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Stabilization by Glutaraldehyde Fixation of Chloroplast Membranes Against Inhibitors of Oxygen Evolution

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With 4 figures

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Summary

Stabilization, by glutaraldehyde fixation, of thylakoid membranes against inhibitors of oxygen evolution was investigated. (1) We show that glutaraldehyde fixation of chloroplasts relieves approximately 50 % of the inhibitory action of Tris-washing, mild heating and $Cd(NO_3)_2$ treatment on unfixed chloroplasts. At least 50 % (if not all) of the inhibition of oxygen evolution by these treatments is therefore suggested to be due primarily to changes in the thylakoid membrane rather than to a direct effect on electron flow. (2) Glutaraldehyde fixation provides different degrees of protection against treatment with chaotropes (NaSCN, $NaClO_4$, guanidine and urea), with the most potent chaotrope (NaSCN) being nearly as effective in inhibiting Hill reaction in fixed as in unfixed chloroplasts. These agents also act primarily through membrane perturbation; NaSCN is a very potent inhibitor, perhaps, because it disrupts water structure at a local level. (3) The impermeable inhibitor trypsin does not inhibit Hill reaction in fixed in contrast to unfixed chloroplasts.

These data are consistent with the idea that the oxygen-evolving intermediate is located in the hydrophobic interior of the membrane.

Key words: Chloroplast thylakoids, Glutaraldehyde fixation, O_2 evolution.

Abbreviations: DCPIP, dichlorophenol indophenol; AS, 12-(9-anthroyl)-stearic acid; DPC, diphenylcarbazide; DCMU, 3-(3′, 4′-dichlorophenyl)-1, 1-dimethylurea; PMS, phenazine methosulfate.

Introduction

Oxygen evolution is inhibited when chloroplasts are heated (KATOH and SAN PIETRO, 1968), aged (MARGULIES and JAGENDORF, 1960), or treated with ultraviolet light (JONES and KOK, 1966), chaotropic agents (CHENIAE and MARTIN, 1966; LOZIER et al., 1971) or proteases (HOMANN, 1966; MANTAI, 1969). Whether the inhibition of oxygen evolution is secondary to changes in the thylakoid membrane is not known. It has been suggested (ARNTZEN et al., 1974; GIAQUINTA et al., 1974) that a high degree of structural integrity of the thylakoid membrane is needed to support a transmembrane electron flow.

MURAKAMI and PACKER (1970) have shown that glutaraldehyde fixed chloroplasts cannot undergo any configurational or large structural changes, although micro-conformational changes may occur. In this paper we present a comparison of effects of permeable and impermeable inhibitors of oxygen evolution on Hill reaction (DCPIP reduction) in fixed and unfixed chloroplasts, in order to understand if the inhibition of O_2 evolution is secondary to gross changes in the thylakoid membrane. Our data suggest that most inhibitors act primarily through changes in the thylakoid membrane and are consistent with the idea that the oxygen-evolving system is located in the hydrophobic interior of the membrane.

Materials and Methods

Chloroplast Preparation

Broken, Class II, chloroplasts were isolated from market spinach *(Spinacia oleracea)* as described earlier (BRAUN and GOVINDJEE, 1972) with minor modifications: phosphate, instead of Tris, buffer was used in the isolation procedure, and $MgCl_2$ was omitted from the homogenizing medium.

Fixation

Chloroplasts were washed twice in the homogenization buffer without sucrose and then suspended in it (to be referred to as washed chloroplasts). Purified glutaraldehyde (Ladd Research Industries, Inc.) was diluted to a concentration of 10 % and added to these washed chloroplasts (150 μg chlorophyll/ml buffer), usually to a final concentration of 1 %. This suspension was stirred at 0 °C in the dark for 5 minutes and then centrifugend at 1500 xg for 10 minutes. The pellet was washed twice and finally resuspended in the homogenizing medium. Formaldehyde was prepared from paraformaldehyde according to PARK (1971) but 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, with the time of incubation extended to 10 minutes.

Acetylation

Washed chloroplasts (100 μg chlorophyll per ml of 10 mM Tris [or Tricine] buffer, pH 8.0, containing 10 mM NaCl) were acetylated by incubating them 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.

Treatments with Permeable Inhibitors

Tris-washing of chloroplasts was done according to YAMASHITA and BUTLER (1968 a) with the incubation in 0.8 M Tris extended to 30 minutes. Fixed and unfixed chloroplasts were heat treated at 50 °C for 5 minutes, as described by YAMASHITA and BUTLER (1968 b). Oxygen evolution inhibition by cadium nitrate was done according to BAZZAZ and GOVINDJEE (1974); these chloroplasts were isolated and fixed in HEPES buffer.

The method of LOZIER et al. (1971) 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 NaSCN, 0.5 M $NaClO_4$, 0.8 M guanidine or 1.0 M urea, followed by washing and resuspension in the homogenization medium.

Treatment with a Non-Permeable Inibitor

Trypsin treatment was done as described by Selman and Bannister (1971); fixation of the thylakoid membranes was done after the chloroplasts had equilibrated in the 10 mM Tris-Cl buffer containing 10 mM NaCl, the same condition which was used when normal chloroplasts were trypsin treated.

Measurements

DCPIP reduction was measured as described earlier (Braun and Govindjee, 1974). Chlorophyll *a* fluorescence was measured according to Shimony et al. (1967) and Papageorgiou and Govindjee (1968), AS fluorescence changes according to Van der Meulen and Govindjee (1974 a), light-induced 90° scattering changes as described by Murakami and Packer (1970) and Van der Meulen and Govindjee (1974 b), proton pumping according to Dilley (1966), and chlorophyll concentration as described by MacKinney (1941).

Results

Tris-washing, Heating, and Cadmium Nitrate Treatments

Table 1 shows rates of DCPIP photoreduction (primarily a PS II reaction under the conditions used) of fixed and unfixed chloroplasts treated with 0.8 M Tris-Cl, pH 8.0, and 0.5 mM $Cd(NO_3)_2$, or heated to 50 °C for 5 minutes. In the case of control chloroplasts, as has been originally shown, each of these treatments severely decreases rates of oxygen evolution; DPC, an artificial electron donor which promotes a DCMU-sensitive electron flow through PS II and bypasses the site of inhibition imposed by these treatments (Vernon and Shaw, 1969), almost completely restores electron flow. (Although we are measuring Hill activity as DCPIP reduction, we are actually referring to reactions prior to the site of DPC donation, i. e., oxygen evolution, and reference to inhibition of oxygen evolution or of Hill activity refers to electron transport between water and the DPC donation site.)

Fixation of chloroplasts with glutaraldehyde alone reduces the rate of Hill reaction shown here and elsewhere (Hallier and Park, 1969 b; Park, 1971). The higher the concentration of glutaraldehyde, the lesser the electron transport rate. (Apparently, glutaraldehyde fixation has multiple inhibitory sites, at least one before and one after the site of DPC donation [Table 1], but the action of «oxygen evolution inhibitors» and not glutaraldehyde fixation was the subject of this study, so this was not investigated further.) Fixation with 0.5 % glutaraldehyde, sufficient to completely fix chloroplasts (see below), protects (see column [B], Table 1) against inhibition of Hill reaction by Tris-washing, heating or $Cd(NO_3)_2$, as percent inhibition in fixed chloroplasts is $\sim$ 50 % that of the unfixed; this loss of electron flow in fixed chloroplasts is also restorable with DPC. If higher (1 % or 2 %; column [A], Table 1) glutaraldehyde concentrations are used, treatment with inhibitors similarly eliminates only $\sim$ 50 % of the electron flow despite the originally much lower rate of electron transport. This suggests a constant protective ability of glutaraldehyde, independent of electron flow.

Table 1: Effect of Tris-washing, heating and cadmium nitrate on electron flow in fixed and unfixed chloroplasts.

Chloroplast treatment	Addition	(A) μmoles $DCPIPH_2$ $(mg\ Chl \cdot hr)^{-1}$	(A) Inhibition %	(B) μmoles $DCPIPH_2$ $(mg\ Chl \cdot hr)^{-1}$	(B) Inhibition %
Unfixed	None	86.3	. . .	98.1	. . .
Unfixed	DPC	131.2	. . .	142.8	. . .
Unfixed, Tris-washed	None	0	100	0	100
Unfixed, Tris-washed	DPC	126.0	4	133.8	6
Unfixed, 50 °C, 5 min	None	6.1	93	8.5	91
Unfixed, 50 °C, 5 min	DPC	102.5	14	124.2	13
Unfixed, 0.5 mM $Cd(NO_3)_2$	None	0	100	0	100
Fixed	None	19.6	. . .	36.8	. . .
Fixed	DPC	33.0	. . .	61.7	. . .
Fixed, Tris-washed	None	10.4	47	18.3	50
Fixed, Tris-washed	DPC	34.7	0	58.4	5
Fixed, 50 °C, 5 min	None	12.8	34	23.3	37
Fixed, 50 °C, 5 min	DPC	33.4	0	59.1	4
Fixed, 0.5 mM $Cd(NO_3)_2$	None	8.8	55	17.7	52

Two ml 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 the 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. (A) Rates represent average of several chloroplast preparations and fixations in 2.0, 1.0, and 0.5 percent of glutaraldehyde. (B) Rates represent an average of measurements for chloroplasts fixed in 0.5 percent of glutaraldehyde.

Characterization of Fixed Membranes

Are all or only 50 % of the chloroplast membranes fixed in our system? Several independent methods show that all chloroplasts are fixed when treated as described in Methods.

(1) Suspension of unfixed chloroplasts in distilled water results in an osmotic shock and breakage of the chloroplast vesicles, while the fixed chloroplasts remained unchanged, as observed with a light microscope.

(2) Figure 1 shows that 0.5 % glutaraldehyde treatment of chloroplasts (suspended to ~ 150 μg Chl/ml) is sufficient to stop these osmotic, configurational changes. In this experiment, osmotic and structural changes in chloroplasts are inferred from percent changes in the ratio of chloroplast absorbance at 680 nm and 546 nm (under conditions when scattered light is included in absorbance measurements). Fixed chlo-

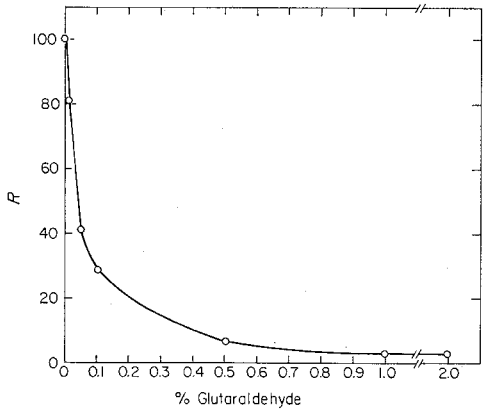

Fig. 1: $R \left[\frac{[A680/A546 \text{ (water)} - A680/A546 \text{ (buffer)}] \text{ 0–2 \% glutaraldehyde}}{[A680/A546 \text{ (water)} - A680/A546 \text{ (buffer)}] \text{ 0 \% glutaraldehyde}} \right] \times 100$ of chloroplasts incubated in various concentrations of glutaraldehyde. Chloroplasts containing 30 μg chlorophyll were placed in a 3 ml volume of 50 mM phosphate buffer or distilled water, and optical density (A) was read in a Cary-14 spectrophotometer without transmission scattering attachment or an integrating sphere.

roplasts, unable to undergo osmotic changes, show similar A 680/A 546 ratios when suspended in distilled water or in 50 mM phosphate buffer, very unlike unfixed membranes, and this provides a simple quantitative assay for determining complete fixation of chloroplast membranes.

(3) Light-induced quenching of Chl *a* fluorescence in the presence of PMS, which reflects structural modification of the thylakoid membranes, has been shown to be absent in glutaraldehyde fixed chloroplasts (Mohanty et al., 1973). Figure 2 shows a concentration curve for the PMS quenching of Chl *a* fluorescence; again, 0.5 % glutaraldehyde is shown to be sufficient to fix chloroplasts. However, we also noted an increase in PMS-induced quenching of Chl *a* fluorescence in the dark with glutaraldehyde fixation. Papageorgiou (1974) has shown that the reduced form of PMS is a better direct quencher of Chl *a* fluorescence than is its oxidized form; he 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 (Hallier and Park, 1969 a), and, thus, the increased dark quenching in glutaraldehyde fixed chloroplasts is attributed to greater accessibility of the (oxidized) PMS to the chlorophyll in the membrane.

(4) Figure 3 shows that 0.5 % glutaraldehyde stops the light-induced 90° light scattering change, while some changes still persist at 0.25 % glutaraldehyde.

Although 0.5 % glutaraldehyde is sufficient to completely fix thylakoid membranes, ordinarily 1 % or even 2 % glutaraldehyde was used, except as indicated in Table 1.

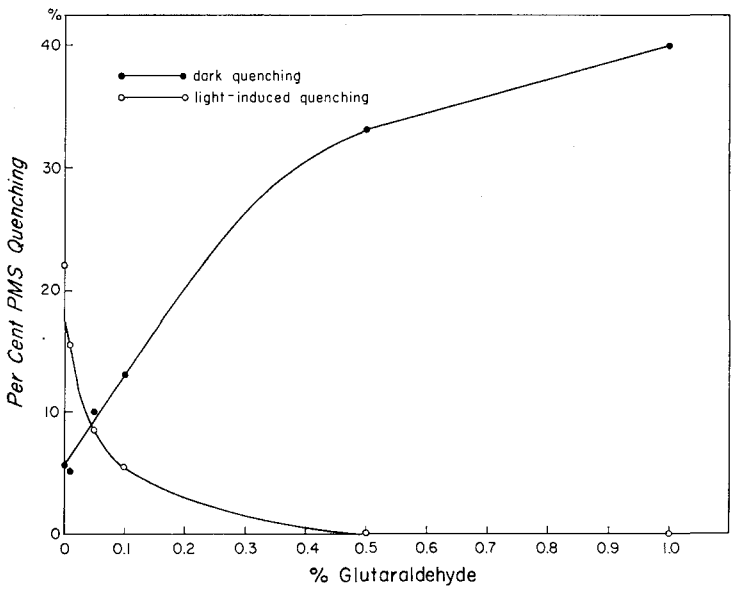

Fig. 2: Percentage of PMS-induced quenching in the dark (●——●) and light (○——○) of chlorophyll *a* fluorescence yield in spinach chloroplasts in the presence of DCMU as a function of glutaraldehyde concentration. First, 5 μM DCMU was added to the chloroplast suspension and relative fluorescence yield was measured. Then, 30 μM PMS was added and after a 3 minute dark equilibration time, 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.

It is certain that under the fixation conditions used here, chloroplasts were completely fixed in all preparations.

Although these chloroplsts are unable to undergo any large conformational or configurational changes as shown by the above tests, microconformational changes can occur. Figure 4 shows light-induced changes in fluorescence yield of the probe AS. This probe binds to the hydrobphobic region of the membrane and has been shown (VANDER MEULEN and GOVINDJEE, 1974; also personal communication) to reflect the «high energy state» or a «conformational state» of the thylakoid membrane. The extent of change of AS fluorescence also reflects the extent of the proton movement into the chloroplasts, which is proportionately reduced in the fixed chloroplasts (Fig. 4). 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.

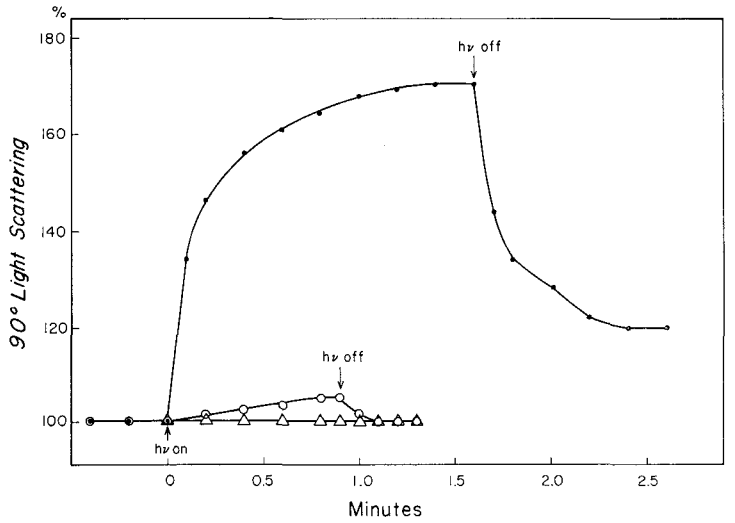

Fig. 3: Kinetics of light-induced 90° light scattering of isolated spinach chloroplasts, incubated in various concentrations of glutaraldehyde, accompanying photoshrinkage in sodium acetate medium. Three ml of sample, containing 10 μg chlorophyll per ml of medium (200 mM Na acetate, pH 6.7, 20 μM PMS), was illuminated with saturating red light (C.S. 2–59 and C.S. 3–73) to see light-induced changes in scattering. 90° 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 % (○——○), and 0.5 % and 1 % (△——△) (same result).

Treatments with NaSCH, $NaClO_4$, Guanidine and Urea (Chaotropic agents)

HATEFI and HANSTEIN (1969) have shown that chaotropic agents cause disruption of biological membranes by weakening the hydrophobic bonds by interrupting the hydrogen bonding structure of water. 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 (HATEFI and HANSTEIN, 1969), LOZIER et al. (1971) found that incubation of chloroplasts with these agents results in an inhibition of electron transport, restorable with artificial electron donors that bypass the oxygen-evolving site. In our unfixed chloroplasts (Table 2), these agents vary in their effectiveness in blocking electron flow through PS II, with NaSCN most efficient and urea the least. Relative degrees of effectiveness, observed here, correlate well with their order of effectiveness in solubilizing membrane bound proteins and in catalyzing the breakdown of membranes (as measured by lipid oxidation by HATEFI and HANSTEIN [1969]). Upon fixation of thylakoids, NaSCN is nearly as effective in the fixed chloroplasts as in the unfixed in stopping oxygen evolution, but urea is not (Table 2). The protection offered by glutaraldehyde fixation is as follows: NaSCN (16 %), $NaClO_4$ (49 %), guanidine (72 %) and urea (86 %).

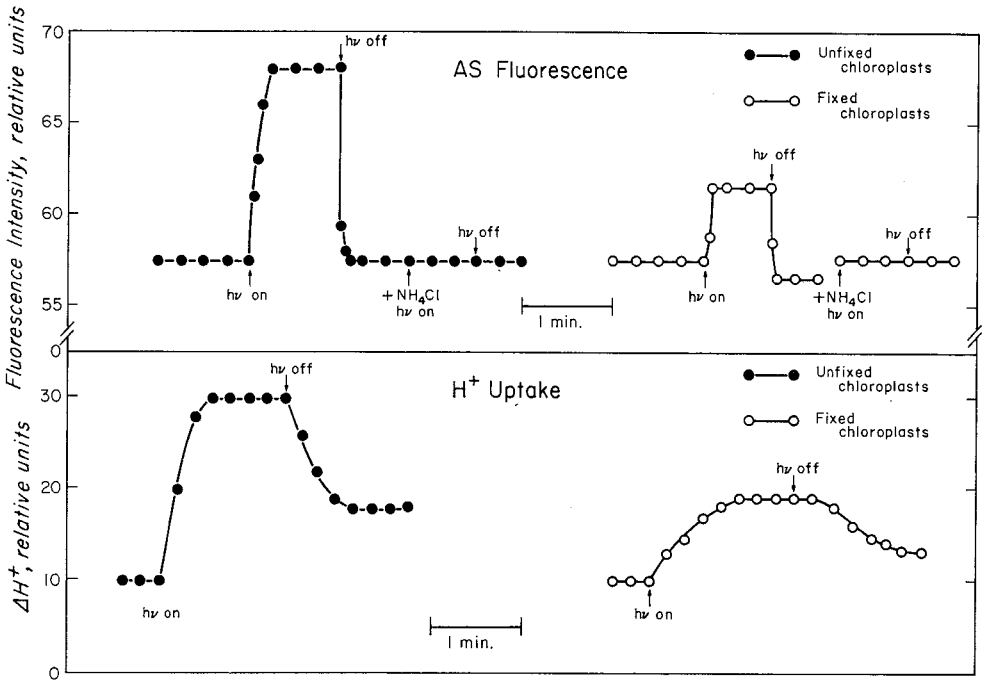

Fig. 4: Light-induced change in anthroyl stearate (AS) fluorescence and H^+ uptake in fixed and unfixed chloroplasts. Fluorescence was measured using a reaction mixture containing 3.3 mM Na phosphate, 3.3 mM $MgCl_2$, 16.7 mM KCl, 10 μM PMS, 15 μg chlorophyll/ml, pH 7.8, in a 3 ml volume; AS concentration 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) 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 heat filter.

Trypsin Treatment

Incubation of unfixed chloroplasts with trypsin is nearly 100 % effective in inhibiting Hill reaction to DCPIP, while the same treatment of fixed chloroplasts results in only a 10 % inhibition (Table 3). These data confirm MANTAI (1970) who had used trypsin treated chloroplasts from fixed whole mature leaves of spinach; in contrast to MANTAI's work, whose conclusions may be questioned, unstacked chloroplasts were intentionally used in our experiments as this is the only condition in which trypsin inhibits oxygen evolution (SELMAN and BANNISTER, 1971).

As glutaraldehyde reacts mainly with the ε-amino groups of lysine (HABEEB and

Table 2: Effect of chaotropic agents on fixed and unfixed chloroplasts.

Chloroplast Treatment	Additions	μmoles $DCPIPH_2$ (mg Chl · hr)$^{-1}$	Inhibition %
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 1; details of treatments are given in Methods.

Table 3: Effect of trypsin on fixed and unfixed chloroplasts,

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

One ml of the trypsin treated or control chloroplasts (see ref. SELMAN and BANNISTER, 1971, for details), containing 30 μg Chl/ml, was 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 1.

HIRAMOTO, 1968), and trypsin cleaves at peptide bonds where the carboxyl group of lysine (or arginine) residues participate (KEIL, 1971), the question arises 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 check 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 (MEANS, 1971). Unlike glutaraldehyde treatment, where a bifunctional aldehyde crosslinks proteins inter- and intra-molecularly (RICHARDS and KNOWLES, 1968) resulting 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 4). Formaldehyde (1, 2 and 5 %, w/v), also reactive with free amino

groups, does not inhibit trypsin action in the chloroplasts (Table 4). 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 (FRAENKEL-CONRAT and OLCOTT, 1968) which could conceivably «fix» membranes. If the membranes were in fact fixed by the formaldehyde, our reasons for using the reagent would be invalidated, so we checked this possibility by our

Table 4. Trypsin inhibition of photosystem II reaction in acetylated (I) and formaldehyde (II) treated chloroplasts.

Chloroplast treatment	Addition	μmoles $DCPIPH_2$ (mg Chl · hr)$^{-1}$	Inhibition %
I. Control	None	108.8	
Control	Trypsin	17.8	84
40 mM Acetic Anhydride	None	72.0	
40 mM Acetic Anhydride	Trypsin	8.9	87
II. 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 3; acetylation and formaldehyde treatment are as in Methods.

Table 5: 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	$\frac{A680nm/A546nm_{(water)}}{A680nm/A546nm_{(buffer)}}$
Control	18	39	1.9
1 % Formaldehyde	14	40	1.8
2 % Formaldehyde	12	. . .	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 1, 2, and 3. (Percent light-induced quenching of Chl *a* fluorescence by PMS for acetylated chloroplasts is 10.5.)

usual methods (Table 5). Chloroplasts were not fixed in the conventional sense of the word (Table 5), and it is probable that at the concentrations used, there are as many available amino groups blocked as with use of 0.5 % glutaraldehyde. Therefore, we conclude that glutaraldehyde is not merely making free amino groups unavailable for trypsin attack.

Discussion

Inhibition of Hill reaction by Tris-washing, heat treatment and $Cd(NO_3)_2$ is, at least, 50 % due to large (destructive) structural changes in the membrane which are eliminated by glutaraldehyde fixation (Table 1); the remaining inhibition that persists in the fixed chloroplasts is due to either: 1. small perturbations of the membrane that can still occur in fixed chloroplasts (as witnessed by changes in AS fluorescence) which also secondarily affect the activity of the oxygen-evolving component, or 2. a direct action of the inhibitor on the oxygen-evolving system. Direct effects might be, for example, the high temperature denaturation and inactivation of an enzyme involved in oxygen evolution (in the case of heat treatment of chloroplasts) or such possible direct effects of Tris as suggested by Yamashita et al. (1971). However, it is unlikely that fixation should hinder such direct effects; therefore, the 50 % inhibition remaining in fixed chloroplasts is suggested to be due to disruptive microconformational changes. It has been shown (Cheniae and Martin, 1966, 1971; Homann, 1968) that heat treatment and Tris-washing of chloroplasts result in a loss of bound manganese from the chloroplasts, concomitant with a loss of oxygen evolution, suggesting that manganese in involved in oxygen evolution. Disruption of the membrane structure could effect removal of loosely bound manganese, as its binding might have rigid structural requirements.

Chaotropic agents, judging from their action in other systems, would also act primarily through membrane disturbance. That the most potent chaotrope NaSCN (Table 2) 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 leading to membrane disturbances which can occur 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 completely stop the Hill reaction in glutaraldehyde fixed chloroplasts; the percent inhibition is 81 % (0.8 M NaSCN), 46 % (0.5 M $NaClO_4$), 21 % (0.8 M guanidine) and 9.5 % (1.0 M urea) (Table 2). (The concentrations of chemicals used here were those used by Lozier et al. (1971) to inhibit oxygen evolution in spinach chloroplasts, and we used the same in our studies.)

The action of trypsin can be either through 1. exclusive and serious membrane perturbation that in turn affects a sensitive component buried in the membrane, or 2. through a macroconformational change in the membrane, induced by interaction of trypsin with the membrane in the dark, which now exposes (subject to attack by

trypsin) a previously buried intermediate involved with oxygen evolution. (This structural change is abolished by glutaraldehyde fixation.) In view of the fact that chaotropic agents can most likely be considered as being membrane disruptive (HATEFI and HANSTEIN, 1969) and they still inhibit fixed chloroplasts, we prefer the latter hypothesis. Moreover, SELMAN et al. (1973) 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. Based on the above data, we suggest that the site of trypsin attack is most likely buried in the membrane, unavailable to this large, impermeable molecule once macroconformational changes are prohibited by glutaraldehyde fixation.

Moreover, we have found (ZILINSKAS, 1975) that two antisera which have been shown to specifically inhibit oxygen evolution in unfixed chloroplasts (BRAUN and GOVINDJEE, 1972, 1974) have no effect on fixed chloroplasts; as these inhibitors are large, impermeable molecules, we feel that their ineffectiveness in the fixed system is due to reasons described above for trypsin.

In conclusion, we suggest that the inhibitors of O_2 evolution in chloroplasts act primarily through effects on micro- and macroconformational changes of the thylakoid membrane – the latter being eliminated by glutaraldehyde fixation.

References

ARNTZEN, C. J., C. VERNOTTE, J.-M. BRIANTAIS, and P. ARMOND: Biochim. Biophys. Acta *368*, 39 (1974).

BAZZAZ, M. B., and GOVINDJEE: Environ. Lett. *6*, 1 (1974).

BRAUN, B. Z., and GOVINDJEE: FEBS Lett. *25*, 143 (1972).

– – Plant Sci. Lett. *3*, 219 (1974).

CHENIAE, G. M., and I. F. MARTIN: Brookhaven Symp. Biol. *19*, 406 (1966).

– – Plant Physiol. *47*, 568 (1971).

DILLEY, R. A.: Brookhaven Symp. Biol. *19*, 258 (1966).

FRAENKEL–CONRAT, H., and R. S. OLCOTT: J. Am. Chem. Soc. *70*, 2673 (1948).

GIAQUINTA, R. T., R. A. DILLEY, B. R. SELMAN, and B. J. ANDERSON: Arch. Biochem. Biophys. *162*, 200 (1974).

HABEEB, A. F. S. A., and R. HIRAMOTO: Arch. Biochem. Biophys. *126*, 16 (1968).

HALLIER, U. W., and R. B. PARK: Plant Physiol. *44*, 535 (1969 a).

– – Plant Physiol. *44*, 544 (1969 b).

HATEFI, Y., and W. G. HANSTEIN: Proc. Nat. Acad. Sci., U.S. *62*, 1129 (1969).

HOMANN, P. H.: Plant Physiol. *42*, 997 (1967).

– Biochem. Biophys. Res. Comm. *33*, 229 (1968).

JONES, L., and B. KOK: Plant Physiol. *41*, 1044 (1966).

KATOH, S., and A. SAN PIETRO: Arch. Biochem. Biophys. *112*, 144 (1968).

KEIL, B.: In: P. D. BOYER (Ed.), The Enzymes, Vol. 3, Hydrolysis and Peptide Bonds, pp. 249–275. Academic Press, New York, 1971.

LOZIER, R., M. BAGINSKY, and W. L. BUTLER: Photochem. Photobiol. *14*, 323 (1971).

MacKinney, G.: J. Biol. Chem. *140*, 315 (1941).
Mantai, K. E.: Biochim. Biophys. Acta *189*, 449 (1969).
– Plant Physiol. *45*, 563 (1970).
Margulies, M. M., and A. T. Jagendorf: Arch. Biochem. Biophys. *90*, 176 (1960).
Means, G. E., and R. E. Feeney: In: Chemical Modification of Proteins, Chapter 5, Holden Day, San Francisco, 1971.
Mohanty, P., B. Z. Braun, and Govindjee: Biochim. Biophys. Acta *292*, 459 (1973).
Murakami, S., and L. Packer: J. Cell Biol. *47*, 332 (1970).
Papageorgiou, G.: In: M. Avron (Ed.), Proc. 3rd. Intern. Congr. Photosynth., Rehovot, Israel, p. 41, 1974.
Papageorgiou, G., and Govindjee: Biophys. J. *8*, 1299 (1968).
Park, R. B.: In: A. San Pietro (Ed.), Methods in Enzymology, Vol. 23, Part A, pp. 248–250. Academic Press, New York, 1971.
Richards, R. M., and J. R. Knowles: J. Mol. Biol. *37*, 231 (1968).
Selman, B. R., and T. T. Bannister: Biochim. Biophys. Acta *253*, 428 (1971).
Selman, B. R., T. T. Bannister, and R. A. Dilley: Biochim. Biophys. Acta *292*, 566 (1973).
Shimony, C., J. Spencer, and Govindjee: Photosynthetica *1*, 113 (1967).
Vander Meulen, D., and Govindjee: FEBS Lett. *45*, 186 (1974 a).
– – Biochim. Biophys. Acta *368*, 61 (1974 b).
Vernon, L. P., and E. R. Shaw: Plant Physiol. *44*, 1645 (1969).
Yamashita, T., and W. L. Butler: Plant Physiol. *43*, 1978 (1968 a).
– – Plant Physiol. *43*, 2037 (1968 b).
Yamashita, T., J. Tsuji, and G. Tomita: Plant and Cell Physiol. *12*, 117 (1971).
Zilinskas, B.: Ph. D. Thesis, University of Illinois, Urbana, Illinois (1975).

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