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The effect of Glutaraldehyde on Light-Induced H^+ Changes, Electron Transport, and Phosphorylation in Pea Chloroplasts

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Introduction

Glutaraldehyde-fixed organelles partially retain electron and energy-transfer activity; chloroplasts retain light-induced electron transport activity[1, 2, 3, 4] and mitochondria retain substrate oxidation.[5] Evidence for the retention of energy-transfer activity has come from the demonstration of electron-transport-dependent H^+ uptake in chloroplasts[2] and H^+ production associated with Ca^{2+} uptake by mitochondria which are still sensitive to inhibition by uncoupling agents.[5] Since mitochondria and chloroplasts given such treatment lose the capacity to manifest volume changes by osmotic perturbations, they also lose the capacity to contract or expand in association with efflux or accumulation of osmotically active ions.[2] However, recent studies of Treffry[6] showed that photochemical reactions at a molecular level, like the photoconversion of protochlorophyllide, are still retained even after glutaraldehyde fixation. Hallier and Park[3, 4] have also studied the effect of aldehyde treatment of chloroplasts on selective inhibition of the photosystems.

An interesting feature of the earlier studies of Utsumi and Packer[5] was that energized calcium uptake of fixed rat liver mitochondria was not accompanied by stimulation of electron-transport activity, whereas unfixed mitochondria generally show stimulation in the presence of ion transport. This capacity to stimulate respiration in mitochondria is known as respiratory control.[7] Stimulation of electron transport in chloroplasts by phosphorylation cofactors was termed photosynthetic control.[8] It was therefore of interest to study the effect of glutaraldehyde treatment on isolated chloroplasts which retain the capacity to manifest photosynthetic control. Moreover, a detailed study of the effect of glutaraldehyde on chloroplast reactions has not yet been reported, and this investigation has revealed some new and interesting features of this activity.

Experimental

Chloroplasts were prepared from pea plants (*Pisum sativum* L. var. Laxton's Superb) grown for two to three weeks in a growth chamber. Peas were grown in moist vermiculite

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and illuminated 12 h out of 24 by five 40-W Ecko daylight fluorescent tubes. Temperatures were 20° C in the light period and 16° C during darkness.

Eight- to fifteen-grams of leaves were ground in a chilled mortar with 40–90 ml grinding medium containing 100 mM choline–chloride, 5 mM TrisHCl, pH 7·8. The homogenate was strained through four layers of absorbent muslin and centrifuged at 5000 × *g* for 2 min. The pellet was washed once in 20 ml cold 100 mM choline–chloride, 0·5 mM Tris resuspending medium (RM) and respun at 5000 × *g* for 2 min. The pellet was finally suspended in 1–2 ml RM to give a chlorophyll concentration of 1·5–3 mg/ml. This gives a preparation of predominantly outer membrane-stripped chloroplasts. Chlorophyll was determined by the method of Arnon.[9]

For aldehyde treatment, 0·5–0·75 ml of chloroplast suspension was used with the appropriate aldehyde concentration in 1–2 ml RM at 0° C. After 6 min, 10–12 ml cold medium were added and the chloroplasts spun at 6000 × *g* for 5 min. The tight pellet was gently rinsed and resuspended in RM using a glass rod. Fresh chlorophyll determinations were made.

Reactions were aerobic and carried out at 20° C. Illumination was from two Aldis 150-W QI 24 (tungsten iodide) projectors giving about 20,000 lux. Red light was from Balzers K6 broad-band interference filters with peak transmission at λ680 nm.

Oxygen production was measured polarographically as described by West and Hill,[10] using a 1-mV Rikadenki recorder. The 5-ml reaction mixtures contained 100 mM choline–chloride, 0·5 mM TrisHCl, pH 7·8, 2 mM $MgCl_2$, 4 mM phosphate, 3 μmoles potassium ferricyanide, 3 μmoles ADP, and 100 μg chlorophyll, unless otherwise stated.

Proton movements were assayed in an adjacent cuvette with a Radiometer combination pH electrode G202C and a PH26 pH meter using the second channel of the recorder on range 1 or 2·5 mV. The 5-ml reaction mixtures contained 100 mM choline–chloride, 0·5 mM TrisHCl, pH 7·8, 2 mM $MgCl_2$, $2{\cdot}4 \times 10^{-5}$ M PMS (phenazine methosulphate), and 100 μg chlorophyll, unless otherwise stated. Calibrations with standard HCl were made before and after the addition of any reagents, e.g. ADP and salts of weak acid anions which changed the buffering capacity of the medium.

PMS-catalysed cyclic photophosphorylation was assayed by the H^+ uptake technique used by Nishimura and Chance.[11] The initial rapid burst of H^+ uptake which occurs independently of the presence of phosphorylation conditions, as stressed by Schwartz,[12] was ignored.

Direct comparisons of cyclic and non-cyclic phosphorylation was made by following the incorporation of ^{32}P into organic phosphates. These were separated from inorganic phosphates by the method of Hagihara and Lardy,[13] 0·1-ml samples from the cuvette were withdrawn at intervals. After removal of inorganic phosphate, 0·5-ml samples were dried on planchettes and the radioactivity counted in a Nuclear Chicago gas-flow counter.

The extent of fixation was assessed from the osmotic properties of the chloroplasts.[2] 0·3-ml samples from the reaction cuvettes were added to 2·7 ml of 0, 50, 100, and 200 mM NaCl solutions. Fixed chloroplasts showed no changes in O.D. at λ546 nm. For comparative purposes the total difference in O.D. of the four samples is given, and is referred to as the extent of fixation.

Glutaraldehyde was obtained from Polysciences Inc., Warrington, Pa. The malondialdehyde (sodium salt) was kindly given by Dr. A. Horton.

Results and Discussion

The effect of phosphorylation cofactors and uncoupling agents on ferricyanide reduction by control chloroplasts, and those completely fixed by 200 mM glutaraldehyde for 6 min is shown in Table I. The normal control chloroplasts had good photosynthetic

TABLE I. Effect of phosphorylation cofactors and uncouplers on ferricyanide reduction in control and fixed chloroplasts

	μmoles oxygen evolved per milligramme chlorophyll per hour					
	(a)		(b)		(c)	
	Control	Fixed	Control	Fixed	Control	Fixed
No ADP plus P_i	55 (4)	37 (3) 67%	61 (4)	44 (3) 72%	42 (2)	30 (1) 71%
Plus ADP plus P_i	90 (3)	38 (3) 42%	148 (2)	44 (3) 30%	76 (2)	29 (1) 38%
Plus NH_4Cl	112 (2)	49 (2) 44%	186 (2)	66 (2) 36%	84 (1)	34 (2) 40%
Plus cyclohexylamine	92 (2)	44 (1) 48%	156 (2)	52 (2) 33%	—	—
Plus methylamine	—	—	—	—	92 (1)	36 (2) 39%
Plus FCCP	103 (2)	54 (2) 52%	152 (3)	57 (2) 38%	—	—

The fixed chloroplasts were treated with 200 mM glutaraldehyde for 6 min. Other conditions are given in the experimental section, with the following additions: NH_4Cl, cyclohexylamine and methylamine, all 2 mM for control chloroplasts and 1 mM for fixed chloroplasts; FCCP, 2×10^{-6} M for control chloroplasts and 1×10^{-6} M for fixed chloroplasts. Figures in parentheses indicate the number of measurements from which the data were averaged. The activities of the fixed chloroplasts are also given as a percentage of those of the control chloroplasts under the same conditions. (a), (b), and (c) were different chloroplast preparations.

control. Electron transport was stimulated 63–140% by ADP plus phosphate, and the addition of the uncouplers gave slightly higher rates of oxygen production. By contrast, photosynthetic control in fixed chloroplasts was completely abolished: ferricyanide reduction was not stimulated by phosphorylation cofactors. However there was a small stimulation by the uncoupling agents. The proportion of the activity retained by the fixed chloroplasts was a maximum of about 70% of the control chloroplasts under non-phosphorylating conditions. This decreased sharply to an average of about 40% under phosphorylating conditions and in the presence of uncouplers.

Table II shows the effect of fixation on light-induced H^+ changes. Data from a number of experiments were averaged. The presence of 2 mM-sodium phosphate increased the

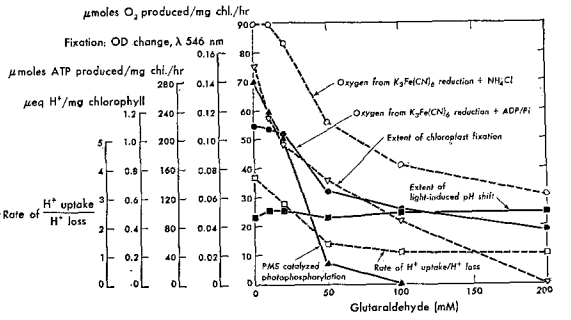

Figure. 1. Effect of glutaraldehyde concentration on chloroplast reactions. Procedure as given in experimental section. Ferricyanide reduction was uncoupled with 1 mM NH_4Cl. The rates of active H^+ uptake and passive H^+ efflux were determined from the initial slopes of the H^+ changes following illumination and the return of the chloroplasts to darkness. Chloroplasts were treated with aldehyde at the concentrations indicated for 6 min at 0°.

values slightly. Fixation reduced the extent of the pH shift by about 10% with a maximum inhibition of 25%, although some preparations showed no inhibition at all (e.g. Fig. 1). The pH changes in fixed chloroplasts were still sensitive to uncoupling agents

TABLE II. Effect of glutaraldehyde fixation on light-induced H^+ changes

	Extent of proton changes (microequivalents H^+ per milligram chlorophyll)	
	Without phosphate	Plus 2 mM phosphate
Control chloroplasts	0·47 (11)	0·50 (6)
Fixed chloroplasts	0·42 (11)	0·45 (6)

	Rates of proton change (microequivalents H^+ per milligramme chlorophyll per minute)					
	Without phosphate			Plus 2 mM phosphate		
	Uptake	Loss	Ratio	Uptake	Loss	Ratio
Control chloroplasts	3·8	1·3	2·98 (8)	4·5	1·6	2·8 (5)
Fixed chloroplasts	3·2	2·3	1·4 (8)	2·5	2·3	1·1 (5)

Conditions are as given in the experimental section. The fixed chloroplasts were treated with 200 mM glutaraldehyde for 6 min. The rates of active H^+ uptake and passive H^+ efflux were determined from the initial slopes of the H^+ changes following illumination and return of the chloroplasts to darkness. Figures in parentheses indicate the number of experiments from which data were averaged.

TABLE III. Effect of weak acid anions on light-induced H^+ changes in control and fixed chloroplasts

	Light-induced H^+ changes (microequivalents H^+ per milligramme chlorophyll)			
Sodium phosphate concentration	2 mM		2 mM	
Sodium succinate concentration	6 mM		20 mM	
Sodium acetate concentration	6 mM		20 mM	
Chloroplasts	Control	Fixed	Control	Fixed
No additions	0·42	0·33	0·41	0·32
Phosphate	0·52	0·38	0·49	0·34
Succinate	0·49	0·42	0·38	0·35
Acetate	0·82	0·43	0·49	0·34
Phosphate plus succinate	0·49	0·30	0·40	0·33
Phosphate plus acetate	0·66	0·37	0·45	0·32
Acetate plus succinate	—	—	0·40	0·28

Conditions are as given in the experimental section. Fixed chloroplasts were treated with 200 mM glutaraldehyde for 6 min.

TABLE IV. Effect of weak acid anions on the rates of active H^+ uptake and passive H^+ loss of control and fixed chloroplasts

	Rates of light-induced H^+ changes (microequivalents H^+ per milligramme chlorophyll per minute)											
Phosphate concentration	2 mM						2 mM					
Succinate concentration	6 mM						20 mM					
Acetate concentration	6 mM						20 mM					
Chloroplasts	Control			Fixed			Control			Fixed		
	Uptake	Loss	Uptake / Loss	Uptake	Loss	Uptake / Loss	Uptake	Loss	Uptake / Loss	Uptake	Loss	Uptake / Loss
No additions	4·2	1·1	3·8	2·7	1·7	1·6	3·1	0·9	3·4	2·3	2·0	1·2
Plus phosphate	5·8	2·0	2·9	2·0	2·2	0·9	3·9	1·8	2·2	1·7	2·4	0·7
Plus succinate	4·7	1·7	2·8	2·5	2·6	1·0	4·7	1·5	3·2	2·4	2·4	1·0
Plus acetate	9·3	2·9	3·2	2·8	2·7	1·0	6·6	1·9	3·5	2·3	2·3	1·0
Phosphate plus succinate	7·2	3·0	2·4	1·4	2·0	0·7	4·8	2·1	2·3	2·1	2·3	0·9
Phosphate plus acetate	8·3	2·9	2·9	1·5	2·0	0·8	4·1	2·8	1·4	1·3	1·8	0·7
Acetate plus succinate	—	—	—	—	—	—	6·0	1·6	3·6	1·6	1·8	0·9
	Average		3·0			1·0			2·8			0·9

Conditions are as given in the experimental section. Fixed chloroplasts were treated with 200 mM glutaraldehyde for 6 min.

and were abolished by 1–2 mM NH_4Cl and cyclohexylamine and by 2–3 $\times$ 10^{-6} M FCCP (carbonylcyanide *p*-trifluoromethoxyphenylhydrazone).

The effect of fixation on the kinetics of the H^+ changes are also shown in Table II. Here a striking result was obtained. Normal chloroplasts showed a rate of active H^+ uptake in the light about three times that of the passive dark efflux. Glutaraldehyde reduced the rate of uptake but stimulated the rate of efflux, giving an average ratio of 1 : 4. This decreases to 1 : 1 in the presence of 2 mM phosphate. The effect of several types of weak acid anion on light-induced H^+ changes in control and fixed chloroplasts are shown in Tables III and IV. The extent of the H^+ changes was slightly increased by 2 mM sodium phosphate and 6 mM sodium acetate and sodium succinate. This was more apparent in control than in fixed chloroplasts. 20 mM acetate and succinate gave no increases. By contrast, the rates of H^+ uptake and loss were stimulated in control chloroplasts by the weak acid anions. With fixed chloroplasts these anions tended to reduce the rate of H^+ uptake but stimulate the passive proton loss, and the ratio of uptake to loss fell to unity or below.

The effect of glutaraldehyde concentration on electron energy transfer parameters in pea chloroplasts is shown in Fig. 1. Several activities were assayed in the same preparation under similar conditions.

The activity most sensitive to glutaraldehyde treatment was photophosphorylation.

This was completely inhibited by 50–100 mM glutaraldehyde. Such concentrations were insufficient to cause fixation which was not complete until 200 mM glutaraldehyde was reached. Cyclic and non-cyclic photophosphorylation were affected to the same extent, as shown in Fig. 2.

The rate of ferricyanide reduction with ADP plus phosphate and also uncoupled with NH_4Cl, progressively decreased with glutaraldehyde concentration. The fixed chloroplasts retained about 35% of their ammonia-stimulated activity. These results are similar to those in Table I, although the fixed chloroplasts from this preparation were more stimulated by NH_4Cl than was usually the case.

The extent of the pH shift was not affected by fixation in this experiment although average values from other experiments indicated about 10% inhibition (Table II).

The ratio of H^+ uptake to H^+ efflux was progressively affected by glutaraldehyde concentration, giving a value approaching unity at 100 mM glutaraldehyde. This curve closely followed the inhibition of phosphorylation.

To distinguish between the specific effects of glutaraldehyde and those of aldehyde in general, the reactions shown in Fig. 1 were repeated after treating the chloroplasts with acetaldehyde or malondialdehyde (Fig. 3). These aldehydes seemed to have little effect on the osmotic properties of the chloroplast, and did not fix. Chloroplast reactions remained unchanged by up to 0·6 M acetaldehyde. Malondialdehyde gave a progressive small reduction in ferricyanide-catalysed electron transport and cyclic photophosphorylation. In this case, however, the rates of change of H^+ remained unaffected.

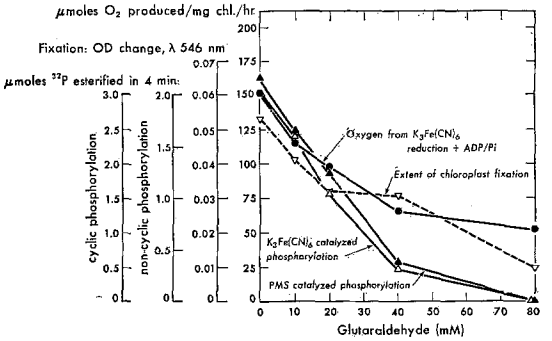

Figure 2. Effect of glutaraldehyde concentration on cyclic and non-cyclic photophosphorylation. Reaction mixtures contained 100 mM choline–chloride, 10 mM TrisHCl, pH 7·8, 2 mM $MgCl_2$, 6 mM phosphate, 2 mM ADP and either $2{\cdot}4 \times 10^{-5}$ M PMS or 6 μmoles of $K_4Fe(CN)_6$. Samples for ^{32}P incorporation measurements were taken after 0, 2, and 4 min illumination. Phosphorylation rates were linear over this period. Chloroplasts were treated with aldehyde at the concentrations indicated for 6 min at 0°.

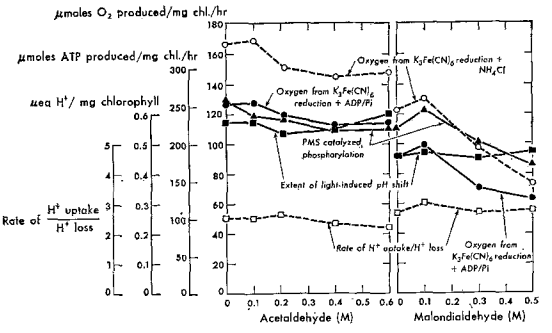

Figure 3. Effect of acetaldehyde and malondialdehyde on chloroplast reactions. Conditions and reaction mixtures as in Fig. 1.

The sensitivity of the phosphorylation reactions to glutaraldehyde suggests a special accessibility either in terms of functional groups or locations of phosphorylation enzymes to glutaraldehyde action. Alternatively, phosphorylation may be dependent on a differentially permeable state of the membranes which is altered by glutaraldehyde before complete fixation occurs. Inhibition of phosphorylation would account for inability of phosphorylation cofactors to stimulate electron transport in fixed chloroplasts. Glutaraldehyde inhibits basal (non-phosphorylating) electron transport and completely

inhibits phosphorylation-stimulated electron transport, although the fixed chloroplasts remain slightly susceptible to stimulation by uncoupling agents. It is difficult to relate this action to that of known phosphorylation inhibitors.

Taken together, the results indicate that glutaraldehyde treatment reduces the capacity of chloroplasts to show a stimulation of either H^+ uptake activity or electron transport activity under conditions which readily do so in control chloroplasts, such as the presence of weak acid or weak base ions or uncoupling agents. This lack of "photosynthetic control" may reflect the inability of treated chloroplasts to change their volume, i.e. to accumulate massive quantities of weak base cations or extrude weak acid anions under conditions of illumination, where the uptake of H^+ shifts the concentration of undissociated forms of these species inside the chloroplasts causing a redistribution of the freely permeable unchanged species across the chloroplast membrane.[2]

These results suggest, as have studies by Horton and Packer[14] with tetraphenylboron, and by Dilley and Rothstein,[15] and Murakami and Packer[16] that chloroplast surface charge may play an important role in the permeability of chloroplasts to ions.

Glutaraldehyde fixation (200 mM) apparently changes the capacity of the chloroplasts for electron transport relative to proton transport. Fixation gives about 37% inhibition of basal (non-phosphorylating) electron transport but only about 10% inhibition of the extent of proton changes. However, if a distinction is made between those changes which are glutaraldehyde resistant and those which are not, results may not be incompatible with the suggestion[17–18] that light-induced electron transport and H^+ gradients are directly linked.

Summary

This investigation has shown the following hierarchy of chloroplast reactions which were susceptible to increasing glutaraldehyde concentration given for 6 min at 0° C.

(1) Cyclic and non-cyclic photophosphorylation was completely inhibited by 100 mM glutaraldehyde.
(2) Reduction in the rate of H^+ uptake, and increase in the rate of H^+ efflux occurred to give a ratio uptake/loss of unity with 100 mM glutaraldehyde.
(3) Volume fixation occurred at 200 mM glutaraldehyde.
(4) Fixed chloroplasts were resistant to stimulation of H^+ and electron transport by weak acid anions and uncouplers.
(5) Light-induced electron-transport activity progressively decreased. About 35% inhibition of the basal (non-phosphorylating) rate and 60% inhibition of the uncoupled rate occurred on fixation.
(6) The extent of the light-induced H^+ changes were reduced only by about 10% upon fixation.

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