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THE DIFFERENTIAL EFFECTS OF SHORT-TIME GLUTARALDEHYDE TREATMENTS ON LIGHT-INDUCED THYLAKOID MEMBRANE CONFORMATIONAL CHANGES, PROTON PUMPING AND ELECTRON TRANSPORT PROPERTIES

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A rapid quench technique utilizing the addition of excess buffer containing free amine groups (Tris, glycylglycine) to the reaction medium has enabled a detailed study of the time-course of glutaraldehyde inactivation on the spinach thylakoid membrane to be undertaken. The following light-induced parameters were inactivated in the sequence: slow transmittance changes (0–5 s) > coupling factor activity (5–20 s) > narrow angle 90° scattering changes (30–60 s). About 20% of PS II activity was lost by this treatment. No effect on activity, proton pumping and proton gradient formation was observed over the time-course studied. A consideration of these effects led to the proposal that the slow, light-induced transmittance changes reflect reversible thylakoid structural changes (unstacking, membrane flattening) in response to electron transport and the consequent proton pumping. The narrow angle 90° scattering changes were considered to reflect directly microconformational structural changes in response to the light-driven proton translocation as previously concluded from other workers.

Introduction

Chemical cross-linking reagents have found much use in probing membrane structure, both as specific modifying agents of the protein or lipid moieties of the membrane; and to stabilize membrane structure for, for example, electron microscopy [1]. One of the most widely used of these compounds is glutaraldehyde, which reacts primarily with free amine groups to form a Schiffs base [1,2], although there are complicating side reactions [1,2].

This reagent has been used extensively in modifying thylakoid structure both in situ [3] and on the isolated membrane [4]. Long incubation times (minimum 5 min) have been used in these studies [5,6]. Although one report has appeared [7] stating the order of inactivation of thylakoid function during glutaraldehyde fixation, no detailed study of the time-course and degree of sensitivity of various thylakoid functions has yet been undertaken. Generally speaking, the functions most affected by glutaraldehyde fixation are the coupling-factor complex and light-induced membrane conformational changes [7,8]. Electron transport and the associated proton pumping are less affected [6,7].

Because of our interest in the mechanism(s) of

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Abbreviations: DAD, 2,3,5,6-tetramethyl-β-phenylenediamine; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCIP, 2,6-dichlorophenolindophenol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; Methylviologen, 1,1′-dimethyl-4-4′-bipyridinium dichloride; Mes, 4-morpholineethanesulphonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PMS, phenazine methosulphate; PS I, Photosystem I; PS II, Photosystem II; Chl, chlorophyll.

coupling electron transport to ATP formation, and the membrane conformational changes associated with this process, we have investigated the short-term affects of glutaraldehyde fixation on coupling-factor activity and light-induced conformational changes. Adopting the protocoll of Dilley and coworkers [9] who have extensively used chemical modifying agents in probing thylakoid structure/function, we have terminated the glutaraldehyde treatment by the addition of an excess of free amine buffer (Tris or glycylglycine). By this procedure treatment times as short as 5 s could be reproducibly carried out.

Materials and Methods

Isolation of thylakoids

Intact chloroplasts were isolated from spinach leaves by standard methods and washed once in isolation medium [10]. Thylakoids were prepared by shocking intact chloroplasts (2–3 mg Chl) into 50 ml 10 mM $MgCl_2$/10 mM Tricine/KOH (pH 7.6) and centrifuging (30 s, 3000 g). The pellet was resuspended in 100 mM sorbitol/10 mM $MgCl_2$/20 mM Tricine/KOH (pH 7.6) to a final concentration of about 2 mg per Chl. All of these operations were performed at 0°C.

Glutaraldehyde treatment

Specially purified glutaraldehyde (grade 1,25% v/v aqueous solution) was obtained from Sigma and stored at −20°C. Reagent purity and concentration was checked by absorption spectroscopy as in Ref. 11.

Glutaraldehyde treatment of thylakoids was based on the recommendations of Papageorgiou [2,11]. Normally a ratio of 65 μmol glut per mg Chl was used, and the treatment was carried out on ice in the dark. After the treatment, the reactants were diluted into 10 ml of a medium containing 100 mM sorbitol/10 mM $MgCl_2$/10 mM Tris/10 mM Tricine (pH 7.6). The thylakoids were centrifuged (45 s, 3000 g), and resuspended in the same medium to a final concentration of about 1 mg Chl per ml.

Photosynthetic measurements

Electron transport measurements ($H_2O \rightarrow$ methylviologen/ferricyanide) were carried out in a Walker type O_2 electrode [12]. Photophosphorylation and light-dependent proton uptake were measured in the same cuvette by alkalization of the medium as described by Dilley [13].

Ninety degree narrow angle light scattering and 9′ aminoacridine fluorescence quenching were measured in an Aminco SPF 500 fluorometer (path length from cuvette to emission photomultiplier about 35 cm). Actinic light was supplied to the top of the cuvette via a Schott KL 1500 fibre optic light source through a Calflex K9 (heat absorbing) and a Schott RG 630 cut off filter. Where necessary the red actinic light (630–800 nm) was attenuated by Schott neutral density filters. The excitation and emission photomultiplier tubes were protected from the actinic light by Corning 4303 blue cut-off filters. Light-induced transmittance changes were either measured in an Aminco DW2 spectrophotometer, or in a laboratory built spectrophotometer [14], which was also used to measure fluorescence and luminescence emission. Appropriate filter combinations were employed to prevent actinic light leaks to the photomultiplier.

Chlorophyll concentrations were measured as in Arnon [15].

Results

The first figure shows the effect of increasing treatment time of glutaraldehyde on various photosynthetic parameters at a constant glutaraldehyde/thylakoid ratio of 60 μmol per mg Chl. Under these conditions there was a rapid ($t_{0.5} \approx 5$ s) inactivation of PMS-mediated cyclic photophosphorylation. Concomitant with this was a removal of most of the phosphorylation-induced stimulation of electron transport as measured by ferricyanide reduction, and an increase in 9′-aminoacridine quenching to the basal level. $NADP^+$ reduction in a thylakoid system reconstituted with 0.1 mM ferredoxin was also progressively inhibited with the same kinetics as photophosphorylation. However, over the time period studied (up to 10 min) there was no effect on basal electron transport ($H_2O \rightarrow$ methylviologen/ferricyanide). Interestingly, there was a loss of between 20 and 30% of uncoupled electron transport parallel to the loss of photophosphorylation. The residual 70–80% of uncoupled electron flow

remained constant over the remainder of the experimental period.

The second figure explores the effect of glutaraldehyde treatment on light-induced transmittance and 90° narrow angle scattering changes at 520 and 540 nm in the presence of the electron acceptor methyl viologen. The slow ($t_{0.5}$ 15–20 s) light induced transmittance changes at 520 and 540 nm were completely abolished by short treatments with glutaraldehyde. The time-course of inhibition ($t_{0.5} < 5$ s) was even more rapid than that of photophosphorylation. In contrast, the fast rise component of the absorbance change appeared relatively unaffected. The 90° scattering component was also progressively inhibited by glutaraldehyde ($t_{0.5} \approx 20$–30 s), but the time-course of inactivation was one order of magnitude less than that of the slow transmittance changes. Thus, after 5 s treatment with glutaraldehyde, the slow transmittance changes were completely abolished whereas the 90° scattering changes were only 20% inhibited.

Fig. 3 examines the relationship between light intensity and linear electron transport in glutaraldehyde-treated thylakoids. About 25% of uncoupled linear electron flow is lost by this treatment, whereas basal electron transport was unaffected. Furthermore, light-limited rates of electron trans-

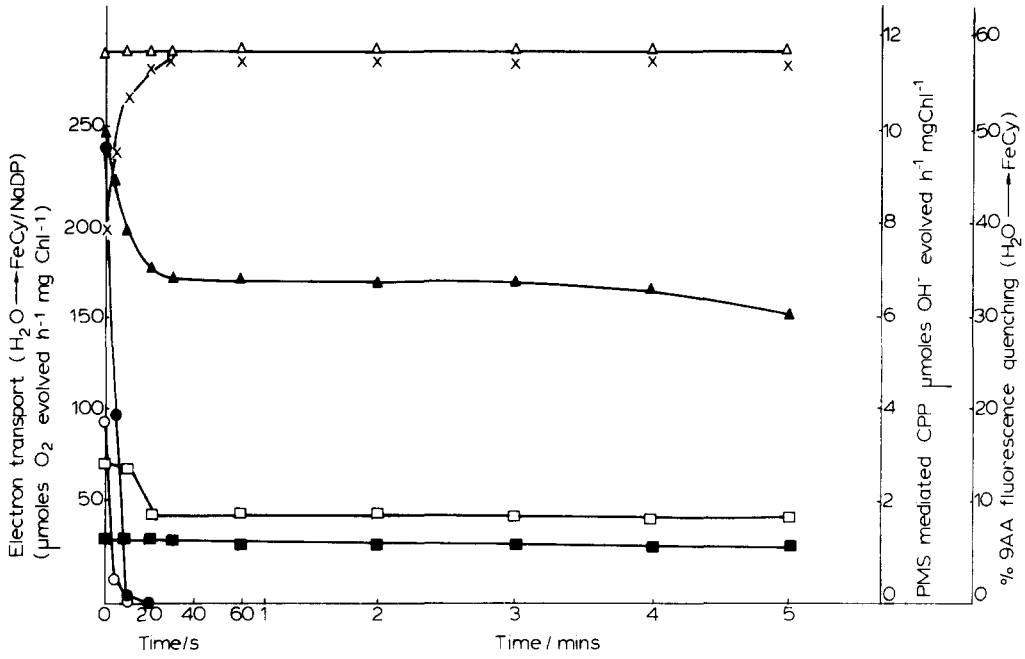

Fig. 1. The effect of short term glutaraldehyde treatment on electron transport, cyclic photophosphorylation and 9′-aminoacridine quenching. *Electron transport.* The medium (1 ml) contained 100 mM sorbitol/10 mM $MgCl_2$/10 mM Tricine/10 mM Tris (pH 7.6)/2 mM ferricyanide (H_2O → ferricyanide), or 0.1 mM ferredoxin/2 mM NADP (H_2O → NADP) for basal electron transport, plus 10 mM KH_2PO_4 and 0.5 mM ADP for phosphorylation, plus 5 mM NH_4Cl for uncoupling, (20°C), 50 μg Chl per ml, actinic light (610–800 nm) intensity 200 $W \cdot m^{-2}$. *Cyclic photophosphorylation.* The medium (1 ml) contained 100 mM sorbitol/10 mM $MgCl_2$/1 mM tricine/KOH (pH 7.8)/1 mM KH_2PO_4/0.5 mM ADP/15 μM PMS 50 μg Chl per ml (20°C). Actinic light intensity (610–800 nm) was 600 $W \cdot m^{-2}$. *9′-Aminoacridine fluorescence quenching.* The medium (1.5 ml) was as for basal electron transport with the addition of 1 μM 9′-aminoacridine, 15 μg Chl per ml (20°C). Fluorescence was excited at 400 nm (1 nm slit width, 2 $W \cdot m^{-2}$) and emitted light measured at 480 nm (40 nm slit width). Actinic light (630–800 nm) was 40 $W \cdot m^{-2}$. All samples were the mean of four replicates, S.D. ±10%. O- - - - - -O, $H_2O \rightarrow NADP^+$ uncoupled electron transport; ■- - - - -■, H_2O → ferricyanide basal electron transport; □- - - - -□, H_2O → ferricyanide coupled electron transport; ▲- - - - -▲, H_2O → ferricyanide uncoupled electron transport; ●- - - - -●, PMS mediated cyclic photophosphorylation; △- - - - -△, H_2O → ferricyanide, basal, 9′-aminoacridine fluorescence quenching; ×- - - - -×, H_2O → ferricyanide, coupled, 9′-aminoacridine fluorescence quenching.

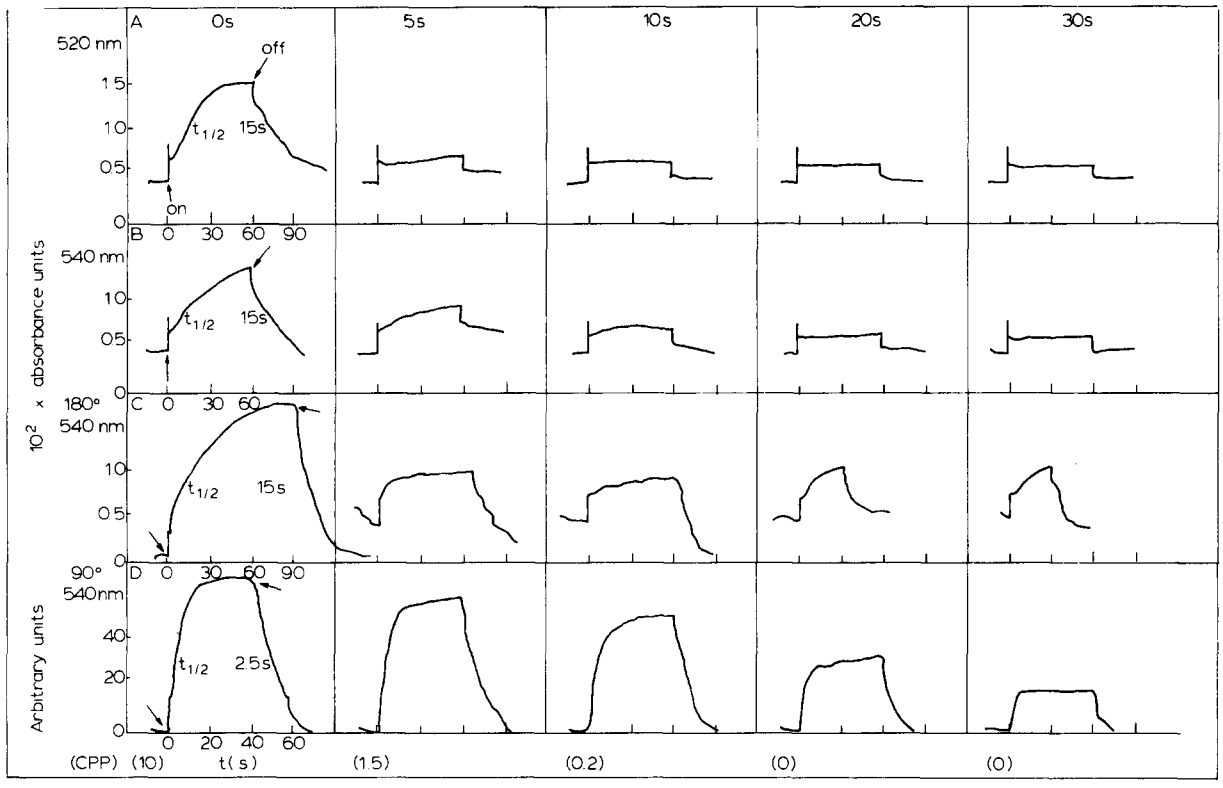

Fig. 2. The effect of short-term glutaraldehyde treatment on scattering and transmittance changes under continuous illumination. (A) and (B). Light induced transmittance changes measured in a laboratory built single beam spectrophotometer [14], at 520 nm and 540 nm. The cuvette (0.75 ml volume) contained basal electron transport medium as in Fig. 1 except 0.1 mM methyl viologen replaced of 2 mM ferricyanide, 15 μg Ch per ml (20°C). Actinic light (630–800 nm) was 40 $W \cdot m^{-2}$. (C). Light-induced transmittance changes at 540 nm measured in an Aminco DW2 spectrophotometer in the single beam mode. The cuvette (2.5 ml volume) contained thylakoids as in (A). Actinic light (630–800 nm) was 50 $W \cdot m^{-2}$. (D). Light-induced 90° narrow angle scattering changes measured at 540 nm in an Aminco SPF 500 spectrofluorometer. The cuvette (1.5 ml) contained thylakoids as in (A). Actinic light (630–800 nm) was 50 $W \cdot m^{-2}$. The excitation (1 nm slit width) and emission (5 nm) were both set to 540 nm for the measurements, and were protected from the actinic light by Corning 4303 blue cut-off filters. The numbers in parenthesis refer to the rate of PMS mediated cyclic photophosphorylation (CCP) at the given treatment time.

port were also unaffected, implying that the primary light-capturing processes were not inhibited by these treatments.

To localize the site of inhibition of elctron transport, the partial reactions for PS II ($H_2O \rightarrow$ DAD/ferricyanide) and PS I (DCPIP/ascorbate $\rightarrow$ methyl viologen) were also measured under a range of light intensities (Figs. 4 and 5). Both basal and uncoupled PS II partial reactions were unaffected by glutaraldehyde treatment. This was also true for basal electron flow through PS I. The uncoupled rates of electron flow through PS I were inhibited by about 30% under light-saturating conditions. No differences were found in the rates of electron transport under light-limiting conditions. Thus, the partial inhibition of linear electron flow was localized somewhere in the PS I region of the thylakoid membrane. As already stated, electron flow from $H_2O \rightarrow NADP^+$ in a reconstituted system was completely inhibited under these conditions, presumably due to inactivation of the ferredoxin $NADP^+$ reductase.

The proton uptake characteristics of linear electron flow ($H_2O \rightarrow$ methylviologen) were investigated to confirm the electron transport results (Figs. 6 and 7). The initial rate of proton uptake at

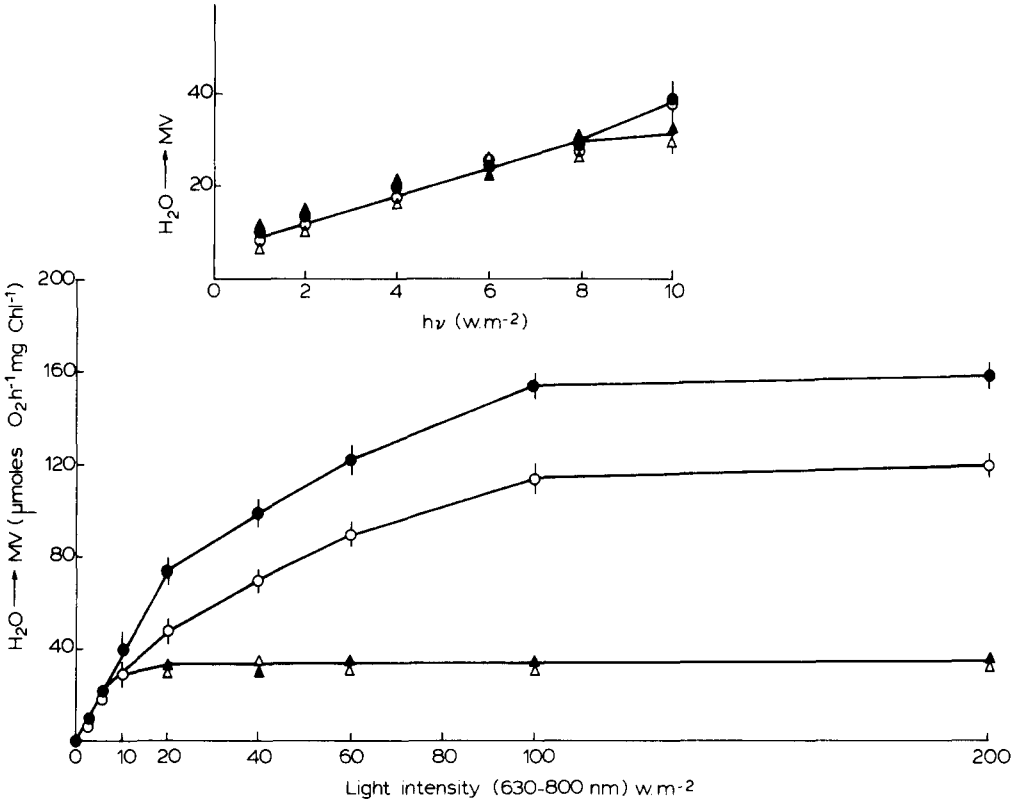

Fig. 3. The effect of a short time glutaraldehyde treatment (20 s) on basal and uncoupled linear electron transport ($H_2O \rightarrow$ methylviologen): the relationship between light intensity and electron flow. Experimental conditions were as in Fig. 1A. Samples were uncoupled where necessary by the addition of 5 mM NH_4Cl in the dark 1 min before illumination commenced. Actinic light intensity (measured with a Yellow Springs model 65A radiometer) was attenuated by Schott neutral density filters. ▲- - - - -▲, basal electron flow; △- - - - -△, basal electron flow, 20 s glutaraldehyde treatment; ●- - - - -●, uncoupled electron flow; ○- - - - -○, uncoupled electron flow, 20 s glutaraldehyde treatment. All points were the mean ± S.D. ($n = 5$).

two different pH values (6.7, 7.6) showed no significant differences between control and glutaraldehyde-treated thylakoids. Similarly, the total amount of proton accumulation was unaffected. So presumably the total internal buffering capacity of the thylakoid was unaffected by this treatment, as was the uptake rate as would be predicted from the electron transport data.

A further prediction from these results would be that the light-driven proton gradient formation would be unaffected by glutaraldehyde treatment. This was confirmed by the data in Fig. 8 showing that total 9′-aminoacridine fluorescence quenching was unaffected by glutaraldehyde treatment. Under light-saturating conditions the total quenching reached a final value of 60%, corresponding to a proton gradient of about 4 pH units. Proton gradient formation was saturated at about 20 $W \cdot m^{-2}$, the same as for basal electron transport and total proton uptake. The initial rate of proton pumping was saturated at slightly higher light intensities (40 $W \cdot m^{-2}$).

In contrast to the total 9′-aminoacridine fluorescence quenching, the kinetics ($t_{0.5}$ rise, and $t_{0.5}$ dark decay) were considerably altered by glutaraldehyde treatment. Under light-saturating conditions, the $t_{0.5}$ rise and dark decay of control thylakoids were 5 and 10 s, respectively. These two parameters exhibited an antiparallel dependence on light intensity. Thus at limiting intensity the

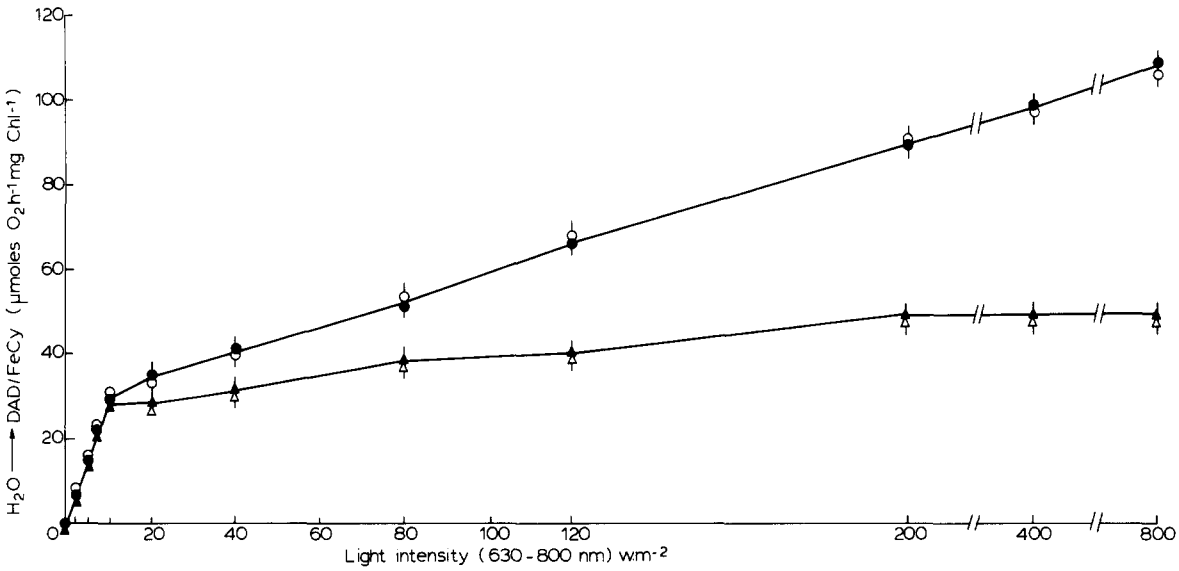

Fig. 4. The effect of glutaraldehyde treatment (20 s) on basal and uncoupled PS II partial reaction ($H_2O \rightarrow$ DAD/ferricyanide): the relationship between light intensity and electron flow. Conditions as in Fig. 1A. Samples were preincubated for 1 min in the dark with 10^{-5} M 1,3-dinitrophenylether of iodonitrothymol, 0.25 mM DAD, potassium ferricyanide 2 mM before illumination. Samples were uncoupled where necessary by the addition of 5 mM NH_4Cl 1 min before illumination. ●- - - - -●, basal electron flow; ○- - - - -○, basal electron flow, 20 s glutaraldehyde treatment; ▲- - - - -▲, uncoupled electron flow; △- - - - -△, uncoupled electron flow, 20 s glutaraldehyde treatment. All values were the mean ± S.D. ($n = 5$).

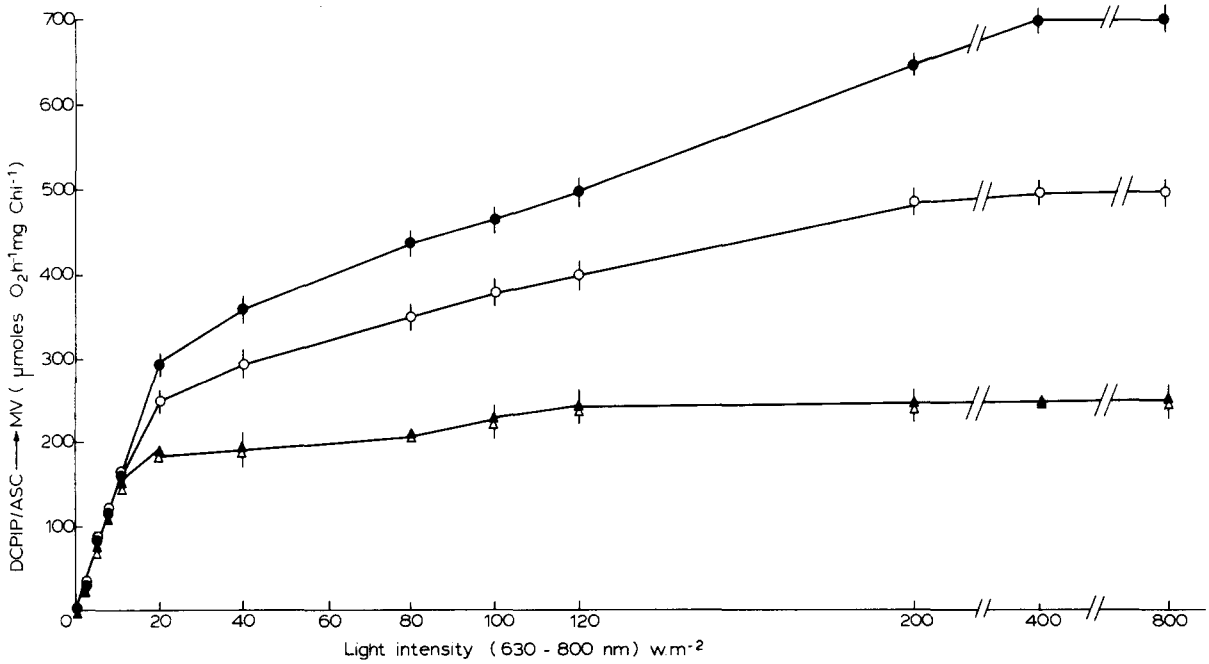

Fig. 5. The effect of glutaraldehyde treatment (20 s) on basal and uncoupled PS I partial reaction (DCIP/ascorbate → methylviologen): the relationship between light intensity and electron flow. Conditions as for Fig. 1A. Samples were preincubated for 1 min before illumination with $5 \cdot 10^{-6}$ M DCMU, 0.1 mM DCIP, 5 mM sodium ascorbate, 0.1 mM methylviologen for 1 min before illumination. Samples were uncoupled where necessary by 5 mM NH_4Cl. ▲- - - - -▲, basal electron flow; △- - - - -△, basal electron flow, 20 s glutaraldehyde treatment; ●- - - - -●, uncoupled electron flow; ○- - - - -○, uncoupled electron flow, 20 s glutaraldehyde treatment. All samples were the mean ± S.D. ($n = 5$).

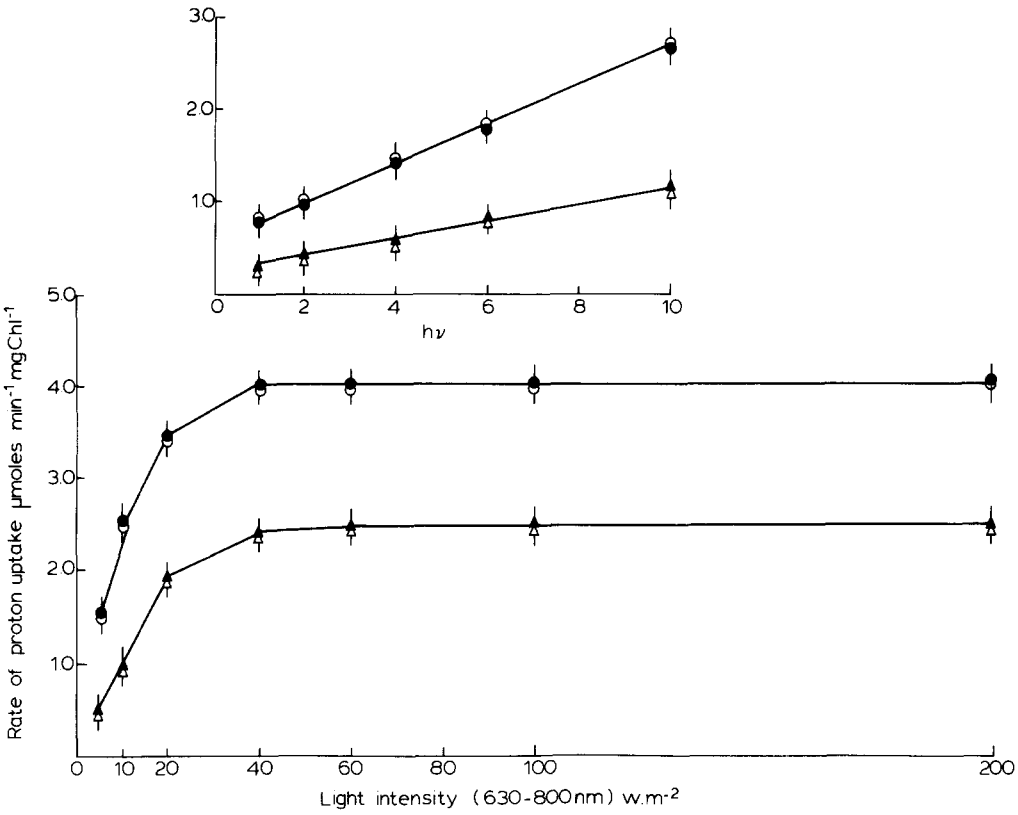

Fig. 6. The effect of glutaraldehyde treatment (20 s) on proton pumping (initial uptake rate) at pH 6.7 and 7.6: the relationship with light intensity for linear electron transport ($H_2O \rightarrow$ methylviologen). The medium (1 ml volume) contained 100 mM sorbitol, 10 mM $MgCl_2$, 1 mM Mes/KOH (pH 6.7) or 1 mM Hepes/KOH (pH 7.6), 50 μg Chl per ml, 0.1 mM methylviologen (20°C). ●-----●, proton pumping pH 6.7; O-----O, proton pumping pH 6.7, 20 s glutaraldehyde treatment; ▲-----▲, proton pumping pH 7.6; △-----△, proton pumping pH 7.6, 20 s glutaraldehyde treatment.

rise time increased and the dark decay time decreased. In complete contrast, these two parameters were both reduced in glutaraldehyde-treated thylakoids ($t_{0.5}$ rise 6 s, $t_{0.5}$ dark decay 7 s under saturating light). Furthermore both parameters increased in parallel under light-limiting conditions. This may well reflect the alterations in membrane surface properties (surface charge characteristics) brought about by glutaraldehyde treatment.

Finally, the kinetics of the chlorophyll fluorescence induction curve, and the luminescence induction curve for samples treated with glutaraldehyde for various times were compared with control thylakoids. Essentially no changes were seen confirming that electron transport from the water-splitting complex, through PS II and the associated proton pumping are unaffected by this treatment (Fig. 9).

Discussion

The most widely accepted model of thylakoid structure involves a vectorial arrangement of the electron-transport chain carriers such that the light-driven electron transport results in the inward accumulation of protons in the intrathylakoid space which involves two components of the electron transport chain, the water-splitting complex and the plastoquinone pool. This leads to $4H^+$ translocated/2e in linear electron flow [16].

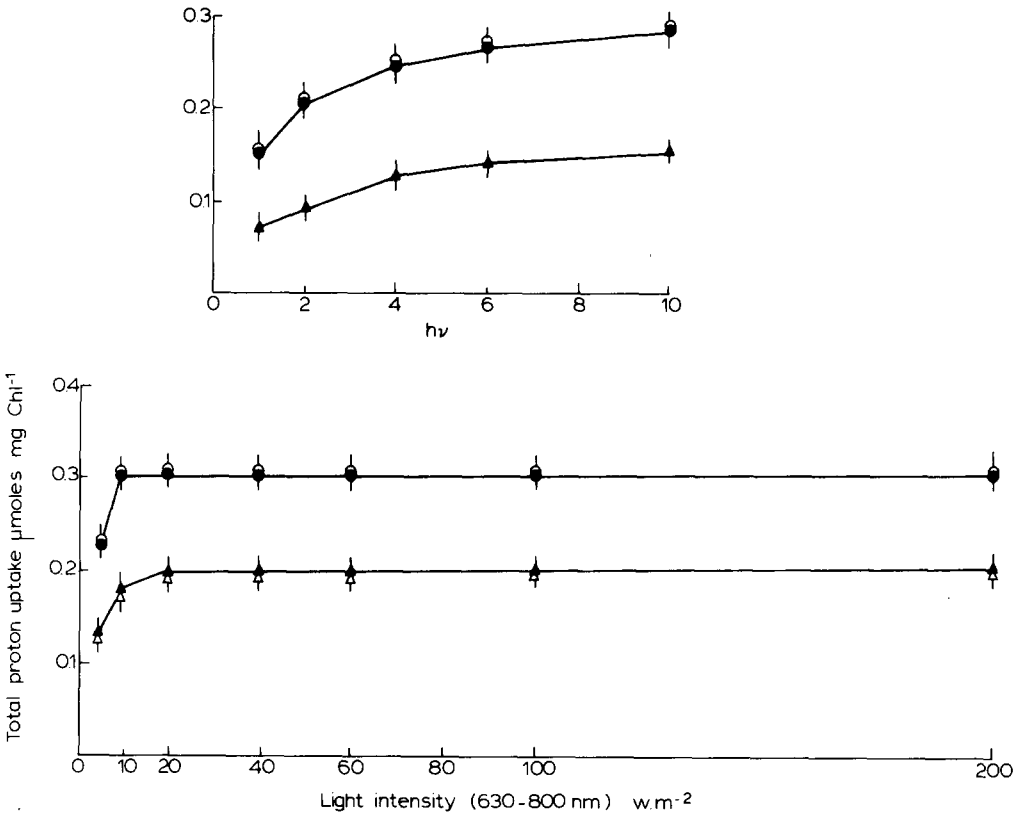

Fig. 7. The effect of glutaraldehyde treatment (20 s) on proton pumping (total amount) at pH 6.7 and 7.6: the relationship with light intensity for linear electron transport ($H_2O \rightarrow$ methylviologen). Conditions as in Fig. 6. All points were the mean ± S.D. ($n = 5$). ●- - - - -●, total proton uptake pH 6.7; O- - - - - -O, total proton uptake pH 6.7, 20 s glutaraldehyde treatment; ▲- - - - -▲, total proton uptake pH 7.6; △- - - - -△, total proton uptake pH 7.6, 20 s glutaraldehyde treatment.

The resultant proton gradient formed, either free in the thylakoid lumen as proposed by the Mitchell chemiosmotic hypothesis [17] or localized in more lipophilic microenvironments as first proposed by Williams [18], is considered to be the major component of the protonmotive force which results in ATP formation by the coupling-factor complex, although the exact coupling mechanism is not yet known [19].

Concomitant with this light-driven proton uptake is a counter exchange of ions (Mg^{2+}) to maintain electroneutrality, and scattering changes which are considered to represent microconformational changes in thylakoid structure [20,21]. These light-dependent scattering changes were first reported by Packer [22], and have been extensively investigated by the groups of Packer [20] and Dilley [21]. The 90° scattering changes have been convincingly correlated with proton pumping [21,23]. Scattering changes measured in the transmittance mode exhibit a more variable response [24,25].

In this report, the initial observation was made that short-time glutaraldehyde treatments selectively inhibited the slow transmittance changes whereas 90° scattering changes were relatively unaffected. This prompted us to investigate the characteristics of glutaraldehyde inactivation in more detail.

Packer et al. [8] were the first to use glutaraldehyde fixation of thylakoid membranes to inhibit the scattering changes while leaving pro-

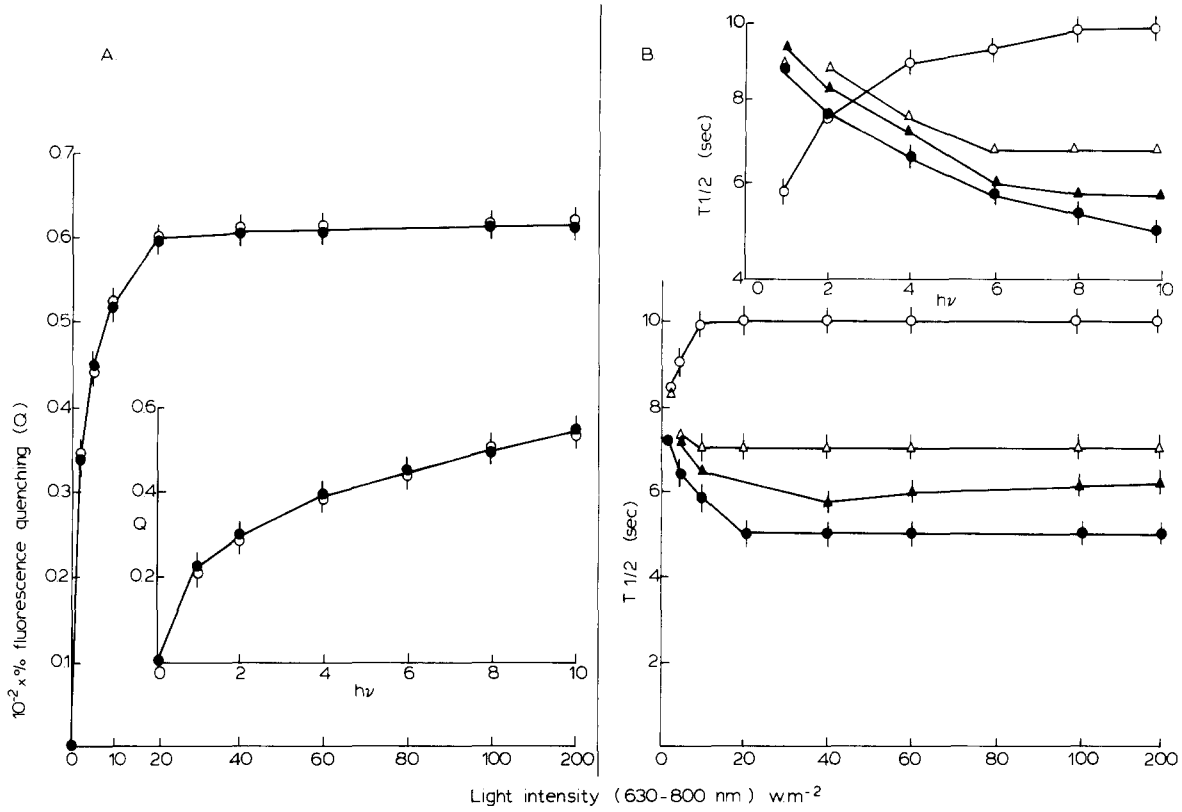

Fig. 8. The effect of glutaraldehyde treatment (20 s) on 9′-aminoacridine fluorescence quenching at pH 7.6: the relationship with light intensity for linear electron transport ($H_2O \rightarrow$ methylviologen). Conditions as in Fig. 1C. (A) Extent of 9′-aminoacridine quenching: ●- - - - -●, control pH 7.6; ○- - - - -○, 20 s glutaraldehyde treatment; (B) Kinetics of 9′-aminoacridine quenching: ●- - - - -●, $t_{0.5}$ rise time control; ○- - - - -○, $t_{0.5}$ dark decay time control; ▲- - - - -▲, $t_{0.5}$ rise time, 20 s glutaraldehyde treatment; △- - - - -△, $t_{0.5}$ dark decay time, 20 s glutaraldehyde treatment. All values were the mean ± S.D. ($n = 5$).

ton-pumping relatively unaffected. In their report long treatment times (5 min) were used. Therefore, in our investigation a time-course 0–10 min was used. In agreement with the recommendations of Papageorgiou [4] a glutaraldehyde/thylakoid ratio of approx. 60 μmol per mg Chl proved optimal for treatment studies.

The functions most rapidly affected by glutaraldehyde treatment of the thylakoid were the slow, light-induced transmittance changes, photophosphorylation and $NADP^+$ reduction. It is known that both the CF_1 part of the coupling-factor complex and FNR undergo light-dependent conformational changes [26,27]. The glutaraldehyde inhibition of photophosphorylation and $NADP^+$ reduction was equally effective when carried out either in the light or the dark. A recent report [28] has shown that limited modification of thylakoid membranes by glutaraldehyde selectively inhibited the P_i-ATP exchange reaction of the coupling-factor complex much more than ATP synthesis or hydrolysis. In agreement with these results, we also observed no extensive crosslinking of membrane proteins (as monitored by SDS-polyacrylamide gel electrophoresis) until after between 1 and 2 min treatment, which paralleled the loss of light-induced 90° scattering changes.

Light-dependent slow transmittance changes have been extensively used by Heber and coworkers both in intact chloroplasts [29,30] and leaves [31,32] as indicators of thylakoid 'membrane energization' in the light [33,34]. Extensive

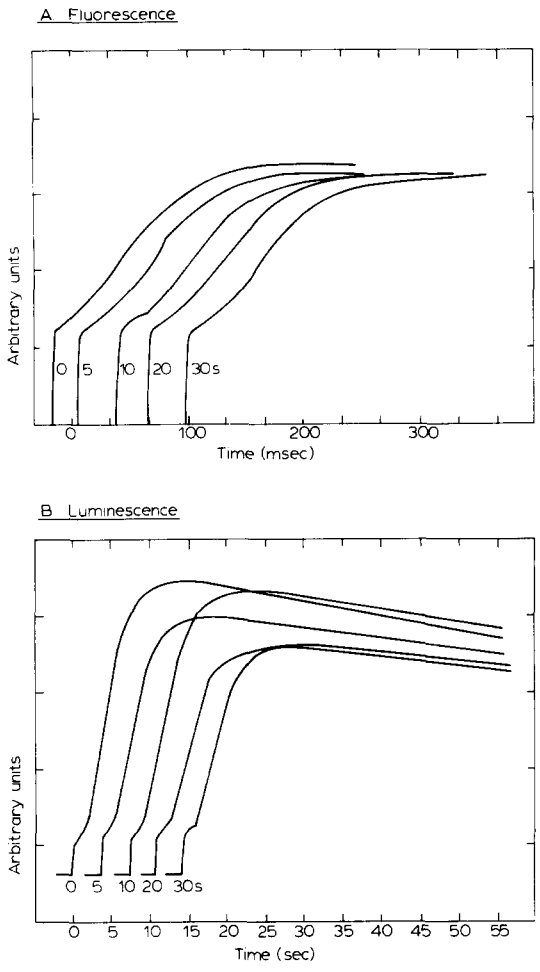

Fig. 9. The effect of short time glutaraldehyde treatments (0–30 s) on the fluorescence induction curve (A) and the luminescence induction curve (B).

evidence has been accumulated for a good positive correlation between these transmittance changes and electron transport, the associated proton pumping and stromal [30] and leaf [32] adenylate levels. These changes usually occur in parallel to, but with a slower $t_{0.5}$ rise, than the narrow angle 90° scattering. Thorne et al. [33] in an elegant investigation of scattering changes in spinach thylakoids correlated the 90° scattering response, and the slow rise component of the 518 nm absorption change with proton uptake. They explained these results as alterations in selective dispersion owing to the conformational changes in the thylakoid (light-dependent decrease in the intrathylakoid distance) first shown in the ultrastructural studies of the Packer group [34]. Thorne et al. [33] measured the 518 nm absorption changes in the dual wavelength mode with 540 nm as the reference wavelength. This eliminates the slow transmittance changes used by the Heber group as an indicator of proton pumping as these have a broad absorption maxima in the region 520–540 nm [31]. It has also been shown by Krause [35] that these transmittance changes only occur in stacked thylakoids, as intact chloroplasts shocked into a medium containing low Mg^{2+} concentrations did not show these effects, although proton pumping was unaffected. There is also an Mg^{2+} affect for the 90° scattering [23], as it is the major counter ion transported out of the thylakoid when protons are pumped in under continuous illumination.

These two phenomena (90° scattering and transmittance changes) cannot normally be separated. The explanation for the 90° scattering changes is in agreement with our results. The transmittance changes, in contrast, have not been satisfactorily explained. The ultrastructural studies of Miller and Nobel [36] and Murakami and Packer [31] show that besides the changes in intrathylakoid distance already mentioned, thylakoids both in situ [36] and in vitro [34,37] undergo larger conformational changes including closer appression of the granal stacks, and membrane flattening. We would tentatively conclude from our data that the slow, light-induced transmittance changes represent these larger macroconformational changes.

Finally, in agreement with Hardt and Kok [6,39], but in contrast to Renger et al. [41], we found no affect of glutaraldehyde treatment on PS II activity. These results also show that there is a 20–30% inhibition of the uncoupled rates of PS I electron transport activity. This region of the electron transport chain is located primarily in the stroma exposed region of the grana [38], and is therefore more available to glutaraldehyde inactivation. Hardt and Kok [6,39] have shown that in longer-time treatments plastocyanin is specifically inhibited. This is unlikely in our experiments as the initial rate of inactivation paralleled the loss of photophosphorylation, and the residual rate remained constant for the rest of the treatment time. P-700 itself is presumably not directly af-

fected as the light-limited rates of electron transport were not affected. Possibly fixation of the membrane led to increased diffusional resistance of the electron carriers, either the plastoquinone pool or the cyt f/b_6 complex. It will be necessary to measure the turnover times of the components of PS I under single turnover conditions as in Hardt and Kok [39], to localize this site of inhibition further. The PS II_β centres which are proposed to lie in the stromal region of the thylakoid were not affected by glutaraldehyde treatment. An analysis of the DCMU fluorescence rise curve as in Ref. 40 revealed no change in the slow rise part of the curve, indicating no inhibition of the β-centres (data not shown).

Basal electron transport and hence proton pumping were unaffected by the glutaraldehyde treatments used in these experiments. Proton gradient formation as indicated by 9′-aminoacridine fluorescence quenching was also unaffected in total although the kinetics ($t_{0.5}$ rise and dark decay were altered). This compound is generally thought to reflect the light-dependent proton gradient [42,43], although the reservations of Kraayenhof [44] that there is an element of dye binding to the thylakoid surface which carries a net negative surface charge under these conditions [45] must be considered. Schapendonk et al. [46] have shown that glutaraldehyde treatment changes the surface charge properties of the thylakoid and abolishes the light/dark transitions which normally occur. This may be the explanation for the observed changes in the kinetics of light-induced 9′-aminoacridine fluorescence quenching and the dark decay observed in our experiments.

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