations, each of the methods described above was used to ascertain complete fixation.

Although these chloroplasts are unable to undergo any large conformational or configurational changes as witnessed by the above tests, microconformational changes can occur. Figure 9 shows light-induced changes in fluorescence yield of the probe 12- (9-Anthroyl stearic acid), AS,

Fig. 9.--Light-induced change in anthroyl stearate (AS) fluorescence intensity and H^+ uptake in fixed and unfixed chloroplasts. Fluorescence was measured using a reaction mixture containing: 3.3 mM NaPi, 3.3 mM $MgCl_2$, 16.7 mM KCl, 10 μM PMS, 15 μg chlorophyll/ml, pH 7.8, in a 3 ml volume; AS concentration was 15 μM. Fluorescence was excited at 366 nm (3.2 nm half-band width, C.S. 7-60 filter) and monitored at 460 nm (6.4 nm half-band width, C.S. 7-60 filter) at 90° to the measuring beam. To measure light-induced changes in AS fluorescence, saturating red light (C.S. 2-59 and C.S. 3-73) was used. A C.S. 4-96 filter was placed before the photomultiplier. H^+ uptake was measured in the same reaction mixture and chlorophyll concentration as used for monitoring AS fluorescence changes. The chloroplasts were illuminated with saturating light passed through a Corning C.S. 3-69 filter plus a heat filter.


Fluorescence Intensity, relative units
70
65
60
55
0
hν off
AS Fluorescence
Unfixed chloroplasts
Fixed chloroplasts
hν off
hν off
hν on
+NH4Cl hν on
1 min.
hν on
hν off
+NH4Cl hν on
ΔH+, relative units
30
20
10
0
hν off
H+ Uptake
Unfixed chloroplasts
Fixed chloroplasts
hν off
hν on
1 min.
hν on

in fixed and unfixed chloroplasts. This probe binds to the hydrophobic region of the membrane and has been shown by VanderMeulen and Govindjee (91) to seemingly reflect membrane conformational changes elicited by a combination of H^+ ion gradient and the membrane potential. The extent of change of the probe fluorescence reflects the extent of the proton movement into the chloroplast, which is proportionately reduced in the fixed chloroplasts (Figure 9). [The H^+ uptake ability of glutaraldehyde fixed chloroplasts, observed by others (see reference 94), is now well established.] As the probe is in the interior of the membrane, it appears, therefore, that small conformational changes in the fixed membranes do occur; however, it is unlikely that macroconformational changes are permitted.

3. Treatments with NaSCN, $NaClO_4$, guanidine and urea (chaotropic agents)

Hatefi and Hanstein (98) have shown that chaotropic agents cause disruption of biological membranes by weakening the hydrophobic bonds in membranes and making more soluble particulate proteins by decreasing the polarity of water molecules. Using the chaotropic ions studied by Hatefi and Hanstein, as well as guanidine and urea which similarly disrupt the structure of water although with lesser efficiency (98), Lozier et al. (43) found that incubation of chloroplasts with these agents results in an inhibition of

electron transport, restorable with artificial donors that bypass the oxygen-evolving site, as well as a release of bound, chloroplastic manganese. In our control chloroplasts (Table 11), these agents vary in their effectiveness in blocking electron flow through PS II, with NaSCN most efficient and urea least of the four reagents tested; these relative degrees of effectiveness correlate well with the reported order of effectiveness of these agents in solubilizing membrane-bound proteins from several biological systems and in destructing membranes (as measured by lipid oxidation) (98). Upon fixation of the chloroplast thylakoids, NaSCN is nearly as effective in the fixed chloroplasts as in the unfixed in stopping oxygen evolution, but urea has lost almost all of its ability to do the same (Table 11). The protection offered by glutaraldehyde fixation is, in increasing order, as follows: NaSCN (16 percent), $NaClO_4$ (49 percent), guanidine (72 percent, and urea (86 percent).

As it was difficult to measure suspected membrane disturbance of the chloroplasts treated with these agents by the usual light scattering techniques, we measured emission spectra of Chl _a_ fluorescence at liquid nitrogen temperature which change depending upon the organization of the pigments on the membrane. An emission spectrum of Chl _a_ fluorescence at 77°K is an indication of the relative concentration of photosystems I and II and of the excitation energy distribution between the two photosystems; fluorescence at 685 nm is

TABLE 11

EFFECT OF CHAOTROPIC AGENTS ON FIXED AND UNFIXED CHLOROPLASTS

Chloroplast Treatment	Additions	μmoles $DCPIPH_2$ (mg Chl·hr)$^{-1}$	% Inhibition of Control
Unfixed	None	66.5	
Unfixed	0.8M NaSCN	2.7	96
Unfixed	0.5M $NaClO_4$	6.0	91
Unfixed	0.8M Guanidine	16.6	75
Unfixed	1.0M Urea	22.6	66
Fixed	None	22.0	
Fixed	0.8M NaSCN	4.3	81
Fixed	0.5M $NaClO_4$	11.9	46
Fixed	0.8M Guanidine	17.4	21
Fixed	1.0M Urea	19.9	9.5

Conditions are as indicated in Table 10; details of treatments are given in Methods.

mainly a measure of PS II and fluorescence at 735 nm of PS I (99, 100). Changes in the relative intensities of fluorescence at 685 nm and at 735 nm may permit us to see some indication of alteration of the membrane induced by these treatments with the chaotropes (perhaps with regard to the structural organization of the chlorophyll molecules of the two pigment systems with respect to each other), as has been indicated for changes caused by monovalent and divalent ions (see e.g., 101 for earlier literature). Urea and thiocyanate cause a significant change in the ratio of 735 to 685 nm fluorescence in the unfixed chloroplasts (Figure 10); a ratio of 3.24 in control increases to 3.65 (in thiocyanate treated chloroplasts) and to 4.18 (in urea treated chloroplasts). This change may be interpreted as an indication of some structural change in the membrane elicited by these agents which leads to a relative increase in excitation energy distribution to photosystem I. In glutaraldehyde fixed chloroplasts, NaSCN, but not urea, similarly increases the relative photosystem I originated fluorescence (Figure 11); the ratio of F735/F685 of 3.65 in fixed chloroplasts increases to 4.06 in NaSCN treated chloroplasts, whereas, it remains low (3.52) in urea treated chloroplasts. This suggests that where there is an inhibition of electron transport (see Table 11), there is also a change in the orientation of the pigment beds in the membrane, a change which is allowed in fixed chloroplasts.

Fig. 10.--Emission spectra of chlorophyll a fluorescence at 77°K of unfixed spinach chloroplasts and chloroplasts treated with NaSCN and urea. The relative fluorescence yield has been normalized at 685 nm. ——, no addition; o——o, treated with 1.0 M urea; Δ——Δ, treated with 0.8 M NaSCN; treatments as described in Materials and Methods. ∿0.5 ml chloroplasts, at 10 μg chlorophyll/ml suspended in 50 mM phosphate buffer, pH 7.2, were cooled quickly in the dark. λ excitation, 435 nm (9.9 nm half-band width); λ observation, variable (3.3 nm half-band width). A C.S. 2-61 filter was placed before the photomultiplier.

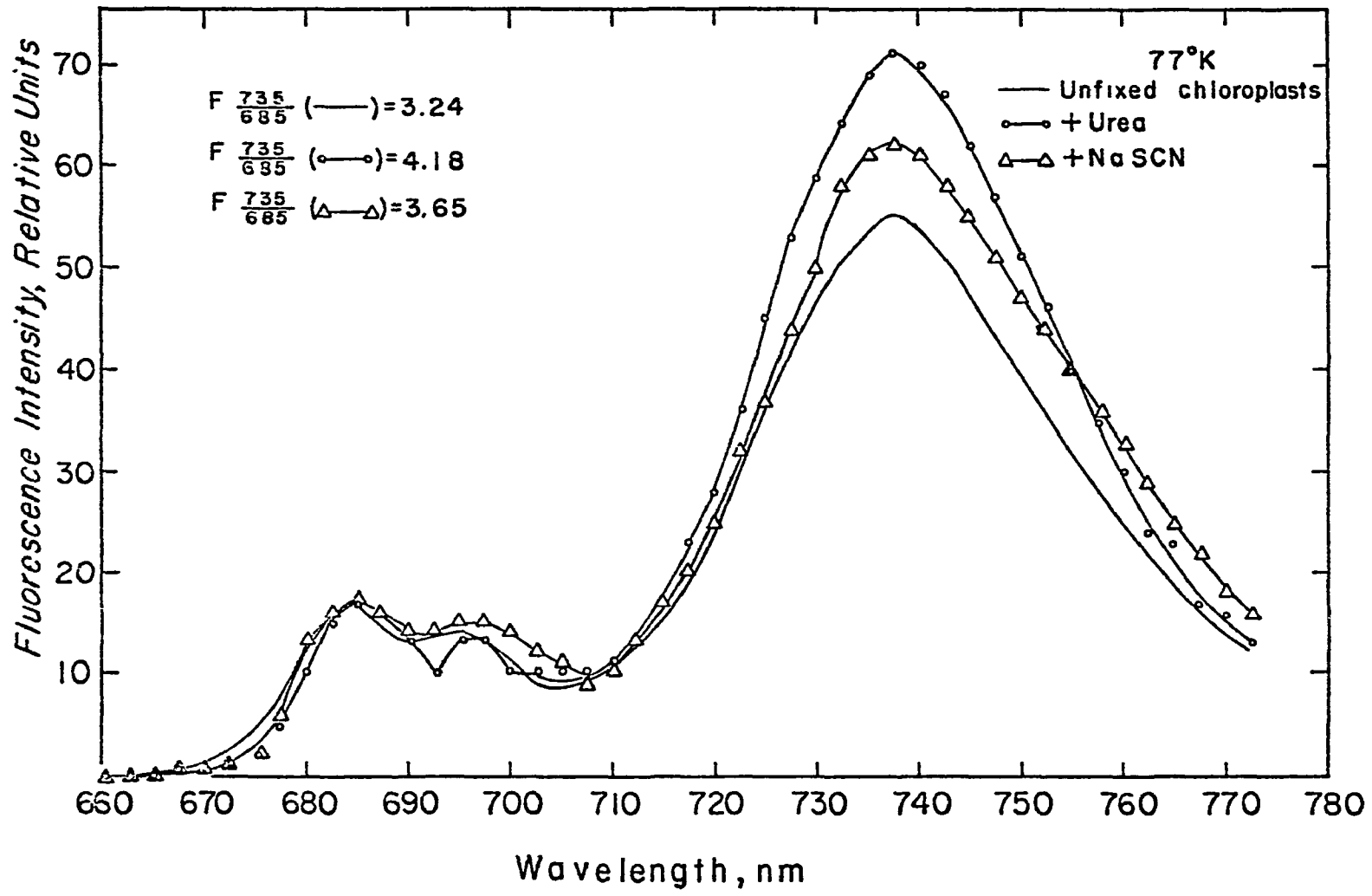
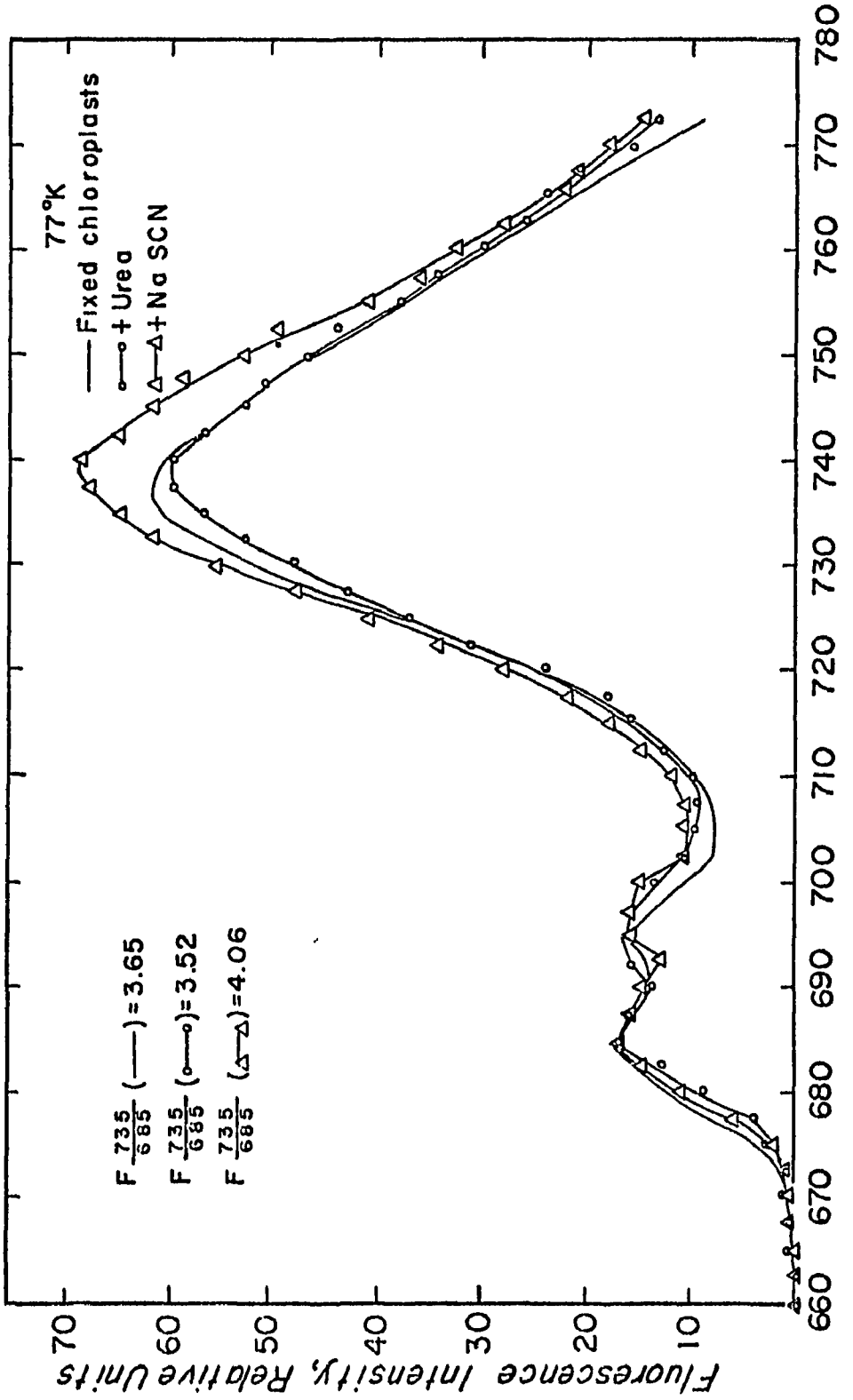

77°K
Unfixed chloroplasts
+ Urea
+ Na SCN
F 735/685 (——)=3.24
F 735/685 (o——o)=4.18
F 735/685 (Δ——Δ)=3.65
Fluorescence Intensity, Relative Units
Wavelength, nm
10
20
30
40
50
60
70
650
670
680
690
700
710
720
730
740
750
760
770
780

Fig. 11.--Emission spectra of chlorophyll *a* fluorescence at 77°K of glutaraldehyde fixed spinach chloroplasts and fixed chloroplasts treated with NaSCN and urea. Conditions as described in the legend of Fig. 10.


77°K
Fixed chloroplasts
+ Urea
+ Na SCN
F 735/685 (——) = 3.65
F 735/685 (o—o) = 3.52
F 735/685 (Δ—Δ) = 4.06
Fluorescence Intensity, Relative Units
Wavelength, nm
10
20
30
40
50
60
70
660
670
680
690
700
710
720
730
740
750
760
770
780

However, in the case of Tris-washed and control chloroplasts, there is no change in the fluorescence emission spectrum when oxygen evolution is effectively eliminated in the unfixed, Tris-washed chloroplasts (102). Since fixation of the chloroplasts by glutaraldehyde eliminated 50 percent of the inhibition due to Tris (Table 10) and we attribute this partial preservation of electron flow to large structural (destructive) changes not allowed once the chloroplasts are fixed, then it might be suggested that Tris-washing and chaotropic agents effect different types of structural changes and that in the case of the chaotropic agents, a correlation may be drawn with the changes as witnessed by the emission spectra and the degree of inhibition of oxygen evolution, but this does not hold true for Tris-induced inhibition.

4. Trypsin treatment

Control and glutaraldehyde fixed chloroplasts treated with trypsin had very different effects on DCPIP photoreduction (Table 12). Close to 100 percent inhibition is obtained with control chloroplasts, while approximately only 10 percent inhibition remains after glutaraldehyde fixation. Mantai (103) found the same effect. However, Selman and Bannister (30) have shown that only swollen and unstacked chloroplasts will show the trypsin inhibition. Mantai fixed mature, whole spinach leaves, and no effort was made to see that the chloroplasts were in the unstacked condition; therefore, his conclusions on the lack of inhibition by trypsin may be invalidated

TABLE 12

EFFECT OF TRYPSIN ON FIXED AND UNFIXED CHLOROPLASTS

Chloroplast Treatment	Additions	μmoles $DCPIPH_2$ $(mg\ Chl \cdot hr)^{-1}$	% Inhibition of Control
Unfixed	None	111.0	
Unfixed	Trypsin	16.4	86
Fixed	None	23.6	
Fixed	Trypsin	22.1	6

One ml of the trypsin treated and control chloroplasts (see ref. 30 for details), containing 30 μg Chl/ml, were added to one ml of reaction mixture containing 10 mM Tris-Cl, pH 7.6, 10 mM NaCl, 45 μM DCPIP and 5 mM NH_4Cl. DCPIP photoreduction was measured as described in Table 10.

by the uncertainty about the state of the chloroplasts. In our experiments, however, chloroplasts prior to and during fixation were suspended in the same buffering conditions used for trypsin incubation of control chloroplasts, i.e., unstacking conditions, as discussed in Methods.

As glutaraldehyde reacts mainly with the ε-amino groups of lysine (104), and trypsin cleaves at peptide bonds where the carboxyl group of lysine (or arginine) residues particpate (105), the question was raised as to whether the protection against trypsin attack offered by glutaraldehyde treatment of chloroplast membranes could simply be explained by a blocking of all the lysine residues by glutaraldehyde such that trypsin can no longer catalyze hydrolysis of the chloroplast proteins. To answer this possibility, we treated chloroplasts with acetic anhydride and formaldehyde and then looked at trypsin effects.

Acetylation has been shown to block free amino groups (106), and unlike glutaraldehyde treatment, where glutaraldehyde is a bifunctional aldehyde which cross-links proteins inter- and intramolecularly (107) and results in structural fixation of the membrane, acetylation permits free structural movement in the membrane. Acetylation of the chloroplasts does not protect the chloroplast proteins from trypsin attack (Table 13). Formaldehyde [at concentrations of 1, 2, and 5 percent formaldehyde (w/v)], which also reacts with free amino

TABLE 13

TRYPSIN INHIBITION OF PHOTOSYSTEM II REACTION IN ACETYLATED (A) AND FORMALDEHYDE (B) TREATED CHLOROPLASTS

	Chloroplast Treatment	Additions	μmoles $DCPIPH_2$ $(mg\ Chl \cdot hr)^{-1}$	% Inhibition of Control
(A)	Control	None	108.8	
	Control	Trypsin	17.8	84
	40 mM Acetic Anhydride	None	72.0	
	40 mM Acetic Anydride	Trypsin	8.9	87
(B)	Control	None	140.0	
	Control	Trypsin	9.2	93
	1% Formaldehyde	None	38.2	
	1% Formaldehyde	Trypsin	1.3	96
	2% Formaldehyde	None	34.0	
	2% Formaldehyde	Trypsin	0.0	100
	5% Formaldehyde	None	8.4	
	5% Formaldehyde	Trypsin	0.0	100

Conditions as indicated in Table 12; acetylation and formaldehyde treatment are as in Methods.

groups, likewise does not inhibit trypsin action in the chloroplasts (Table 13). Formaldehyde is a monofunctional aldehyde, so it cannot cross-link proteins in the manner that glutaraldehyde does. However, if steric conditions are favorable, the aminomethyol groups formed can condense with certain other functional groups to form methylene bridges (108) which could conceivably "fix" membranes. If the membranes were, in fact, fixed by the formaldehyde, this would invalidate our reasons for using the reagent, so we checked this possibility by our usual methods (Table 14). It does not appear likely that these chloroplasts are fixed in the conventional sense of the word (Table 14), but certainly at the concentrations used, at least as many available amino groups are blocked as with 0.5 or one percent glutaraldehyde. Therefore, it is quite doubtful that glutaraldehyde is merely making free amino groups unavailable for trypsin attack.

5. Treatment with antisera against photosystem II particles and an extract from frozen and thawed chloroplasts

Similar to the protection against the deleterious effects of trypsin on oxygen evolution by glutaraldehyde fixation, Table 15 shows that the two antisera shown previously (26, 27 and Chapter II) to specifically inhibit water oxidation reactions no longer are effective when large conformational changes in chloroplast membranes are immobilized with

TABLE 14

INDICATIONS FOR ABSENCE OF FIXATION BY FORMALDEHYDE OF CHLOROPLAST MEMBRANES

Chloroplast Treatment	% Light-induced PMS Quenching of Chl *a* Fluorescence	% Light-induced 90° Light Scattering Change	A680nm/A546nm (water) / A680nm/A546nm (buffer)
Control	18	39	1.9
1% Formaldehyde	14	40	1.8
2% Formaldehyde	13	--	1.7
5% Formaldehyde	8	0	1.9
1% Glutaraldehyde	0	0	1.06

Conditions for formaldehyde treatment as indicated in Methods. Conditions for measurement of the parameters above given in legends of Figures 6, 7, and 8. (Percentage of light-induced quenching of Chl *a* fluorescence by PMS for acetylated chloroplasts is 10.5.)

TABLE 15

EFFECT OF ANTISERA AGAINST PS II PARTICLES AND AN EXTRACT FROM FROZEN AND THAWED CHLOROPLASTS IN FIXED AND UNFIXED CHLOROPLASTS

Treatment	Additons	μmoles $DCPIPH_2$ $(mg\ Chl \cdot hr)^{-1}$	% Inhibition of Control
Unfixed	None	216	
Unfixed	Control Serum	94	
Unfixed	PS II Particle Antiserum	80.5	13.4
Unfixed	Extract Antiserum	75.3	19
Fixed	None	63.7	
Fixed	Control Serum	38.0	
Fixed	PS II Particle Antiserum	38.0	0
Fixed	Extract Antiserum	37.4	2

Reaction conditions as in Table 10. Prior to measurement of DCPIP reduction, chloroplasts and sera were incubated in the dark at 0°C for 15 minutes (3 μl serum/μg Chl). Prior to making these measurements, experiments as described in Tables 2, 3 and 5 were repeated to show that these antisera were specifically exhibiting oxygen evolution.

glutaraldehyde. The low level of inhibition in the unfixed chloroplasts by the antisera has been interpreted to be most likely due to a limited accessibility of the antiserum for the antigenic site in the membrane, whether that site be the oxygen-evolving intermediate itself or some structural component perhaps complexed with the oxygen-evolving intermediate. Apparently, glutaraldehyde fixation renders the appropriate antigen completely unavailable to the antibodies.

6. Diazonium benzene sulfonic acid treatment

As discussed above, DABS is a small, impermeable reagent which reacts with histidine, tyrosine, cysteine and lysine amino acid residues (24). As shown in Table 16, there is approximately a 60 percent decrease in the amount of inhibition resultant from incubation of the chloroplasts with this compound in the light (as compared to dark incubation) when the chloroplasts are fixed with glutaraldehyde. However, the electron transport rate of the glutaraldehyde fixed chloroplasts (with DABS incubation in the dark) is also approximately 60 percent of the corresponding rate in the unfixed chloroplasts. Giaquinta et al. (24, 25) have shown that electron flow through PS II is necessary for the DABS effect on oxygen evolution to be realized; the lower the rate of electron transport, attained by decreasing the intensity of actinic light or by using suboptimal concentrations of DCMU, the lower was the percent inhibition by DABS treatment. If the same

TABLE 16

EFFECT OF LIGHT INCUBATION OF DABS IN FIXED AND UNFIXED CHLOROPLASTS

Chloroplast Treatment	Additions	μmoles $DCPIPH_2$ $(mg\ Chl \cdot hr)^{-1}$	% Inhibition of Control
Unfixed	DABS in dark	164.0	
Unfixed	DABS in light	105.5	36
Fixed	DABS in dark	61.7	
Fixed	DABS in light	53.7	13

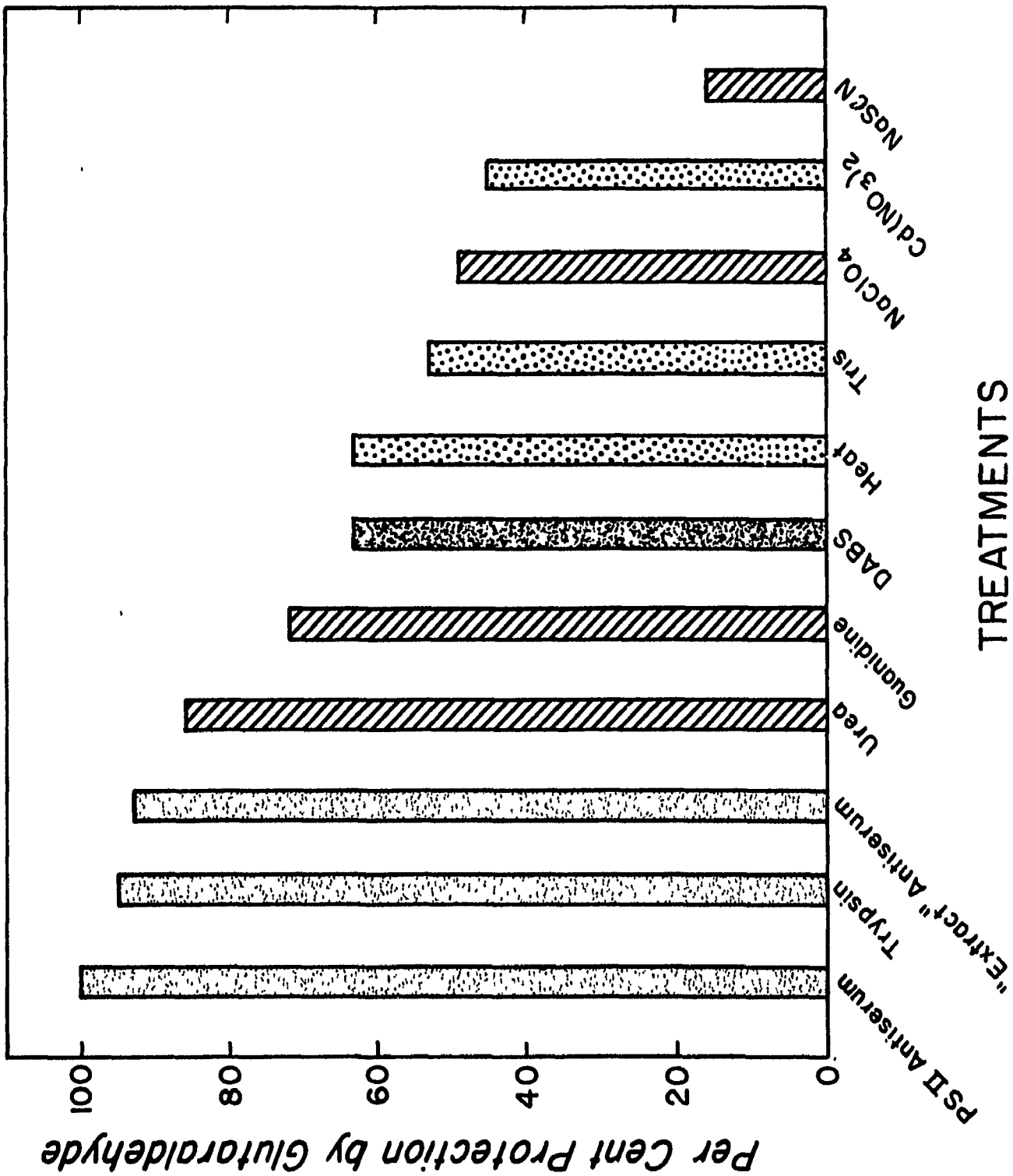
Reaction conditions as in Table 10 with 5 mM NH_4Cl added; details of treatments as given in Methods.

correlation between electron transport rates and DABS inhibition can be extended to the glutaraldehyde data, then one may believe that the light-potentiated DABS inhibition is as effective in fixed chloroplasts as it is in unfixed membranes. Giaquinta (personal communication) has unpublished data that is in contrast to ours in that the extent of light-potentiated inhibition by DABS is the same in fixed and unfixed chloroplasts. However, that is difficult to understand in the light of their experiments with DCMU and varying light intensity as Hill activity is decreased considerably with fixation.

D. Discussion

Glutaraldehyde fixation of chloroplasts appears to, at least, depress the inhibitory action specifically at the level of oxygen evolution of a variety of treatments of chloroplasts. In the case of permeable (or physical) inhibitors tested, leaving aside chaotropic agents for the moment, fixation of chloroplast membranes relieves the inhibition by 50 percent (Figure 12). Since it is well-known that glutaraldehyde fixation does not allow large structural changes in the membrane to occur, it might be concluded that Tris-washing, treatment with the heavy metal cadmium, and mild heating of chloroplasts primarily cause a perturbation of the membrane that secondarily results in inhibition of oxygen evolution. It has been shown (51, 109) that heat treatment and Tris-washing of chloroplasts result in loss of

Fig. 12.--Percentage protection by glutaraldehyde of chloroplasts against inhibition of oxygen evolution by various treatments. Impermeable inhibitors: trypsin (0.13μg/μg Chl), antiserum against extract from repeatedly frozen and thawed chloroplasts (3μg/μg Chl); chaotropic agents: NaSCN (0.8 M), $NaClO_4$ (0.5 M), guanidine (0.8 M), urea (1.0 M); other treatments: Tris-washing (0.8 M, pH 8.0), $Cd(NO_3)_2$ (0.5 mM), heating (50°C, 5 min.); DABS (2 mM). Other conditions as indicated in Tables 10-12, 15, and 16.


Per Cent Protection by Glutoraldehyde
0
20
40
60
80
100
PSΠ Antiserum
Trypsin
"Extract" Antiserum
Urea
Guanidine
DABS
Heat
Tris
NaClO4
Cd(NO3)2
NaSCN
TREATMENTS

bound manganese from the chloroplasts, concomitant with a loss of oxygen evolution, experiments of which type have supported the generally held notion that manganese is intricately involved in oxygen evolution. It can be easily visualized that disruption of the membrane could effect removal of loosely bound manganese, as the binding of manganese might require rigid structural requirements. As glutaraldehyde fixation is only 50 percent efficient in protecting these chloroplasts' oxygen-evolving capacity against these treatments, the amount of structural change that is permitted once the chloroplasts are stabilized with glutaraldehyde (as witnessed by the changes in AS fluorescence) might be sufficient to allow some membrane disturbance to occur. An alternate explanation is that the remaining inhibition is due to a direct effect on the oxygen-evolving intermediate.

The chaotropic ions, "inorganic anions which favor the transfer of apolar groups to water" (98), are very potent inhibitors of oxygen evolution in unfixed chloroplasts. Sodium thiocyanate is nearly as effective an inhibitor of water oxidation in fixed chloroplasts as it is in unfixed chloroplasts (Figure 12). Its persistent effectiveness in fixed chloroplasts indicates that water structure-breaking phenomena and the subsequent effect seen on the oxygen-evolving apparatus can still occur. This breakdown of local water

structure and weakening of hydrophobic interactions in the membrane where oxygen-evolving intermediates are situated are sufficient to impair the operation of this particular component, while other agents that are less effective (in other biological systems as well as unfixed chloroplasts) perhaps require a greater involvement with general disorganization of bulk water structure. The effect of NaSCN, therefore, can loosely be termed as one of membrane disturbance primarily (which then secondarily affects oxygen evolution), with the membrane destruction occurring in the locality of the oxygen-evolving intermediate, showing resistance to glutaraldehyde fixation because of the nature of the potency of the agent's water structure-breaking capacity. As one uses less potent chaotropes, fixation preserves to a greater extent the activity of the chloroplasts; we have found that the order of effectiveness (Figure 12) of inhibition of oxygen evolution in fixed chloroplasts (SCN^- < ClO_4^- < guanidine < urea) correlates well with the potency of these reagents to act as chaotropes in a number of systems treated (98). In addition, chaotropes of the urea-guanidine class appear to act by both breaking down the solvent structure of water as well as specifically binding to certain groups in the affected molecule, resulting in a weakening of the noncovalent bonds in this molecule (110). Both guanidine and urea are considerably less effective in inhibiting oxygen evolution in fixed systems, and

it may be suggested that the cross-linking of the protein by glutaraldehyde may prevent the usual specific binding of urea and guanidine, or alternatively, its binding may not disrupt the native configuration of the component when the membrane is fixed. These arguments, however, cannot be considered established as it is known by data reported in this communication and elsewhere (86) that small conformational changes do persist in glutaraldehyde fixed membranes.

Thus, it is shown that the permeable (or physical) class of oxygen-evolution inhibitors seems to act, at least partly, by disrupting membrane structure; in the case of chaotropes, the more potent agents cause sufficient damage at a microstructural level as to persist in their effectiveness in fixed chloroplasts.

The inhibitory effects of three of the impermeable oxygen evolution inhibitors, trypsin, an antiserum against photosystem II particles and an antiserum against an extract from repeatedly frozen and thawed chloroplasts [both antisera having been shown previously to specifically inhibit oxygen evolution (26, 27)], are completely eliminated by glutaraldehyde fixation (Figure 12). There are two possible interpretations of this data: (1) that trypsin and the two antisera cause large destructive changes in the membrane which secondarily affect a sensitive component buried in the membrane that is involved with oxygen-evolution, and

glutaraldehyde fixation does not permit this destructive effect, or (2) that the addition of trypsin or antisera to unfixed chloroplasts in the dark results in macro-conformational change in the membrane which repositions specific sites in the oxygen-evolving intermediate (or protein in complex with it) so that they are now accessible to those impermeable inhibitors, and glutaraldehyde fixation does not allow this structural change.

In view of the fact that chaotropic agents can most likely be considered as being membrane disruptive (98) and they still inhibit fixed chloroplasts, we prefer the latter hypothesis. Moreover, Selman et al. (31) have presented electron micrographs of chloroplasts treated with trypsin, in the manner that they (and we) do it, which show that trypsin treatment does not change the morphology of the chloroplast membranes. This further supports the conclusion that trypsin incubation (for 10 minutes) does not result in a general breakdown of chloroplast structure, although precaution must be exercised as with all interpretations drawn from electron micrographs. Also, it has been shown (35) with animal membranes that mild proteolysis leads to redistribution of certain glycoproteins in these membranes. Based on the above data, we suggest that whatever site the trypsin and antisera are attacking (and as stated previously, one cannot eliminate the possiblity of its being a site of some protein

complexed to the oxygen-evolving intermediate, as opposed to the intermediate itself), that site is most likely buried in the membrane, unavailable to large, impermeable molecules once the macroconformational changes are prohibited by glutaraldehyde fixation.

The effect of DABS (Figure 12) on glutaraldehyde fixed membranes is the most difficult piece of information to assimilate into this series of experiments. It is thought to be an impermeable molecule, although direct experimental proof to this effect is lacking in the chloroplast system and its impermeability is only implied from work with other membrane systems. In fact, it has been suggested (Dr. Charles Arntzen, personal communication) that its light-potentiated inhibition is simply a consequence of its being electrophoresed across the thylakoid membrane; if this be the case, our data can be explained as a direct inhibition of the oxygen-evolving intermediate by the DABS binding. However, Giaquinta et al. (25) feel that it is unlikely that the light potentiation of the DABS inhibition of oxygen evolution is caused by electron-flow dependent electrophoresis of DABS into the membrane, on the basis of the absence of DABS inhibition with PMS-catalyzed cyclic electron flow around PSI.

As pointed out above, the light-potentiated DABS inhibition may be taken to be the same in fixed and unfixed chloroplasts if one is to take into account the lower

electron transport rate during light incubation with DABS in the fixed chloroplasts. It is known that glutaraldehyde fixed chloroplasts do not undergo light-induced conformational changes as measured by 90° light scattering (86, data presented here). However, Giaquinta et al. (25) suggest that the light-induced change is not the usual change measurable by 90° light scattering, resultant from H^+-ion accumulation, but rather a "conformational change" dependent only upon electron flow through PS II. In these abstract terms, it is difficult to visualize what effect fixation would have in allowing such a change to occur. It must be emphasized that DABS inhibition must be light-potentiated, while trypsin and antisera inhibition is seen in unfixed chloroplasts incubated with these proteins in the dark. We have suggested that the effect of trypsin and antisera addition to the membrane in the dark brings the "site" in contact with these impermeable agents. Fixation does not allow this same dark effect to occur; we must assume that this (dark) change in the membrane brought about by the presence of trypsin or the antisera is a larger structural change than is induced by light, and fixation only allows the latter.

In conclusion, we suggest the following interpretations of our data:

1. Inhibition of oxygen evolution by Tris-washing, heat treatment and $Cd(NO_3)_2$ is at least 50 percent due to

large structural and destructive changes in the membrane (that are stopped by glutaraldehyde fixation); the remaining inhibition (that persists in the fixed system) is due to either smaller perturbations of the membrane that can still occur in fixed chloroplasts or a direct action of the inhibitor on the oxygen-evolving intermediate.

2. Chaotropic agents, judging from their action in other systems, would be expected to act primarily through membrane disturbance and secondarily on oxygen evolution. That the most potent chaotrope is almost as effective in fixed as in unfixed chloroplasts is consistent with the suggestion that its potency is caused by severe local water structure-breaking phenomena, i.e., membrane disturbance, that can be seen even in chloroplasts where only microstructural changes occur. As the potency of the chaotrope decreases, the smaller local effects on water are not sufficient to stop oxygen evolution, and the bulk water structure-breaking effects on the membrane are stopped by glutaraldehyde fixation.

3. The action of trypsin and of the two antisera used can be either through (a) exclusive and serious membrane perturbation that in turn affects a sensitive component buried in the membrane, or (b) through a macroconformational change in the membrane, induced by interaction of these inhibitors with the membrane in the dark, which now exposes (subject to attack by trypsin and antisera) a previously buried

intermediate involved with oxygen evolution. (This structural change is abolished by glutaraldehyde fixation.) We favor the latter hypothesis, in that chaotropic agents which certainly disrupt membrane structure are largely still effective in glutaraldehyde fixed chloroplasts. These data do not distinguish between these impermeable inhibitors acting directly on the oxygen-evolving intermediate itself or on some proteinaceous component complexed to it, but, whatever the case, the intermediate is probably embedded in the hydrophobic interior of the membrane.

CHAPTER IV

SILICOMOLYBDATE AND SILICOTUNGSTATE MEDIATED DCMU-INSENSITIVE PHOTOSYSTEM II REACTION: ELECTRON FLOW, CHLOROPHYLL a FLUORESCENCE AND DELAYED LIGHT EMISSION CHANGES

A. Introduction

Girault and Galmiche (16) and Giaquinta et al. (17) have recently shown a dichlorophenyldimethylurea (DCMU) insensitive oxygen evolution from PS II in the presence of silicotungstate (SiTu) and ferricyanide (FeCy), and silicomdybdate (SiMo) and ferricyanide, respectively. Based on the fact that methylviologen, a PS I acceptor of very low redox potential, did not support a DCMU-insensitive oxygen evolution mediated by silicotungstate, the suggestion was made (1) that silicotungstate acts by altering membrane properties in such a manner as to allow ferricyanide (an acceptor of higher redox potential) to accept electrons between the PS II reaction center and the DCMU site of inhibition, i.e., directly from Q, the primary electron acceptor of PS II. Giaquinta et al. (2) supported similar conclusions with their data on the methylviologen-silicomoloybdate couple. Such a clearcut photosystem II partial reaction would greatly simplify many research problems in photosynthesis, and, therefore, it is

necessary to ascertain by other independent measurements that indeed one is measuring a DCMU insensitive photosystem II electron transport.

We have confirmed previous observations that oxygen evolution persists in the presence of DCMU with both ferricyanide and either silicomolybdate or silicotungstate present. We studied more carefully the silicomolybdate rather than the silicotungstate mediated oxygen evolution, as the rates of reactions were considerably higher in the former than in the latter case. We found that electron transport stops within one minute after the addition of both DCMU and silicomolybdate; monitoring, therefore, only initial rates after silicomolybdate addition can be used for electron flow from H_2O to this chemical. Secondly, silico-compounds are shown to be sufficient to sustain electron flow without the addition of ferricyanide; again, this reaction is DCMU insensitive for only a limited time. Silicomolybdate appears to be acting as an electron acceptor itself.

If these silico-compounds were indeed accepting electrons directly from Q, then one might predict that the variable chlorophyll a fluorescence yield [which reflects the level of reduced Q (111)] and delayed light emission [which reflects the back reaction between Q^- and Z^+, where Z is the electron donor to reaction center II (112)] in the presence or absence of DCMU would be severely depressed as Q would be kept in the

oxidized state. The data reported in this chapter, however, show that in addition to suppressing variable fluorescence, there is a remarkable decrease in "constant" fluorescence ("0" level), with concentrations of silicomolybdate and silicotungstate greater than 25 μM. At concentrations lower than 25 μM of the silico-acids where oxygen evolution is supported for only a brief time, the constant level of fluorescence is no longer depressed, yet the variable fluorescence decreases in proportion to the concentration of the silico-compounds; addition of DCMU in these cases only slightly raises the fluorescence maximum.

Measurements of delayed light emission of chloroplasts treated with concentrations of silicomolybdate that only affect the variable fluorescence yield also give support to the fluorescence and oxygen data which show that these compounds are accepting electrons directly from Q.

B. Materials and Methods

Chloroplast isolation from market spinach or romaine lettuce was as described previously (26), but with phosphate buffer of similar concentration and pH substituted for the Tris-Cl buffer; fresh, rather than frozen, chloroplasts were used in this study. Chlorophyll estimation was made according to the method of MacKinney (59).

Oxygen evolution was measured with a Clark electrode, using a Yellow Springs oxygen monitor (model 53) and an

Esterline Angus recorder (model E11015). The time course of chlorophyll a fluorescence was measured according to the method of Munday and Govindjee (113) with a spectrofluorometer described elsewhere (61). The method for measuring delayed light emission was essentially that of Jursinic and Govindjee (114).

Light-induced absorbance changes were measured at 540 nm using the split beam difference spectrophotometer of Sybesma and Fowler (115). The measuring monochromator was set at 540 nm (half-band width, 6.6 nm), and a 540 nm Farrand interference filter (half-band width, 1.6 nm) was placed before the photomultiplier to eliminate the blue actinic light (Farrand interference filter 480, half-band width, 5.5 nm).

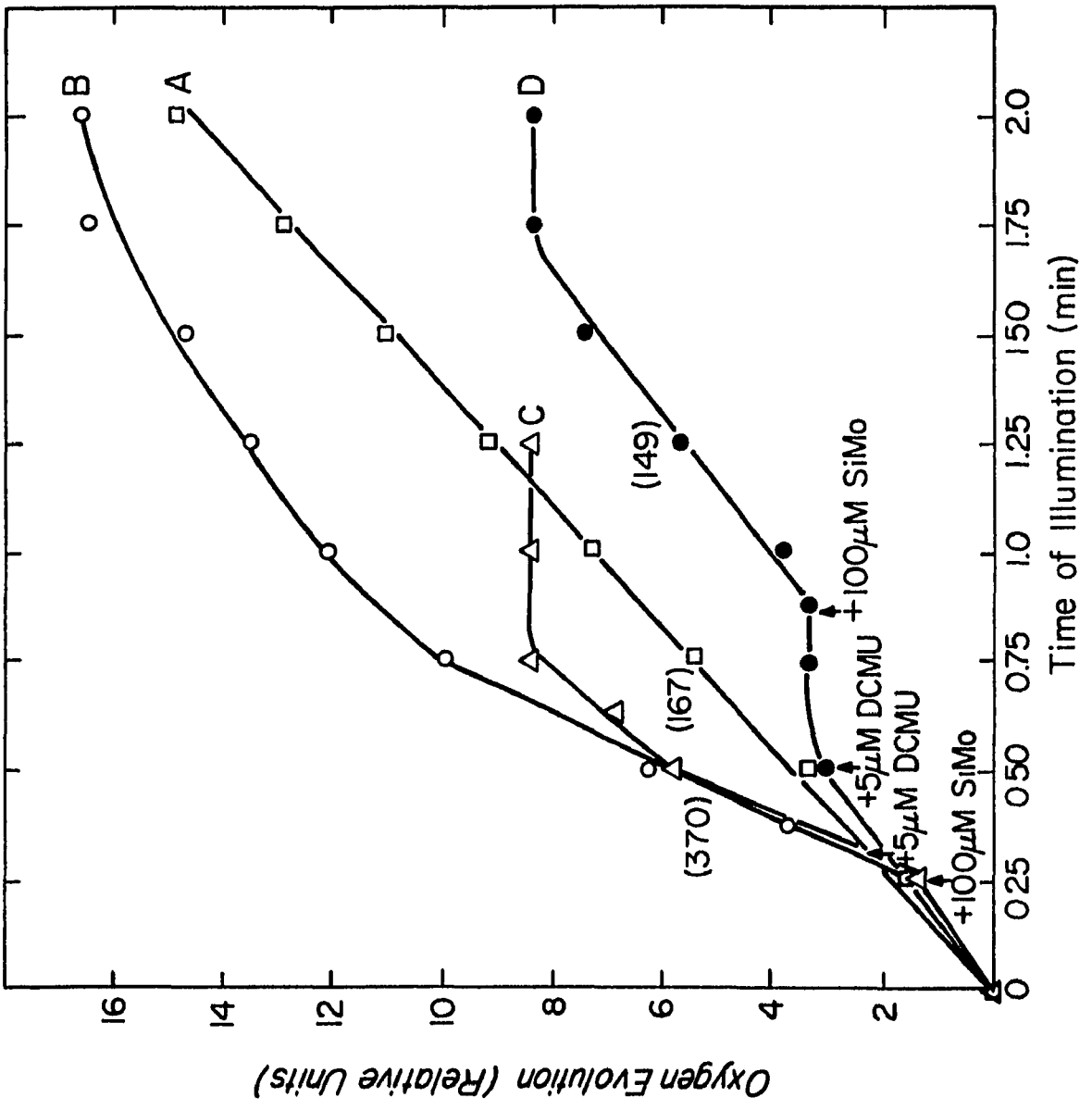
Other details are given in the legends of the figures and tables.

C. Results

1. Silicomolybdate and silicotungstate as electron acceptors: electron flow

As indicated in Figure 13, curve D, silicomolybdate can restore oxygen evolution to DCMU inhibited electron flow to ferricyanide, as already shown by Giaquinta et al. (17). However, these rates are initial rates, as after 45 seconds after addition of the silicomolybdate, the rate of oxygen evolution begins to level off and is immeasurable at one minute after addition. It is not a case of time-dependent

Fig. 13.--Ferricyanide-mediated oxygen evolution of spinach chloroplasts in the presence or absence of silicomolybdate and DCMU. The reaction mixture contained in 1.7 ml: 100 mM KCl, 5 mM $mgCl_2$, 20 mM Tricine-KOH (pH 8.1), 0.5 mM FeCy and chloroplasts equivalent to 35 µg Chl/ml. A, □——□ , FeCy reduction, no additions; B, o——o, 100 µM SiMo added at 0.25 seconds; C, Δ——Δ , 100 µM SiMo added at 0.25 seconds and 5 µM DCMU added directly thereafter; D, FeCy as acceptor with 5 µM DCMU added at 0.5 seconds, followed by SiMo at 0.85 seconds. Numbers in parentheses are initial rates of O_2 evolution expressed in µ equivalents $(mg\ Chl \cdot hr)^{-1}$.


Oxygen Evolution (Relative Units)
Time of Illumination (min)
0
0.25
0.50
0.75
1.0
1.25
1.50
1.75
2.0
2
4
6
8
10
12
14
16
A
B
C
D
(370)
(167)
(149)
+100μM SiMo
+5μM DCMU
+5μM DCMU
+100μM SiMo

photoinactivation of the chloroplasts as there is no change in the rate of oxygen evolution supported by ferricyanide until after several minutes (curve A). Neither is it a case of limiting concentrations of silicomolybdate, as when no DCMU is present, the rate is still measurable beyond 45 seconds for some time, although it does begin to slow down (curve B). Moreover, addition of a second aliquot of silicomolybdate will almost completely restore the original rate to these chloroplasts, while similar additions to DCMU treated chloroplasts will not restore oxygen evolution.

As can be seen in Figure 13, curve D, rates of oxygen evolution as restored by silicomolybdate to DCMU treated chloroplasts are approximately the same as those rates with ferricyanide alone. Addition of silicomolybdate to chloroplasts evolving oxygen with ferricyanide as electron acceptor results in an increased electron transport rate (curve B). As shown in curve C, if the silicomolybdate addition is followed shortly thereafter with DCMU addition oxygen evolution is completely inhibited in approximately 30 seconds.

Ferricyanide need not be present to see oxygen evolution with silicomolybdate; in fact, as seen in Table 17, the rate with silicomolybdate alone (added in the light) is more than double that of the control rate with ferricyanide. It is interesting to note that the illumination of the chloroplasts in the acceptor-less reaction mixture prior to addition of

TABLE 17

SILICOMOLYBDATE-MEDIATED OXYGEN EVOLUTION

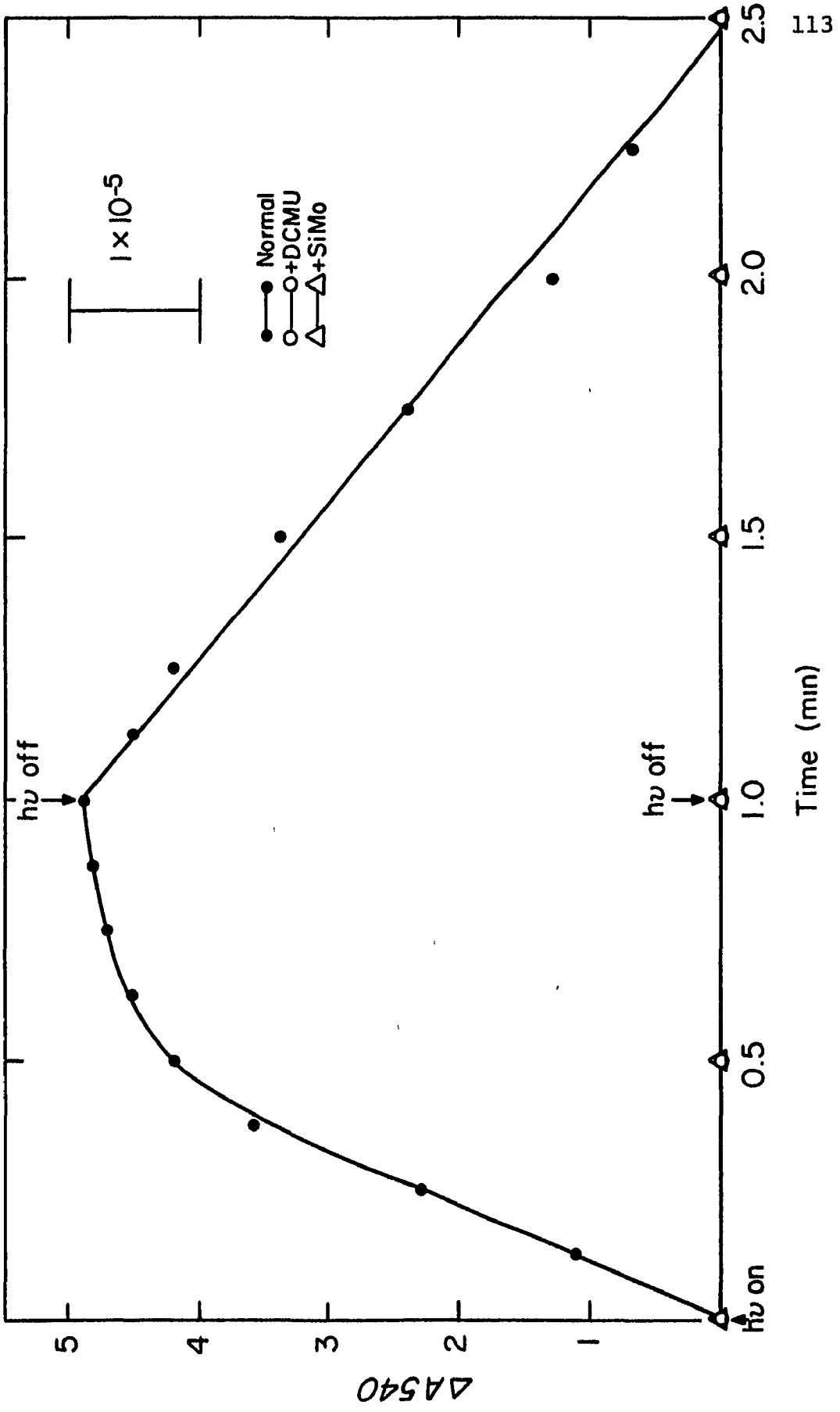
Chloroplasts	Electron Acceptor	Treatment	Oxygen Evolution μ equiv. (mg Chl·hr)$^{-1}$
Normal	FeCy added in dark	None	103
Normal	FeCy added in light	None	107
Normal	SiMo added in dark	None	82
Normal	SiMo added in light	None	250
Normal	SiMo added in light	DCMU added in dark (prior to SiMo)	98
Normal	SiMo added in light	DCMU added in light (prior to SiMo)	156
Normal	SiMo added in light	DCMU added in light (after SiMo)	250
None	SiMo added in light	None	0
Heat Treated 5 min, 50°C	FeCy added in dark	None	0
Heat Treated 5 min, 50°C	SiMo added in light	None	0

The reaction mixture contained in 1.7 ml: 100 mM KCl, 5mM $MgCl_2$, 20 mM Tricine-KOH (pH 8.1) and chloroplasts equivalent to 35 μg Chl/ml. Where indicated, 0.5 mM FeCy, 100 μM SiMo and 5 μM DCMU are added. The rates represented are initial rates.

the silicomolybdate yields a much higher rate of electron transfer than if the compound is added to dark-adapted chloroplasts (Table 17), while this is not the case for ferricyanide-mediated electron flow. Also, addition of DCMU to the chloroplasts in the dark followed by addition of silicomolybdate in the light considerably decreases the initial rate of DCMU-insensitive oxygen evolution as compared to the rate when DCMU is added in the light after silicomolybdate addition.

As has been adequately documented (see reference 116), illumination of chloroplasts, even in acceptor-less reaction mixtures results in a reversible absorbance change at 540 nm, an indication of conformational and configurational changes in the membrane resulting from electron flow. Chloroplasts used in all experiments reported in this communication showed this light-induced reversible absorption change at 540 nm (Figure 14). We would like to suggest that silicomolybdate alone because of its large size can perturb the membrane to some extent to make more accessible Q for direct reduction. However, if the chloroplasts are first exposed to light prior to silicomolybdate addition, the light-induced change in the structural organization of the membrane might result is a repositioning of some of the electron transport components in the membrane such that Q is more exteriorly located on the membrane; in this manner Q can more efficiently donate its electrons to silicomolybdate, which might explain the three-

Fig. 14.--Light-induced absorbance change at 540 nm in the presence and absence of DCMU or silicomolybdate. Reaction mixture as in Table 17 with chloroplasts equivalent to 35 µg Chl/ml contained in a 1 mM pathlength cuvette. ●——●, normal chloroplasts, no additions; o——o, 5 µM DCMU;Δ——Δ, 100 µM SiMo. Additions made to fresh, dark-adapted samples. Instrumental conditions as in Materials and Methods.


ΔA540
1
2
3
4
5
hυ on
hυ off
hυ off
0.5
1.0
1.5
2.0
2.5
Time (min)
1 × 10-5
Normal
+DCMU
+SiMo

fold increase in electron transport rates when silicomolybdate is added to illuminated rather than dark-adapted chloroplasts. Addition of DCMU to dark-adapted chloroplasts results in the elimination of the light-induced absorbance change at 540 nm as shown in Figure 14, and a rate of oxygen evolution with silicomolybdate added in the light (after dark DCMU addition) not greatly different from the rate seen with dark addition of the acceptor (Table 17), which can be explained by the model described above. If DCMU and then the silicomolybdate are added to illuminated chloroplasts, the rate of oxygen evolution is intermediate between the rate seen when silicomolybdate is added to illuminated and dark-adapted chloroplasts, which might be understood in terms of the light-induced structural change of the chloroplast membrane being slowly relaxed with DCMU addition, resulting in a somewhat decreased availability of Q for silicomolybdate. DCMU added to chloroplasts already evolving oxygen with silicomolybdate does not alter the initial rates, simply showing the initial insensitivity of silicomolybdate reduction to DCMU.

Figure 14 also shows the addition of silicomolybdate to dark-adapted chloroplasts results in the elimination of the light-induced absorbance change at 540 nm, as is the case with DCMU. As we know that electron transport through PS II is indeed occurring, we must postulate one of two possibilities: (a) that electron flow from Q to some component in the intersystem electron transport chain or in PS I is necessary to

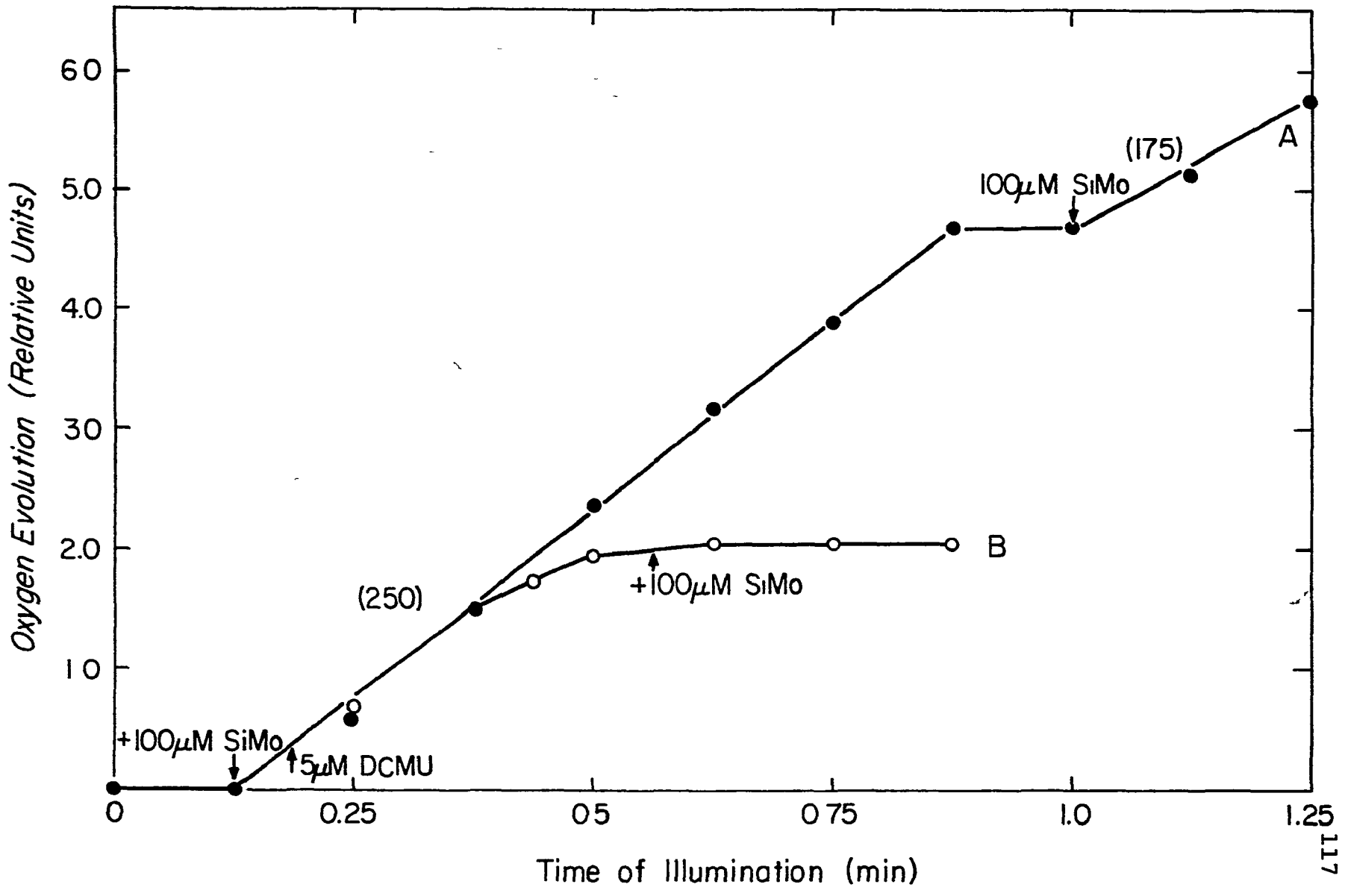
support the structural changes that lead to 540 nm absorption changes, or (b) that the absence of phosphorylation, for reasons not yet clearly understood, in the silicomolybdate system (17) stops the light-induced absorption changes.

Silicomolybdate and silicotungstate used alone are not catalyzing some light-induced, unprecedented type of oxygen evolution independent of photosynthesis, as illumination of the reaction mixture either in the absence of chloroplasts or in the presence of chloroplasts heated to 50°C for five minutes, which have been shown to have an inhibited water oxidation reaction (48), does not produce any oxygen, as shown in Table 17.

Figure 15 shows that as with ferricyanide and silicomolybdate used in conjunction, DCMU will inhibit the Hill reaction supported by silicomolybdate alone shortly after 30 seconds of DCMU addition. As in the case of ferricyanide and silicomolybdate used with DCMU, the second addition of silicomolybdate restores oxygen evolution only to those chloroplasts that have not seen DCMU (curve A versus curve B).

Data presented for silicomolybdate essentially have been confirmed for the silicotungstate system (data not shown). Relatively low concentrations of silicotungstic acid (20 μM) can partially relieve the DCMU inhibition of oxygen evolution with ferricyanide as electron acceptor, and the oxygen evolution is DCMU-insensitive only for a limited time. Silicotungstate can also act alone in supporting oxygen evolution, with

Fig. 15.--Silicomolybdate-mediated oxygen evolution of spinach chloroplasts in the presence and absence of DCMU. Reaction mixture as described in the legend of Fig. 13 with ferricyanide omitted; 5 μM DCMU added to sample B only. Numbers in parentheses are initial rates of O_2 evolution expressed in μ equivalents $(mg\ Chl \cdot hr)^{-1}$. The control rate of oxygen evolution in the water to ferricyanide reaction (minus DCMU and silicomolybdate) was 103 μ equivalents $(mg\ Chl \cdot hr)^{-1}$ in this particular chloroplast preparation.


Oxygen Evolution (Relative Units)
6.0
5.0
4.0
3.0
2.0
1.0
0
0.25
0.5
0.75
1.0
1.25
Time of Illumination (min)
+100μM SiMo
5μM DCMU
(250)
+100μM SiMo
B
(175)
100μM SiMo
A

rates, using 100 μM silicotungstate, approximately one-half of those with the same concentration of silicomolybdate. Table 18 shows rates of oxygen evolution mediated by silicomolybdate; very low concentrations of silicomolybdate support oxygen evolution, although clearly the time in which electron transport is sustained is severely limited with the lower concentrations of the acceptor.

2. Chlorophyll a fluorescence

In the presence of DCMU, the fluorescence yield of broken chloroplasts rapidly rises to a maximum due to an accumulation of reduced Q and remains so throughout illumination (117). Addition of ferricyanide to the DCMU treated chloroplasts results in only a small decrease in the fluorescence yield due to the filter effect of the colored acceptor; this is expected as ferricyanide accepts electrons after the DCMU block (data not presented here). As first shown by Malkin and Kok (118), ferricyanide added to normal chloroplasts keeps Q in the oxidized state and the fluorescence level remains low (close to F_o), until the ferricyanide is reduced, after which the maximum fluorescence level (F_∞) is finally reached as Q becomes reduced. Only in the case where the electron acceptor is autoxidizable, does the fluorescence level remain low, as shown by Mohanty et al. (94) with methylviologen. As we had no reason to believe that silicomoloybdate is autoxidizable as it seems to become concentration limiting as it is reduced

TABLE 18

SILICOMOLYBDATE-MEDIATED OXYGEN EVOLUTION IN THE ABSENCE OF FERRICYANIDE

Silicomolybdate Concentration (μM)	Oxygen Evolution (μequiv. (mg Chl·hr)$^{-1}$
0	0
5	102
10	124
25	209
50	246
100	240
200	231
300	192

The conditions are described in the legend of Table 17. SiMo in concentrations given was added in the light. Only initial rates are reported. The control rate with electron flow to ferricyanide was 96 μequiv. (mg Chl·hr)$^{-1}$.

in mediating oxygen evolution, we expected a fluorescence transient similar to that seen for ferricyanide, but DCMU-insensitive.

We looked at the Chl a fluorescence induction in the presence of the silico-compounds and DCMU to arrive at an independent measurement for the proposed electron acceptor role for the silico-compounds (i.e., before the DCMU block and directly from Q). It was, however, surprising to find that silicomolybdate or silicotungstate at 100 μM concentration (the same concentrations we routinely used to measure oxygen evolution) is a very potent quencher of Chl a fluorescence in the chloroplasts; as expected, DCMU does not affect this quenching, as shown in Figure 16 (A). It is interesting to note that neither silicomolybdate nor silicotungstate has any quenching effect on Chl a extracted with acetone, as indicated in Table 19. The dramatic quenching phenomenon observable in in the chloroplast appears to rely upon the Chl a as being bound in the membrane as some chlorophyll-protein complex. This is quite unlike the quenching properties of other well-studied chemicals, such as dinitrobenzene which effectively quenches chlorophyll fluorescence both *in vivo* and *in vitro* (119). At lower concentrations of silicomolybdate, as shown in Figure 16, the constant fluorescence (F_o) and the rise in fluorescence corresponding to the photochemical reaction alone (F_o to F_i) are not altered by the silicomolybdate, while the

Fig. 16.--Time course of chlorophyll a fluorescence yield in spinach chloroplasts with and without silicomolybdate and DCMU. Fluorescence was measured at 685 nm (half-band width, 6.6 nm). (A): ●——●, normal chloroplasts without any addition; o——o, 5 μM SiMo; ■——■, 100 μM SiMo (with and without 5 μM DCMU); Δ——Δ, normal chloroplasts with 5 μM DCMU; □——□, 5 μM SiMo and 5 μM DCMU. (B): ●——●, normal chloroplasts without any addition; o—o 25 μM SiMo; Δ——Δ, normal chloroplasts with 5 μM DCMU; □——□, 25 μM SiMo and 5 μM DCMU. Excitation, broad-band blue light (C.S. 4-96 and C.S. 3-73); [Chl], 70 μg in 2 ml buffer, 20 mM Tricine-KOH (pH 8.1), containing 100 mM KCl and 5 mM $MgCl_2$; chloroplasts dark-adapted before each measurement.

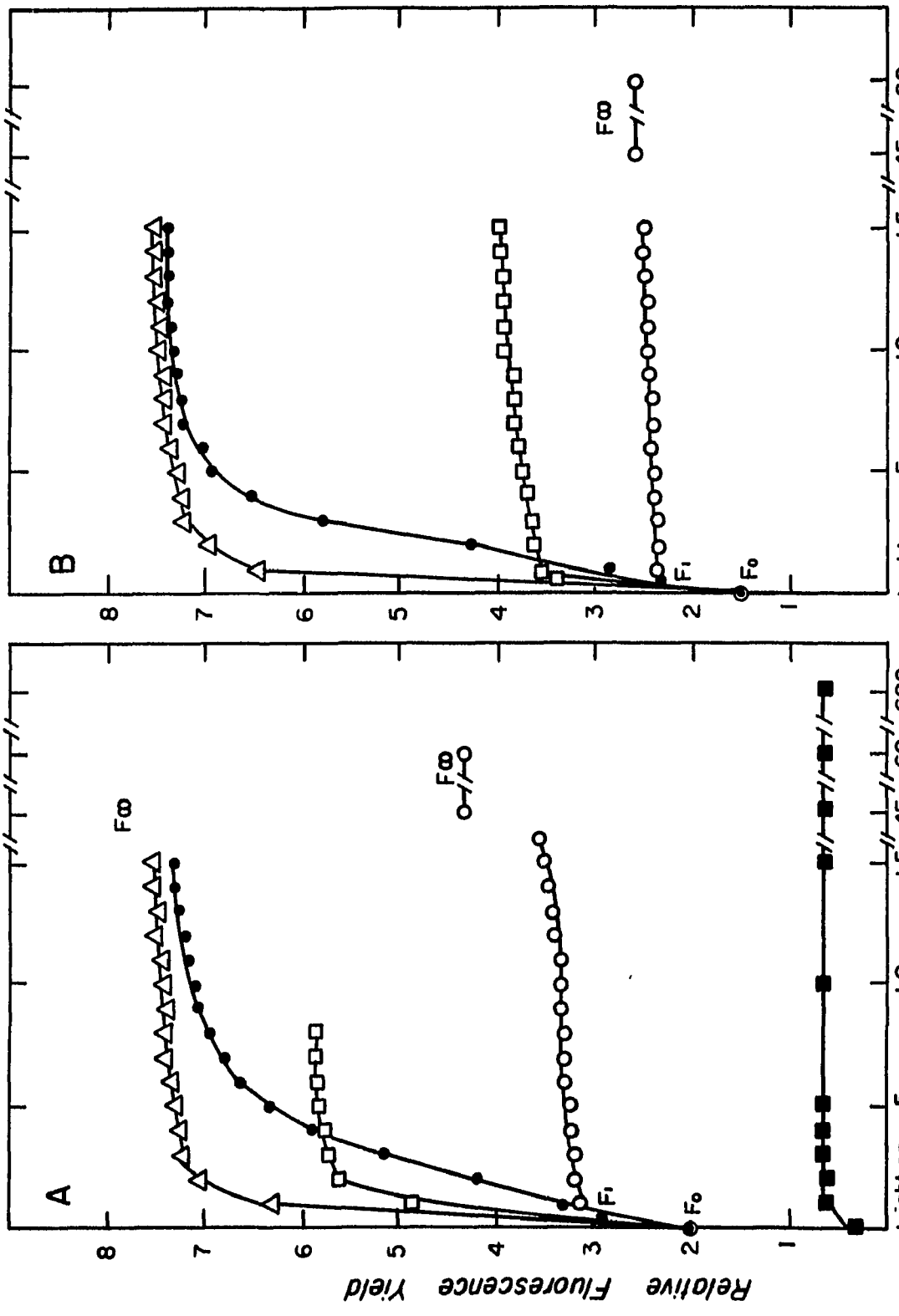

A
B
Fω
Fω
Fω
F₁
F₁
F₀
F₀
Light on
Light on
Relative Fluorescence Yield
Time of Illumination, (sec)
1
2
3
4
5
6
7
8
.5
1.5
45
60
600
10
15
45
60

TABLE 19

EFFECT OF SILICOMOLYBDATE AND SILICOTUNGSTATE ON CHLOROPHYLL *a* FLUORESCENCE *IN VITRO*

Additions	Relative Fluorescence Yield at 685 nm
None	53.5
100 µM SiMo	55.0
100 µM SiTu	54.0

Chlorophyll extracted from spinach chloroplasts with 80 percent acetone and subsequently centrifuged to remove non-chlorophyllous materials; 70 µg Chl in 2 ml of 80 percent acetone used as sample. Instrumental conditions as in legend of Fig. 16.

rise to F_∞ is much slower, and the maximum level as seen in the control chloroplasts is not even met when DCMU is added. Effects on fluorescence kinetics are shown for both 5 and 25 μM silicomolybdate additions to illustrate the different degrees of quenching of the variable fluorescence level. Percentage variable fluorescence of the total fluorescence may vary in different chloroplast preparations, as in the case here; therefore, it is mandatory to use appropriate controls for each measurement. As shown in Figure 17, a 1 μM concentration of silicomolybdate or silicotungstate does not significantly affect the fluorescence transient, while concentrations above 5 μM and below 25 μM do not affect the fluorescence at the O level but do have a large quenching at the P (F_∞) level, and at these concentrations, DCMU only partially restores the maximum level of fluorescence. Concentrations of silicomolybdate and silicotungstate above 25 μM greatly depress the O level of fluorescence. These observations parallel the quenching properties described for dinitrobenzene (119), although there are some differences noted here.

Lavorel and Joliot (120), using moderately quenching concentrations of dinitrobenzene, found that the sigmoidal O to P rise typical of normal chloroplasts changes to exponential with dinitrobenzene addition, and they have extended this data to support the connected units model for energy migration. Figure 18 shows an oscilloscope trace of Chl a fluorescence of normal and 25 μM silicomolybdate treated samples; the

Fig. 17.--Percentage quenching of chlorophyll _a_ fluorescence yield at F_o, and at $F_\infty - F_o$ (in the presence and absence of 5 μM DCMU) as a function of silicomolybdate concentration. To calculate percentage quenching of the variable fluorescence yield in the presence of SiMo plus DCMU, the constant fluorescence was assumed to be the same in the presence of DCMU as in the silicomolybdate treated chloroplasts. Conditions as indicated in the legend of Fig. 16.

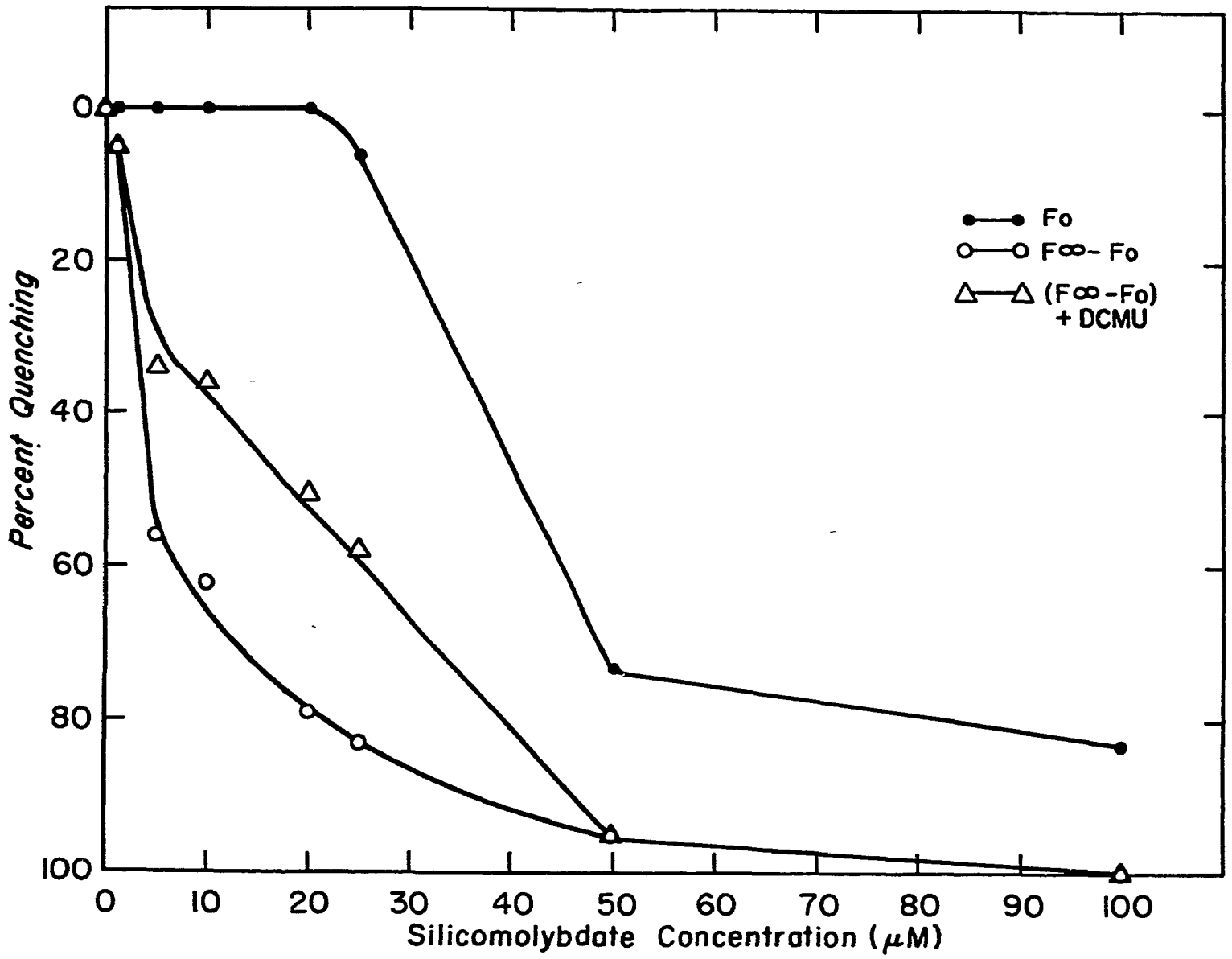
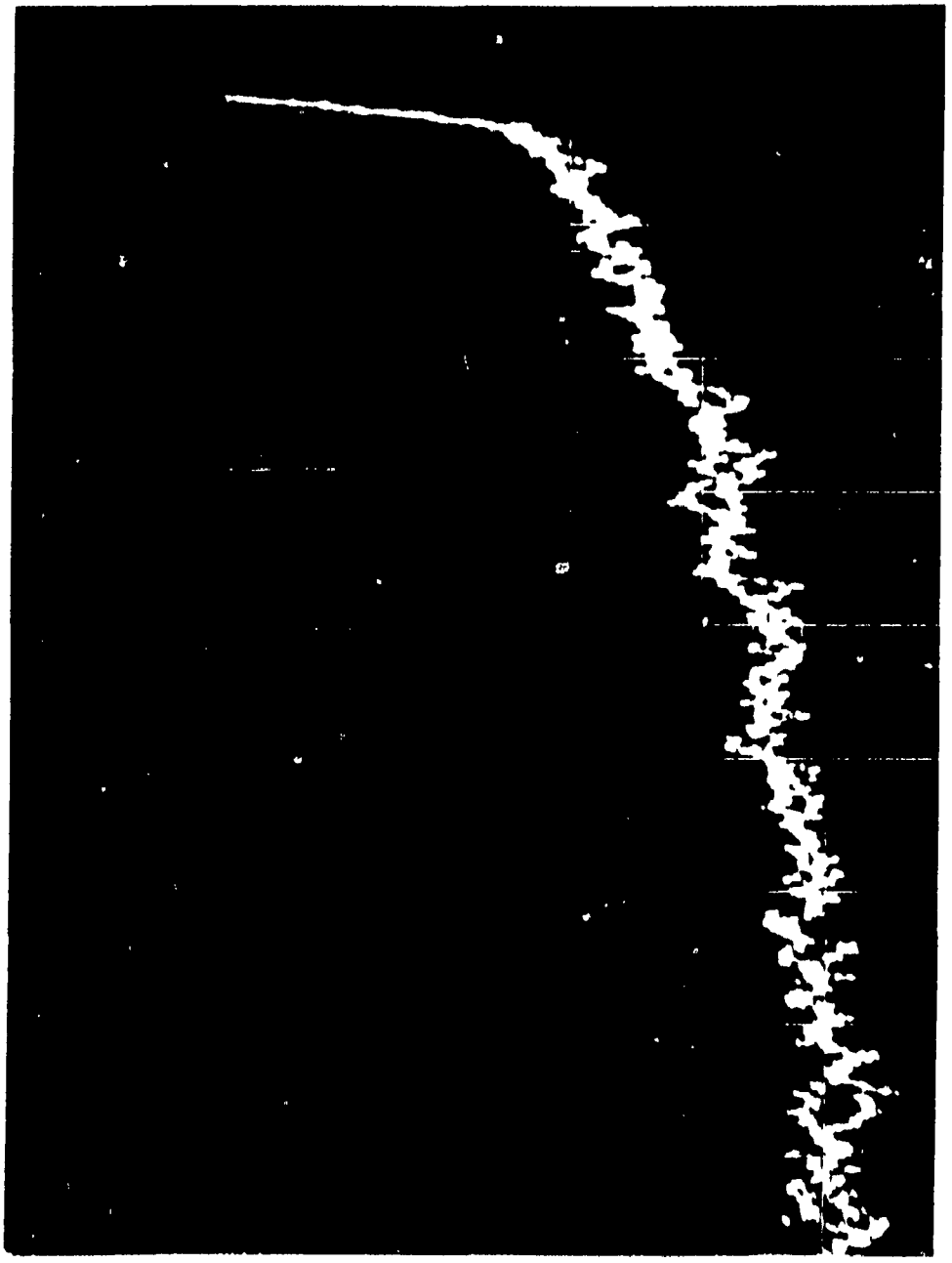

Percent Quenching
0
20
40
60
80
100
0
10
20
30
40
50
60
70
80
90
100
Silicomolybdate Concentration (μM)
Fo
F∞- Fo
(F∞-Fo) + DCMU

Fig. 18.--Time course of chlorophyll *a* fluorescence yield in spinach chloroplasts plus 25 μM silicomolybdate superimposed upon each other. Oscilloscope setting: 0.2 volts/division; 5 m sec/division. Conditions as in Fig. 15.

Fluorescence Yield



Time

photographic film was purposefully exposed twice to emphasize the fact that the two transients are virtually identical. Equally superimposable induction curves in the first 40 msec after onset of illumination were obtained with control chloroplasts compared with the same chloroplasts treated with 5 or 10 μM silicomolybdate (data not shown). The significant differences in the shapes of the two induction curves in the case of the dinitrobenzene treated chloroplasts are lacking here. (Whether this data presents new possibilities for alternative models for energy migration is not very clear to the author and, thus, will not be discussed further.)

Etienne and Lavergne (119), working with dinitrobenzene, separated the action of dinitrobenzene on the basis of concentration into a quencher of the photochemical (O → I) and thermal (I → P) phases of fluorescence. They found that the quenching was irreversible as far as the thermal rise was concerned but reversible with respect to the photochemical rise. We found that both phases were affected irreversibly with 100 μM silicomolybdate or silicotungstate, as indicated in Table 20, by incubating the chloroplasts with the silicocompounds for five minutes in the dark, followed by centrifugation and resuspension of the chloroplasts in the same volume of buffer.

Lastly, we looked briefly at the effect of cations on the degree of quenching by the silicomolybdate. Papageorgiou and Argoudelis (121) reported that high concentrations of

TABLE 20

IRREVERSIBILITY OF THE QUENCHING EFFECTS OF THE 100 μM SILICO- COMPOUNDS

Treatment	Additions	Relative Fluorescence at F_o	Relative Fluorescence at F_∞
I. Normal	None	33.5	97.5
	100μM SiMo	3.5	6.5
	100μM SiTu	4.0	7.5
II. Washed*	None	34.0	94.0
	100 μM SiMo	3.0	6.5
	100 μM SiTu	4.5	8.0

Conditions as in legend of Fig. 15 with 100 μM additions of the silico- compounds given to the control chloroplasts (I.) as indicated above.

*Washing was done by preincubating the chloroplasts, suspended to 35 μg Chl/ml in the usual reaction mixture, with 100 μM SiMo or SiTu for 5 minutes in the dark at 0°C, followed by centrifugation at 1000 × g for 10 minutes, and resuspension of the chloroplasts in the same volume of buffer. The control was washed in the same manner without any additions.

divalent cations increased the quenching capabilities of dinitrobenzene by enhancing the hydrophobicity of the membrane and thereby making chlorophyll more accessible to the quencher. This appears also to be the case with silicomolybdate and silicotungstate, although lesser concentrations of divalent cations and significant amounts of monovalent cations can be more effective than the extremely high concentrations of $MgCl_2$ Papageorgiou and Argoudelis used in quenching Chl *a* fluorescence both at the constant and variable levels, as indicated in Table 21.

It is obvious that the apparent chemical quenching of these silico-compounds at high concentrations makes it impossible to compare the effects of DCMU on leveling, within short time, of the initial rates of oxygen evolution. From parallel oxygen measurements, if these compounds were not acting as direct chemical quenchers, one might expect fluorescence to rise when the original 100 μM silicomolybdate becomes limiting, i.e., within two minutes after its addition. Also the fluorescence level remains low after DCMU addition when we have shown that there is a delayed sensitivity to DCMU in oxygen evolution. Indeed, these contradictions existing between the fluorescence and oxygen data simply point out that these compounds are not acting as simple acceptors such as ferricyanide but have the additional effect of directly quenching fluorescence.

TABLE 21

EFFECT OF SALTS ON THE QUENCHING OF CHLOROPHYLL *a* FLUORESCENCE BY 100 μM SILICOMOLYBDATE

Reaction Mixture	Additions	Relative Fluorescence at F_o	Relative Fluorescence at F_∞
20 mM Tricine, 100 mM KCl, 5 mM $MgCl_2$ (usual reaction mixture)	None	18	72
	100 μM SiMo	3	6
2 mM Tricine	None	14.5	61.5
	100 μM SiMo	7	16
2 mM Tricine, 0.5 M $MgCl_2$	None	44	60
	100 μM SiMo	5	5
2 mM Tricine, 50 mM $MgCl_2$	None	16	88
	100 μM SiMo	10	19

Conditions as in legend of Fig. 16. Additions and reaction mixtures as indicated.

3. Delayed light emission

Table 22 shows the effect of silicomolybdate on quenching the intensities of the slow component (100 msec after cessation of illumination) of delayed light emission from spinach chloroplasts. If silicomolybdate does in fact accept electrons directly from Q, then the electron donor to PS II, Z, can become oxidized and accumulate charges, but Q will be oxidized very quickly by the silicomolybdate. The elimination or quenching of the slow component of delayed light with the addition of silicomolybdate can be interpreted to be due to the inhibition of the back reaction between oxidized donor Z^+ and reduced acceptor Q^-, as has been the interpretation with hydroxylamine and DCMU treated chloroplasts (122-124). In fact, this experiment will provide the role of Q in delayed light emission as hydroxylamine data does for Z.

One hundred μM silicomolybdate eliminates delayed light emission, and DCMU addition has no effect on the delayed light. As the concentration of silicomolybdate is decreased, the percent quenching of delayed light emission is likewise decreased, and finally at one μM concentrations, the intensity of delayed light is not appreciably less than that of the control. DCMU added after silicomolybdate appears to slightly increase the intensity of delayed light emission that has been quenched by concentrations of silicomolybdate that have an

TABLE 22

EFFECT OF SILICOMOLYBDATE ON DELAYED LIGHT EMISSION AT 100 msec FOLLOWING CESSATION OF ILLUMINATION

Silicomolybdate Concentration (μM)	% Quenching of Delayed Light	% Quenching of DLE with DCMU added
100	100	100
50	89	89
25	85	75
10	75	62
5	55	51
1	5	3

One ml of sample, containing 35 μg Chl in reaction mixture as described in Table 18, was illuminated for 15 sec with blue light (Corning filter: C.S. 4-96). The intensity of the exciting light was ∿10 K ergs cm^{-2} sec^{-1}. Delayed light emission was measured ∿100 msec after cessation of the illumination. Additions were made in the dark; DCMU, in 5 μM concentrations, was added to the chloroplasts in the dark after silicomolybdate addition.

effect only on the variable yield of fluorescence. The above results (in Table 22) thus provide an experimental suggestion for Q to be indeed playing a role in delayed light emission, as the hydroxylamine data (122-124) did for Z. Furthermore, the percentage quenching of delayed light emission by silicomolybdate may reflect the average rate of silicomolybdate reduction during illumination prior to delayed light measurement.

D. Discussion

Three lines of evidence presented in this communication indicate that both silicomolybdate and silicotungstate can act as direct electron acceptors from PS II before the DCMU site of inhibition: (1) silicomolybdate and silicotungstate mediated oxygen evolution in the presence of DCMU, but in the absence of additional electron acceptors, (2) the effects of the compounds on Chl a fluorescence induction curves, and (3) the effects of the compounds on delayed light emission.

The first and most direct indication is that both silicomolybdate and silicotungstate can accept electrons flowing from water in the absence of ferricyanide and that this electron transport is DCMU-insensitive for a limited period of time. We have shown that if the silicomolybdate or silicotungstate mediated oxygen evolution in the presence or absence of ferricyanide is to be used, one must necessarily be concerned with initial rates only, as the incipient insensitivity to DCMU is only short-lived. The leveling of oxygen-evolving rates in

the absence of DCMU at concentrations less than 50 μM appears to be due to limiting concentrations of the acceptor, as further additions of the silico-compounds repeatedly restore the original rate. At higher concentrations of silicomolybdate and silicotungstate, this leveling probably also reflects the interference of these chemicals in the transfer of excitation energy among the chlorophyll molecules as witnessed by the dramatic quenching effects on "constant" fluorescence, manifested the moment the chloroplasts are illuminated, while the decrease in oxygen evolution is only seen after many seconds.

Fluorescence data also support the suggestion that these silico-compounds accept electrons directly from Q. In these studies, one must look only at the chlorophyll a induction curves of samples to which only low concentrations of silico-compounds have been added, as high concentrations ($\geq$ 50 μM) greatly suppress the constant (F_o) level of fluorescence, apparently acting as direct chemical quenchers of fluorescence. The slower kinetics of the variable rise in fluorescence and the proportional quenching of the maximum fluorescence level with increasing silicomolybdate concentrations suggest that silicomolybdate is behaving as an electron acceptor. DCMU somewhat decreases the quenching of F_∞, but not sufficiently to return the fluorescence level to that seen with DCMU treated control chloroplasts. These data raise two questions if one

is to compare these findings with those for oxygen evolution. First, we find that oxygen evolution with low concentrations of silicomolybdate levels within a short time, suggesting as mentioned above, that all silicomolybdate is reduced; it is not clearly understood why the fluorescence rise to F_∞ is not met after several minutes of illumination, as might be expected if one is to draw an analogy with the effect of other non-autoxidizable acceptors; eventually, when all the acceptor is reduced, F_∞ should be the same as that in the control sample. One very simple explanation might be that fluorescence is a much more sensitive measure of electron flow than is the use of the concentration electrode, and very low levels of oxygen evolution may be read as zero, while one can still see changes in fluorescence. Secondly, the fact that DCMU can somewhat decrease the quenching of fluorescence by silicomolybdate may possibly show that although electrons preferentially flow to silicomolybdate, as the compound becomes reduced, there may be additional electron flow diverted to the "A" pool and addition of DCMU blocks this electron transport.

Delayed light is one additional, independent confirmation of the action of silicomolybdate as electron acceptor from Q. Quenching of delayed light emission by silicomolybdate (and silicotungstate, data not presented here) is a reflection of the fact that these silico-compounds keep Q in the oxidized

state so that there can be no back reaction between reduced Q and oxidized Z to produce delayed light. The illumination time of the chlorplasts in the presence of silicomolybdate was 15 seconds, so in very low concentration ranges of silicomolybdate, it is conceivable that the lessened quenching seen may be due to the fact that most of the acceptor is in this reduced state. DCMU again has an effect comparable to that seen in fluorescence, relieving some of the quenching of delayed light emission by silicomolybdate at concentrations lower than 50 μM. It is interesting to note that this effect is opposite to that seen in our control chloroplasts, where DCMU decreases the intensity of the slow-component of delayed light.

The fact that silicomolybdate or silicotungstate can support oxygen evolution in the absence of any (additional) electron acceptor suggests to us that the previously proposed mode of its action, i.e., altering membrane properties such that ferricyanide can accept electrons directly from Q, cannot be the situation, at least, not in all cases. Both silicomolybdate and silicotungstate are extremely large molecules, and therefore, probably non-penetrating. As shown, cations effectively increase the quenching of chlorophyll a fluorescence by these compounds, which suggests that an increased hydrophobicity of the membrane permits better accessibility to the chlorophyll. Preillumination of acceptor-less chloroplasts

prior to addition of the silicomolybdate results in a three-fold greater rate of oxygen evolution as compared to rates when silicomolybdate is added to dark-adapted chloroplasts, suggestive of a light-induced conformational change in the membrane that makes the silico-compounds more accessible to Q.

CHAPTER V

SUMMARY AND CONCLUDING REMARKS

This thesis deals with a study of the functioning and localization of the photosystem II (PS II) components in the thylakoid membrane through use of immunochemical methods (Chapter II), membrane fixatives (Chapter III), and the electron acceptors silicomolybdate and silicotungstate (Chapter IV).

A. Use of Antisera to Photosystem II

We have prepared antisera against PS II particles and an extract from repeatedly frozen and thawed chloroplasts which specifically (but only partially) inhibit the reactions involved with oxygen evolution (Tables 2, 3, and 5). PS II particles were used as an antigenic source as they were enriched in the oxygen-evolving intermediate and lacked the immunogenic PS I components (7, 8, 9). Several other chloroplast-derived preparations were also made, based on suggestions in the literature that these treatments might release some component involved with oxygen evolution (41, 52, 53); one of these simpler antigens, a soluble extract from repeatedly frozen and thawed chloroplasts, induced

production of antibodies which inhibited oxygen evolution in the same manner as did the PS II particle antiserum. The level of inhibition is quite low, approximately 15 percent, as has been the case with other antisera inhibiting PS II reactions [found independently by others (20, 28, 29) with different antigens]. This may be due to a limited accessibility of the antigen in the chloroplasts to the antibodies and/or to the weak affinity of the antibody-antigen interactions (assuming that the antigen has been somewhat altered from its native state during extraction, and, therefore, the antibody elicited against it will not strongly bind the native chloroplast components).

With regard to the accessibility question, we looked at agglutination conditions that are common for both types of antisera in broken chloroplasts and system II particles (Tables 8, 9). This type of study becomes quite complicated by the fact that both PS II particles and the soluble extract contained several immunogenic substances, each of which elicited antibody production; this is quite unlike the simple system where Berzborn (7), using antibodies prepared against purified ferredoxin-NADP-reductase, was able to definitively state that this enzyme is localized in a cavity on the chloroplast thylakoid surface. Direct agglutination of chloroplast lamellae was seen with addition of either type of antiserum, indicative of an outside localization of some antigenic substance to which an antibody has been made. Additional

agglutination is seen when anti-rabbit immunoglobulin is added to chloroplasts incubated with either antiserum, indicative of yet another common site, located within a cavity, at least partly accessible to an antibody but not available for agglutination unless a bivalent anti-immunoglobulin bridges two antibody molecules already attached to chloroplast thylakoids. The same indirect agglutination is seen with photosystem II particles incubated with the extract antiserum or PS II particle antiserum (although in the latter case, one must subtract the direct agglutination seen only with the particle antiserum). Under the conditions used, at least some of the chloroplast sites are partly exposed to allow the antibody to act with them, although it is impossible to distinguish between the site involved in direct and indirect agglutination of chloroplasts.

The question now raised is what the inhibitory antibody is actually binding to, i.e., what the antigenic component is that elicited such an antibody. If we rely on the information provided by measurements of the electric field (125-126) which is generated by a charge separation across a membrane, which, in turn, requires that the positive charge accumulation be on the inner face of the membrane, then the oxygen-evolving intermediate must be localized deep inside the membrane. Similarly, the work of Fowler and Kok (22) which shows that protons released in water oxidation are

deposited inside the membrane causes one to question whether the oxygen-evolving intermediate should be even partially accessible to antibodies. Is the antibody against the oxygen-evolving intermediate or something complexed to it, which being altered by antibody binding, in turn, affects the activity of the intermediate itself? Is the oxygen-evolving component a transmembrane protein, similar to those described by Lenard and Singer (34), with the charge-accumulating and proton-releasing portion of the molecule being situated at the far inside end? Is the effect of the antiserum simply a reflection of membrane damage secondarily effecting inhibition of oxygen evolution? Our attempts to answer these questions are described below.

Analysis of the soluble extract used in immunization neither negated nor established the presence of a "manganoprotein," the suspected oxygen-evolving intermediate (see 38); EPR measurements (Figure 5) suggest that the extract contains, in expected quantities, manganese complexed to some substance, but we cannot assume that it is the oxygen-evolving intermediate. Isolation of a fraction enriched in this signal might be fruitful. Thus, from this line of investigation, one cannot answer the question as to whether the antibody is binding to the oxygen-evolving intermediate or something else. However, one can rule out the possibility that if the

"complex" idea is considered, the antibody is not against chlorophyll in that complex, as the extract does not contain immunogenic quantities of that pigment.

B. Use of Membrane Fixatives

We then directed ourselves to the question asked above of whether the antiserum, as well as other inhibitors of oxygen evolution, might simply act by disrupting membrane structure which secondarily affects a sensitive component in the membrane, i.e., the oxygen-evolving intermediate. This component has generally been thought to have a strict structural requirement in the membrane, as oxygen evolution is the least resistant chloroplast reaction to a number of treatments. We have shown that fixation of chloroplasts with glutaraldehyde relieves 50 percent of the inhibitory action of Tris-washing (0.8 M, pH 8.0), mild heating (50°C, 5 min.) and $Cd(NO_3)_2$ (0.5 mM) treatment on unfixed chloroplasts (Table 10). Glutaraldehyde fixation provides different degrees of protection to treatment with chaotropes (NaSCN, $NaClO_4$, guanidine and urea), with the most potent chaotrope (NaSCN) being nearly as effective in inhibiting oxygen evolution in fixed chloroplasts as in unfixed (Table 11). The impermeable inhibitors trypsin and the two antisera described above do not inhibit oxygen evolution in fixed chloroplasts in contrast to unfixed (Tables 12, 15).

We interpret our data to suggest that at least 50 percent (if not all) of the inhibition seen by several inhibitors of oxygen evolution [Tris-washing, heating and Cd $(NO_3)_2$] is due to membrane disturbance primarily rather than a direct effect on the oxygen-evolving intermediate. Chaotropes also act first through membrane perturbation and then on oxygen evolution, with NaSCN being effective in fixed chloroplasts because of its potency in disrupting water structure at a local level.

One must consider the following facts: (1) chaotropes can certainly be considered to be membrane disruptive (98) and they still inhibit oxygen evolution in fixed chloroplasts, (2) electron micrographs of trypsin treated chloroplasts, (2) electron micrographs of trypsin treated chloroplasts show no general breakdown in membrane structure (31), and (3) very mild proteolysis of animal membranes shows gross relocalization within these membranes of certain types of glycoproteins, called lectins, whose surface localization is easily detectible because of their hemagglutinating properties (35). Based on the above information, we would like to believe that trypsin or antibodies do not cause large destructive changes (stopped by glutaraldehyde fixation) in the thylakoid which secondarily affect a sensitive component buried in the membrane that is involved with oxygen evolution. Rather, a more probable explanation is that addition of trypsin or antisera to unfixed chloroplasts results in macroconformational changes

in the membrane which reposition specific sites in the oxygen-evolving intermediate (or something complexed to it) so that they are now accessible to those impermeable inhibitors (and glutaraldehyde fixation does not allow this change).

With this in mind, it is now possible to reconcile the seeming contradiction (see Trebst, 4) existing between the ability of the impermeable trypsin (30, 31) and the several antisera (26 - 29) to inhibit oxygen evolution, with the data on the creation of an electric field across the thylakoid (125-126) and proton release in water oxidation (22), both of which require an internal positioning of the oxygen-evolving intermediate. Although this component can be thought to be inside the thylakoid now, one cannot definitely state that the oxygen-evolving intermediate itself is being directly affected by the trypsin or antisera, rather than something in complex with it. Antibodies can be inhibitory of certain enzymes by binding either at the active site or at some other site in the molecule, because of the conformational change occurring in the enzyme (127). If the oxygen-evolving intermediate is intimately complexed to some other component, e.g., the pigment-protein complex of the reaction center, then antibody binding to the latter may possibly affect oxygen-evolving capacity; the

rather indirectness involved may be an additional explanation for the low level of inhibition seen by antibodies.

C. Use of Silicomolybdate and Silicotungstate

Lastly, we have examined the availability of Q, the primary electron acceptor of PS II, to certain artificial electron acceptors. It appears that silicomolybdate and silicotungstate do not modify membrane organization in such a way as to allow the hydrophilic acceptor, ferricyanide, to accept electrons directly from Q, as suggested previously (16, 17). These two silico- compounds themselves accept electrons directly from Q, as we show by data on electron flow, chlorophyll a fluorescence transients, and delayed light emission. Either of these compounds can accept electrons directly from Q in a DCMU insensitive electron transport; however, the DCMU insensitivity is only short-lived, so initial rates must be used exclusively (Figure 15). High concentrations of these silico- compounds act as direct chemical quenchers of chlorophyll a fluorescence, but lower concentrations which also mediate oxygen evolution affect only the variable component of fluorescence in a manner suggestive of its electron accepting capabilities (Figures 16, 17); measurements of delayed light emission confirm the conclusions made from the fluorescence data and also show the role of Q in delayed light emission as hydroxylamine data (122-124) have shown the role of Z, the electron donor of PS II (Table 22).

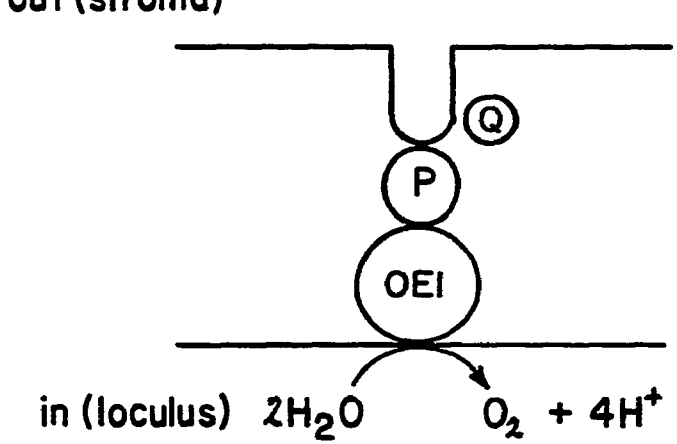
As it is thought that Q is not exposed to the hydrophilic region of the membrane although being close to the thylakoid exterior (18, 19), and these very large inorganic molecules cannot penetrate the membrane, there is probably some membrane alteration by simple addition of these compounds to the membrane, making them more accessible to Q; the availability can be increased by additional light-induced conformational changes in the chloroplasts (Table 17, Figure 14).

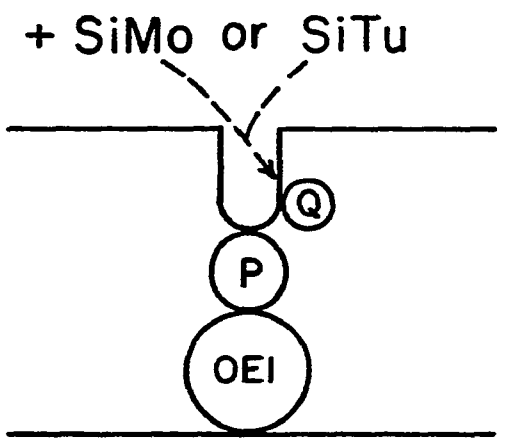
D. Working Hypothesis

For the sake of future discussions, we present a tentative working model (Figure 19) for the organization of photosystem II components in the membrane, based on the data presented in this thesis as well as a synthesis of research done in other laboratories. At least the charge accumulating and proton releasing portion of the oxygen-evolving intermediate must be localized on the inner surface of the thylakoid membrane to concur with the data of Fowler and Kok (22, 126), and Witt and Zickler (125). Similarly, to allow a separation of charges across the membrane to generate an electric field, Q, the primary electron acceptor, must be localized on the opposite side of the membrane, although not necessarily exposed to the chloroplast matrix (18, 19). To incorporate both the data of Arntzen et al. (21) and

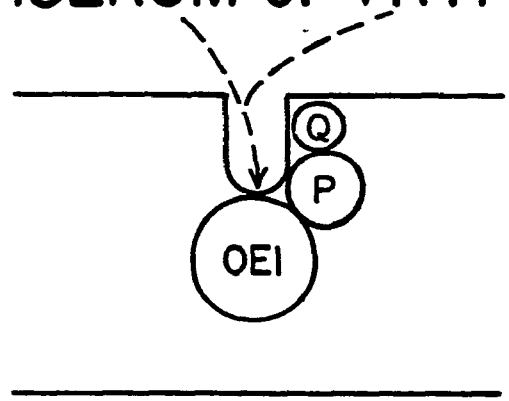
Fig. 19.--Tentative working model for the localization of photosystem II components in the thylakoid membrane. Left top, unfixed chloroplasts membrane (normal condition) or glutaraldehyde fixed thylakoid to which PS II particle antiserum, antiserum against frozen and thawed chloroplasts, or trypsin has been added. Right top, unfixed chloroplast thylakoid with antiserum or trypsin. Left bottom, chloroplast thylakoid with addition of silicomolybdate (SiMo) or silicotungstate (SiTu) in the dark. Right bottom, chloroplast thylakoid with addition of silicomolybdate or silicotungstate in light. In the models, OEI represents oxygen-evolving intermediate(s); P, reaction center of PS II; and Q, the primary electron acceptor of PS II (see text for explanation).

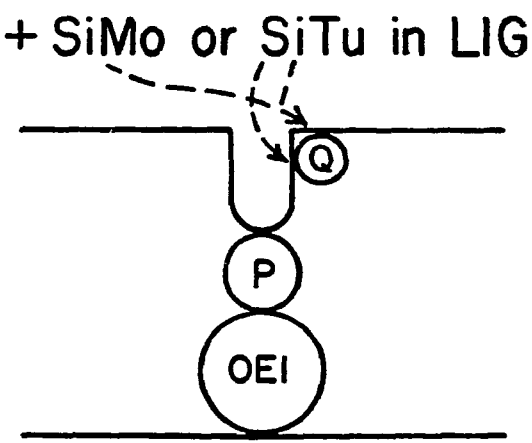
UNFIXED
or
FIXED + ANTISERUM / TRYPSIN





UNFIXED
+
ANTISERUM or TRYPSIN





Radunz et al. (20) the latter of which shows that antibodies to chlorophyll a cause only an indirect agglutination of chloroplast lamellae, P_{680} is tentatively placed close to the surface of the thylakoid membrane but localized at the bottom of a cavity.

Our data support the aforementioned localizations of the oxygen-evolving intermediate and Q. The fact that antibodies and trypsin, both being impermeable, are able to impair water oxidation can be construed as being due to a relocalization of the attacked site upon addition of antisera or trypsin to the chloroplasts; the reasons for such a proposal are given in detail above (Chapter III). We accept the necessity of the proton releasing and charge accumulating portion of the oxygen-evolving intermediate being on the inner side of the membrane and feel that it is unlikely, considering the distance the molecule must transverse to be in contact with impermeant modifiers, that either trypsin or the antisera are inhibiting by direct attack at the active site of the intermediate; the possibility is greater that these molecules are binding a "closer" site on the oxygen-evolving intermediate or even some part of the reaction center complex with which it is in close association, thus inhibiting oxygen evolution through adverse conformational change in the molecule.

On the basis of data showing the ability of silicomolybdate and silicotungstate to accept electrons directly from Q

and their increased ability to do so after a light-induced conformational change in the membrane, we corroborate previous suggestions (18, 19) that the primary electron acceptor is localized close to the surface of the thylakoid membrane.

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VITA

Barbara Ann Zilinskas was born on September 21, 1947 in Waltham, Massachusetts. She earned her high school diploma in 1965 from Waltham High School and obtained a Bachelor of Arts degree in Biology (magna cum laude) in 1969 from State College at Framingham (Massachusetts). From 1969 through 1972, she was a National Aeronautics and Space Administration fellow at the University of Illinois, receiving a Master of Science degree in Botany in 1970. She took leave of her graduate studies from September, 1972 until June of the following year while assisting in anthropological research in Peru. After that time, she served as a part time research and teaching assistant for the Botany department.

She is a member of the Society of Sigma Xi and is co-author of the following publications:

1. "Antibodies Against an Intermediate on the Water Side of Photosystem II of Photosynthesis." FEBS Letters, 25, 143-146 (1972).

2. "Chlorophyll Fluorescence Characteristics of System I Chlorophyll a Protein Complex and System II Particles at Room and Liquid Nitrogen Temperatures." Plant and Cell Physiology, 13, 81-91 (1972).

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