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STABILIZATION BY GLUTARALDEHYDE OF HIGH-RATE ELECTRON TRANSPORT IN ISOLATED CHLOROPLASTS

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SUMMARY

Treatment of isolated chloroplasts with glutaraldehyde affects their ability to photoreduce artificial electron acceptors. The remaining rate of O_2 evolution approaches zero with methyl viologen, is low with ferricyanide, but nearly normal with lipophilic Photosystem II acceptors, like oxidized *p*-phenylenediamine and oxidized diaminodurene. Since Photosystem I donor reactions are also affected, a specific site of inhibition of electron transport to Photosystem I is indicated. At the same time, glutaraldehyde prolongs the longevity of the chloroplasts stored in dark. In control samples the half-life of Photosystem II activity varied between 5 days at 4 °C and 1 day at 25 °C. Glutaraldehyde treatment increased these half times approx. 3-fold. The glutaraldehyde doses required to induce inhibition and stabilization were very similar.

INTRODUCTION

Bifunctional reagents which immobilize proteins such as glutaraldehyde, are used commonly in the field of enzyme stabilization [1, 2].

Park et al. [3–7] have reported that isolated chloroplasts and algae fixed with glutaraldehyde maintain their light-induced electron transport activities longer than untreated ones, albeit at much diminished rates. Photosystem II activity of DPIP reduction as well as Photosystem I activity with methyl viologen as acceptor and reduced DPIP as donor, were considerably reduced. On the other hand Park et al. [3] found that in glutaraldehyde-treated chloroplasts the remaining Hill reaction activity survived at 4 °C more than 24 days compared to 3 days in unfixed material. In addition this photochemical activity showed increased resistance to detergent. Hallier and Park [4] further observed that glutaraldehyde made whole algae permeable to electron acceptors such as DPIP and methyl viologen.

Packer et al. [9] used the disappearance of the light-induced absorbance changes, which reflect volume changes of the chloroplasts, as the measure of the "completeness" of fixation. They showed that the PMS-mediated, light-induced

Abbreviations: DAD_{ox}, oxidized diaminodurene; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DMQ, 2,5-dimethyl-*p*-benzoquinone; DPIP, 2,6-dichlorophenolindophenol; PD_{ox}, oxidized *p*-phenylenediamine; PMS, *N*-methylphenazonium methosulfate.

proton uptake was only slightly inhibited by glutaraldehyde treatment, although both cyclic and non-cyclic photophosphorylation were already inhibited at low concentrations [10].

This paper reports further observations on the stability of glutaraldehyde-treated chloroplasts and attempts to overcome the severe loss of electron transport activity reported in the earlier studies.

PROCEDURES

Spinach chloroplasts were prepared as described previously [11]; Hill reaction activity was measured polarographically. The reaction medium contained 0.4 M sucrose, 50 mM NaCl, 5 mM $MgCl_2$, and 50 mM Tris or Tricine buffer at pH 7.4 or, occasionally, pH 8.0. Unless stated otherwise 30 mM methylamine was added as an uncoupler. O_2 evolution was measured using either 20 μM methyl viologen or 1.5 mM ferricyanide as electron acceptor. 0.6 mM PD_{ox} or DAD_{ox} was used in addition to the ferricyanide, with the omission of methylamine [12]. Photosystem I activity was monitored by the reduction of 20 μM methyl viologen in the presence of 5 μM DCMU and 30 μM methylamine using 2 mM sodium ascorbate and 50 μM DPIP (or 0.5 mM DAD) as the electron donor system. This activity, as well as the above water $\rightarrow$ methyl viologen activity, was monitored as light-induced O_2 uptake via H_2O_2 formation.

Whole chloroplasts were prepared according to Morgenthaler et al. [13]. Blue green algae *Anacystis nidulans* and *Anabaena cylindrica* and the green algae *Chlamydomonas reinhardi*, *Chlorella vulgaris*, and *Scenedesmus* sp. were cultured as described previously [14, 15]. After 40–48 h of growth cells were harvested by centrifugation, washed, and resuspended in 30 mM phosphate buffer at pH 7.4. The reaction mixtures used for the assay of O_2 evolution coupled to electron acceptors were the same as those used for spinach chloroplasts. The O_2 evolution coupled to CO_2 uptake was assayed in a 0.1 M $NaHCO_3$ solution through which 3 % CO_2 was bubbled for about 2 min

Specially purified glutaraldehyde was purchased from Sigma as a 25 % solution (lot 55C-5044) and stored at -18 °C. The appropriate concentration was obtained by dilution with 20 % sucrose solution and the pH was adjusted to 7.0 with 0.01 M NaOH. The routine fixation procedure was as follows: To a volume of fresh chloroplasts in 0.4 M sucrose, 50 mM NaCl, 50 mM Tris buffer, pH 7.4, containing 3–4 mg chlorophyll per ml, an equal volume of glutaraldehyde solution (final concentration 5 %) was added slowly. The suspension was shaken during 5 min in an ice bath and then the glutaraldehyde was removed by two washings: the suspension was diluted approx. 10 times with a solution of 0.4 M sucrose, 50 mM NaCl, and 50 mM Tricine at pH 7.4, and centrifuged. The chloroplasts were finally resuspended and stored in 2 ml of the same solution. One type of control chloroplasts was treated with the same volume of sucrose/NaCl/Tris or 20 % sucrose solution instead of glutaraldehyde and subjected to the same washing procedure. The original non-treated chloroplasts provided another type of control material. The chloroplasts were stored in absence of electron acceptors at 4 °C in a cold room. For storage at higher temperatures we used a controlled temperature shaker bath. Shaking was always used in the experiments at temperatures $>$ 4 °C.

RESULTS

Effect of fixation on electron transport activities

Fixation by glutaraldehyde causes a decrease of the electron transport activities of the chloroplasts. Fig. 1 shows how the rate of O_2 evolution, observed with various electron acceptors, varies with the concentration of the fixative. Ferricyanide reduction activity dropped fast in the concentration range $\leqslant 1\,\%$ but then approached a constant level of approx. 12 % of the control rate. The inhibition was half maximal with 0.6 % glutaraldehyde. With methyl viologen as the electron acceptor, the inhibition was still more severe and no significant residual rate was observed. The intensity dependence of the rate of the Hill reaction coupled to ferricyanide reduction is shown in Fig. 2A. In the fixed chloroplasts, the maximal rate was inhibited to 17 % of the control rate. In extremely weak light, however, the inhibition became less severe, declining to approx. 50 %.

We also measured the effect of fixation upon the activity of Photosystem I, viz. the reduction of methyl viologen with reduced DPIP or DAD as electron donors. As shown for DPIP in Fig. 2A, we found a degree and type of inhibition which was comparable to that of the overall Hill reaction with ferricyanide. In comparison, DAD yielded higher (3.5 times) rates in the unhibited system, but identical rates in treated material (i.e. a relatively stronger inhibition).

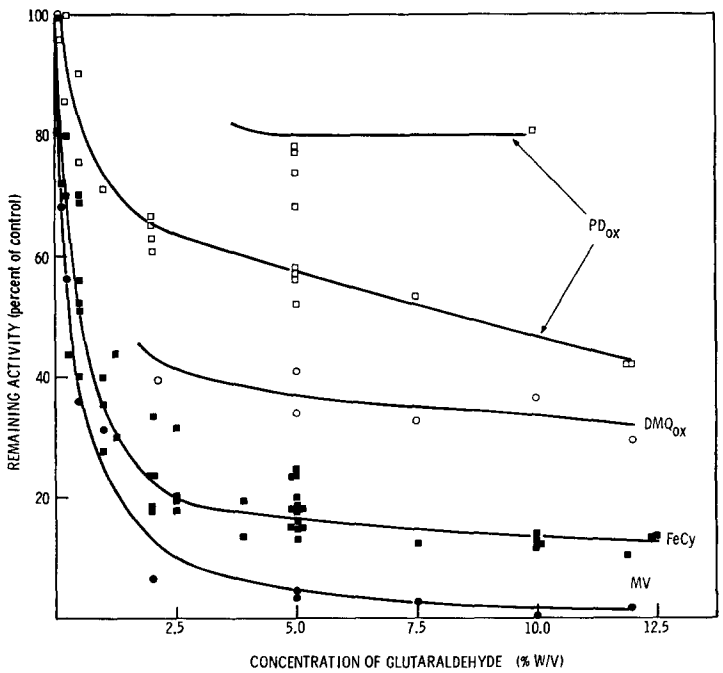


Fig. 1. Effect of glutaraldehyde concentration upon the rate of O_2 evolution using various electron acceptors. Fixation time 5 min. Data were obtained in several experiments made on different dates; the activity of the controls (100 %) varied somewhat and was usually highest with PD_{ox}. Temperature 22 °C.

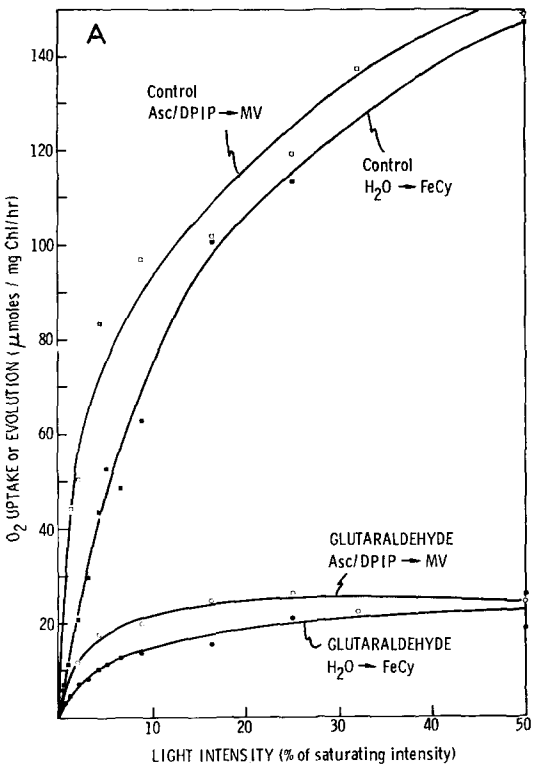


Fig. 2. For legend see opposite page.

Surprisingly, O_2 evolution was much less inhibited when we used lipophilic electron acceptors such as PD_{ox}, DAD_{ox}, and DMQ_{ox}, which, according to Saha et al. [12] bypass Photosystem I. Figs. 1 and 2B illustrate that in the presence of PD_{ox} the fixed chloroplasts showed 60–80 % of the control rate in strong light; also the quantum efficiency of PD_{ox} reduction in the fixed chloroplast was quite high, 90–100 % of the control. With the PD_{ox} and DAD_{ox} assays the degree of inhibition was rather sensitive to pH and the presence or absence of methylamine. For instance, using DAD_{ox} we found 40 % of the control rate at pH 9.0, and approx. 85 % of the control rate in the pH range 7.5–5.5.

A straightforward interpretation of the data in Figs. 1 and 2 is that glutaraldehyde blocks electron transport to (or through) Photosystem I and that the residual activity reflects the rate with which the various reagents can accept electrons directly from Photosystem II. For methyl viologen, this rate is zero; for PD_{ox}*, it is nearly normal. With ferricyanide it is 10–20 % of this rate and with DMQ_{ox}, approx. 30 % of the maximal velocity.

Presumably lipophilic acceptors penetrate more readily through the thylakoid

* The highest rates of O_2 evolution we have seen with this acceptor using various conditions were $\leq$ 530 O_2/chlorophyll per h. They exceeded the normal "uncoupled" rates with methyl viologen or ferricyanide, but by no more than 1.5 times. We therefore wonder whether PD_{ox} truly accepts electrons before the normal rate-limiting step (as tacitly implied by Saha et al. [12]).

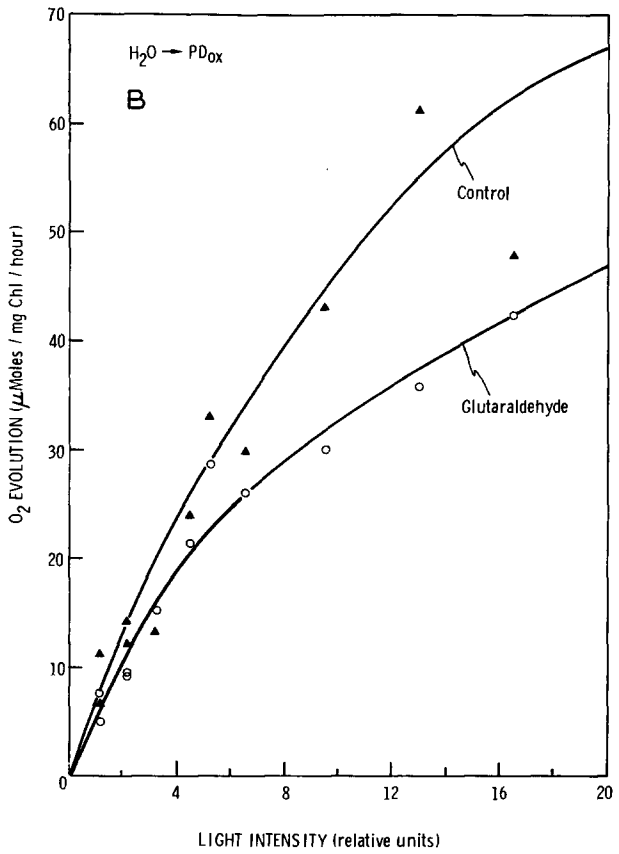
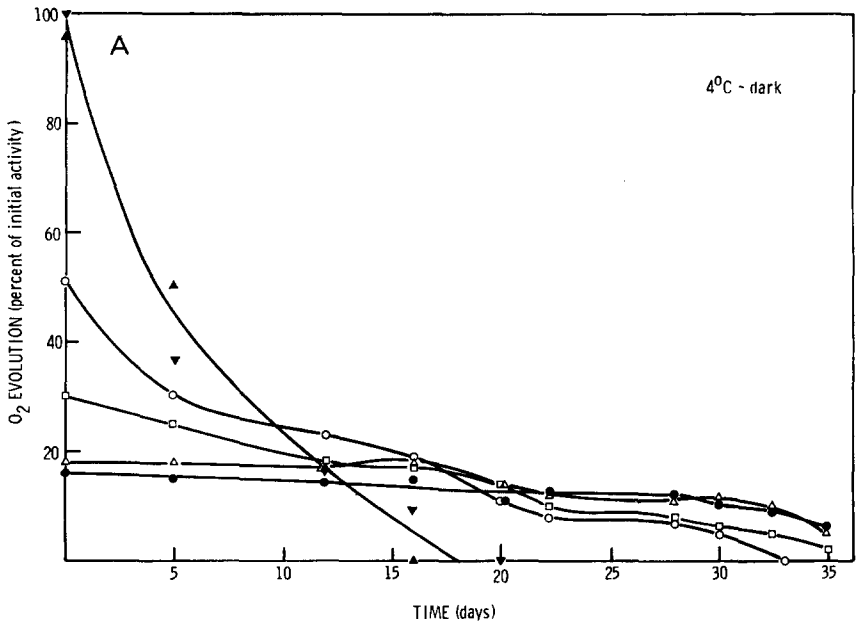


Fig. 2. Dependence of the rate of electron transport on the light intensity in fixed and in control chloroplasts at pH 7.4 and 22 °C. Fixation with 5 % glutaraldehyde during 5 min. (A) O_2 evolution coupled to ferricyanide reduction and O_2 uptake coupled to methyl viologen reduction using the ascorbate DPIP donor system were measured as described in Procedures. Maximal rates for O_2 evolution were 160 and 27, and for O_2 uptake 150 and 17 μmol/mg chlorophyll per h in the control and in the fixed samples, respectively. (B) O_2 evolution coupled to reduction of PD_{ox}. Maximal rates: 180 and 130 μmol O_2/mg chlorophyll per h in the control and in the fixed samples, respectively. No uncoupler was present.

membrane to the site of electron donation and, as illustrated in Table I through other membranes as well. Table I illustrates that these agents also readily penetrate the outer membrane of whole chloroplasts and blue green algae. Whole chloroplasts, which maintain their outer membrane during isolation, exhibit very low rates of O_2 evolution coupled to the reduction of ferricyanide. Breaking the outer membrane by osmotic shock makes these chloroplasts more permeable to ferricyanide and increases the rates of O_2 evolution 7–9 times [13, 16]. Table I shows that the intactness of the chloroplasts apparently did not prevent PD_{ox} and DAD_{ox} to sustain high rates. In whole cells of *An. nidulans* and *A. cylindrica*, PD_{ox} and DAD_{ox} substained high rates of O_2 evolution, considerably exceeding the rates of photosynthesis and ferricyanide reduction. However, in a few experiments made with the green algae



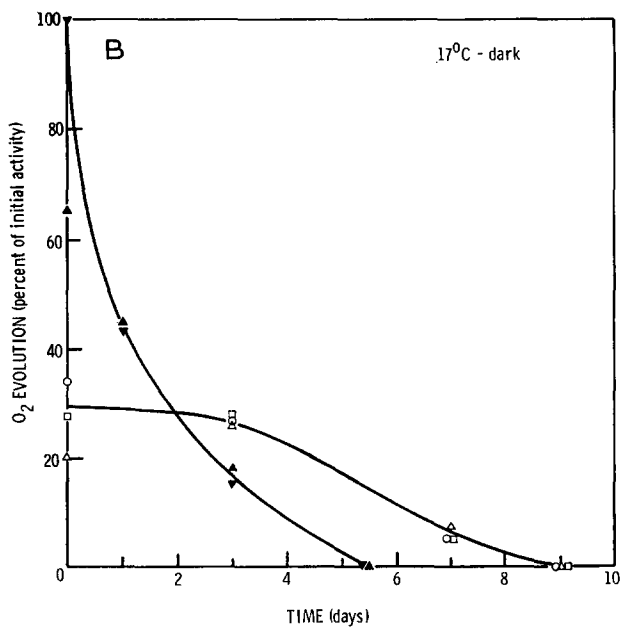


Fig. 3. Decay of O_2 evolution activity in chloroplasts stored in the dark. Assay with ferricyanide at pH 7.4 and 22 °C. ▼, non-treated chloroplasts; ▲, chloroplasts "treated" with sucrose/NaCl/Tris. Fixation during 10 min with glutaraldehyde: ○, 0.5 %; □, 1.25 %; △, 2.5 %; ●, 5.0 %. (A) Storage at 4 °C without shaking. Initial control rate 202 μmol O_2/mg chlorophyll per h. (B) Storage at 17 °C with shaking. Initial control rate 318 μmol O_2/mg chlorophyll per h.

TABLE I

O_2 evolution activities in whole cells of *An. nidulans* and *A. cylindrica* and in whole spinach chloroplasts with different electron acceptors. Rates are expressed in μmol O_2/mg chlorophyll per h and are average values of 2–4 observations.

Material	Electron acceptor				
	CO_2	PD_{ox}	DAD_{ox}	Ferricyanide	
Anacystis nidulans	103	304	180	–	
Anabaena cylindrica	208	440	315	–	
		Intact	Intact	Intact	After breakage
Whole chloroplasts		157	185	29	184

Scenedesmus sp., *Cl. vulgaris*, and *Chl. reinhardi*, no O_2 evolution was observed with these acceptors, probably indicating the impermeability of these cells.

Stability of Hill reaction activity at different temperatures

Initially, we repeated the experiments of Park et al. [3] who reported the stability of fixed chloroplasts at 4 °C. The data shown in Fig. 3A agree with their observations. In the control samples, the ferricyanide reduction activity declined monotonically with a half time of approx. 5 days. As discussed above, treatment with 2.5–5 % glutaraldehyde caused a severe loss of the rate. However, this surviving activity remained stable during more than 30 days. Treatment with lower concentrations of glutaraldehyde yielded higher rates immediately after fixation, but as seen in Fig. 3A the decay curves appeared biphasic, the lifetime of the "extra" activity being rather short and comparable to that of the control. No significant extension of the lifetime was obtained by varying the "exposure time" to the fixative, or the pH of the fixation medium between 6 and 8.

The effect on the stability of storage at higher temperature (17 °C) is illustrated in Fig. 3B. The kinetics of activity loss in the controls and in the fixed materials are quite similar to those seen at 4 °C, but in both cases, the decay is approx. five times faster. Storage at 25 °C further accelerated the decay half-life (approx. 1 day in the controls), but did not affect the mode of decline.

Figs. 4A and 4B show similar experiments in which activity was assayed with PD_{ox} as the electron acceptor. The rates immediately after fixation were higher and the decay curves monotonic. Glutaraldehyde slowed down the decline of the initial activity 2.5–3-fold at both temperatures. In the final phase of the decay of the fixed material, the rates with ferricyanide and PD_{ox} tend to become identical (compare Fig. 4A to Fig. 3A) as if the same rate limitation appeares in both assays. Evidently the damage reflected in the PD_{ox} decay curve occurs in the abbreviated electron transport chain from water to PD_{ox}. When most of the Photosystem II chains have become inoperative, electron flow to the quinone pool becomes slow enough to be handled by ferricyanide.

It has long been known that Photosystem I activity is relatively stable in vitro. In our experiments its half-life was approx. 10 times longer than that of Photosystem

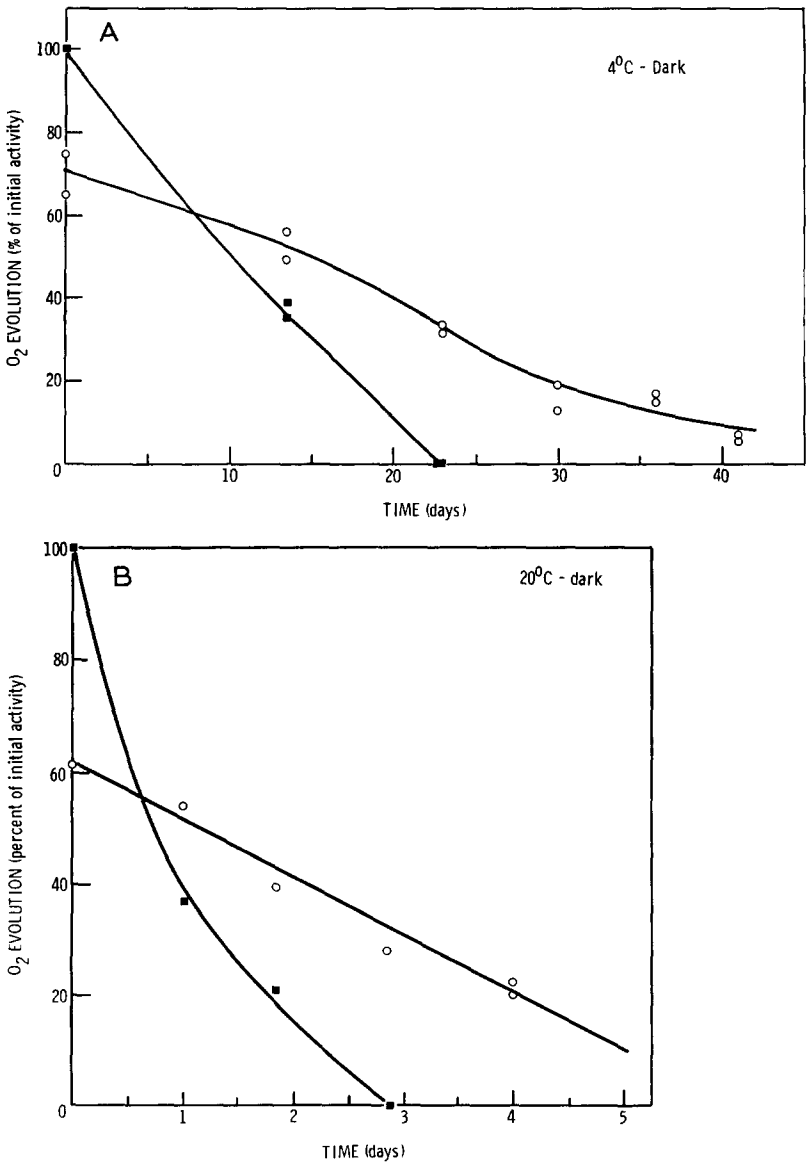


Fig. 4. Decay of O_2 evolution activity of chloroplasts stored in the dark. Assay with PD_{ox} without uncoupler at pH 7.4 and 22 °C. ■, control chloroplasts (treated with sucrose/NaCl/Tris); ○, fixed with 5 % glutaraldehyde during 5 min. (A) Storage at 4 °C without shaking. Initial control rate 181 μmol/mg chlorophyll per h. (B) Storage at 20 °C with shaking. Initial control rate 183 μmol O_2/mg chlorophyll per h.

II. Glutaraldehyde treatment severely inhibits the Photosystem I assay (see Fig. 2A) but still enhances the stability of the remaining activity. For instance, at 22 °C in the dark the half-life of the activity was more than 11 days in fixed chloroplasts as compared to about 5 days in the control.

This 2–3-fold increase of half-life was also observed in other oxygen-evolving systems, those assayed with lipophilic acceptors, for instance. With *Anacystis* cells stored in dark at 21 °C, fixation by a 5 min exposure to 5 % glutaraldehyde increased the half-life of this activity from 4.5 days to 7 days. In intact chloroplasts stored in the dark at 4 °C, this fixation increased the half-life of O_2 evolution from 3.5 to 5.5 days. In chloroplast particles prepared by breaking the fixed chloroplasts with a Yeda press (140 kg/cm^2, two successive passages), the half time of O_2 evolution was 8 days at 4 °C compared to 3.5 days in particles from control chloroplasts.

DISCUSSION

Some of the serious problems encountered by Park et al. [3–7] in their studies on the glutaraldehyde fixation of chloroplasts and algae were the reduced rates and efficiencies of both Photosystem II and Photosystem I. A satisfactory way to bypass this rate limitation and to obtain high rates of O_2 evolution even at high light intensity was found by using lipophilic electron acceptors (Figs. 1, 2A and 4).

As shown in Table I we found that lipophilic acceptors readily permeate into whole cells of blue-green algae and intact chloroplasts (but not green algae). This is similar to the well-documented permeability of benzoquinone in algal cells (c.f. Warburg and Luttgens [17]). Saha et al. [12] suggested that the lipophilic acceptors are reduced via a site close to Photosystem II, whereas hydrophilic acceptors such as ferricyanide and methyl viologen are reduced by Photosystem I. The residual rates in Fig. 1 in this paper probably reflect the velocity with which the various acceptors can react with the photoreductant of Photosystem II. This also explains the high (46 %) residual rate of DPIP reduction which was reported by Oku et al. [8].

It seems a bit surprising that Photosystem II and the O_2-evolving system (as well as Photosystem I, to be discussed in a later paper), which one suspects to contain several proteins and to require membrane integrity, are oblivious to > 10 % glutaraldehyde. Our data indicate that the inhibition caused by glutaraldehyde is not due to a general "fixation" of the membrane structure possibly yielding a decreased permeability to hydropilic agents. Rather, the inhibition appears to be due to a specific interaction with a carrier in the electron transport chain between the two photoacts or with Photosystem I. In a subsequent paper we will present evidence for the second alternative.

Although different samples of glutaraldehyde were used, the "dose" (concentration × exposure time) of glutaraldehyde needed to decrease the electron transport rate from water or from reduced DPIP to methyl viologen is quite similar to that reported by West and Packer [10] (approx. 0.5 % for 5 min, see Fig. 1). These authors further reported rather similar dose requirements for the inhibition of the light-induced scattering changes and other sensitive chloroplast reactions. Peculiarly, as illustrated in Fig. 5, the stabilizing and inhibiting effects of glutaraldehyde showed a very similar dependence upon concentration (and time of exposure). In Fig. 5 both effects are half maximal at a concentration of approx. 0.6 % glutaraldehyde. Quite possibly this similarity is more than coincidental and indicates that the mechanism(s) underlying the two actions are closely correlated.

Finally, concerning the stabilizing effect of glutaraldehyde and considering the fact that at room temperature the half-life of chloroplast activity is only a few hours,

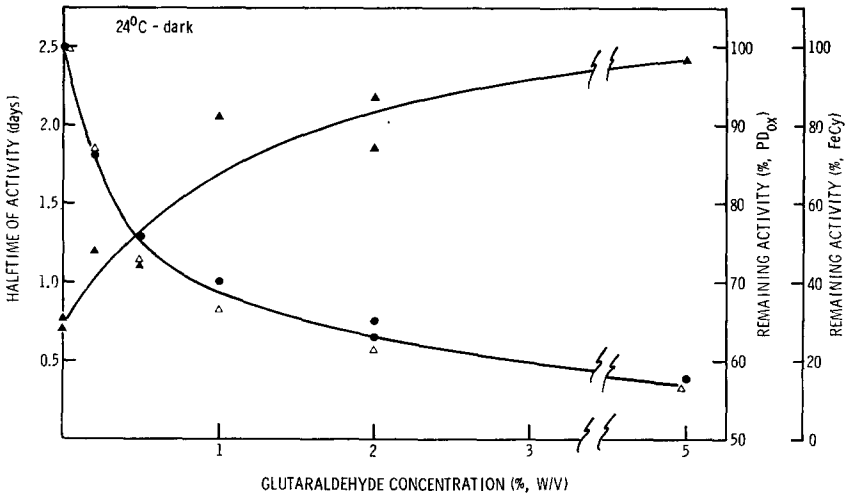


Fig. 5. Effect of glutaraldehyde concentration during a 5 min fixation on: (1) the activity remaining immediately after treatment, assayed with PD_{ox} (●) and with ferricyanide (△); (2) the half time of the decay of activity during storage in the dark at 21 °C with shaking, assayed with PD_{ox} (▲).

the observed 2–3-fold gain of half-life (at the price of a rate inhibition) may not appear very impressive. However, this gain is real and compares favorably with the small effects of imidoester bifunctional reagents such as were recently reported by Packer et al. [18]. In all likelihood, stabilization of entire organellae such as chloroplasts will require several measures simultaneously.

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