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LIGHT-DEPENDENT STRUCTURAL CHANGES IN THE LAMELLAR MEMBRANES OF ISOLATED SPINACH CHLOROPLASTS: MEASUREMENT BY ELECTRON MICROSCOPY

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SUMMARY

1. The distance between the lamellar membranes of isolated spinach chloroplasts fixed by glutaraldehyde–acrolein in the dark or after varying periods of illumination was measured by electron microscopy.

2. The interlamellar distance decreased on illumination by 18.6% (215 ± 19 to 175 ± 24 Å).

3. The dependence of structural change on illumination time was similar to the dependence of two-stage photophosphorylation on illumination time, suggesting a relationship between structural change and the generation of high energy intermediate(s) in ATP formation.

4. The light-dependent decrease in interlamellar distance was 68% inhibited by $1 \cdot 10^{-4}$ M carbonyl cyanide *m*-chlorophenylhydrazone.

5. The light-dependent increase in 90° light scattering by isolated chloroplasts was accompanied by 11.0% decrease in interlamellar distance.

INTRODUCTION

Illumination causes a decrease in spacing between the lamellar membranes of the spinach chloroplast; this has been demonstrated both by electron microscopy on positively stained material[1–3] and by the freeze–etch technique[4]. Measurement of this light-dependent structural change by electron microscopy was recently reported by MURAKAMI AND PACKER, both for isolated spinach chloroplasts in different light scattering states[5] and for chloroplasts in whole algal cells[6].

In this study, we attempted to obtain a quantitative comparison between structural change and the generation of early, light-dependent intermediates in photophosphorylation. The illumination time dependence of structural change, measured by electron microscopy, was compared with the illumination time dependence of high energy intermediate (X_e) formation in the two-stage photophosphorylation of HIND AND JAGENDORF[7]. The effect of the photophosphorylation uncoupler, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), on structural change was also

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; X_e, high energy intermediate in photophosphorylation.

determined. In addition, the illumination time dependence of the light-induced light scattering response was measured and compared with the illumination time dependence of structural change.

This work has been described in detail in a thesis[8] and in abstract form in *Proc. Natl. Acad. Sci. U.S.*, 66 (1970) 246.

MATERIALS AND METHODS

Chloroplasts were isolated from market spinach by a procedure modified from KALBERER *et al.*[9]. Electron microscopy showed that most of the chloroplasts had lost their outer membranes; the inner membrane system generally was intact but was partially fragmented into single grana. Chlorophyll was determined by the method of VERNON[10].

The illumination time dependence of X_e formation was measured by post-illumination formation of ATP as previously described[7], except that reactions were terminated by addition of $HClO_4$, and the uptake of ^{32}P labelled inorganic phosphate into organic phosphate was determined as described by LINDBERG AND ERNSTER[11].

Chloroplasts were fixed for electron microscopy by rapid addition of a glutaraldehyde–acrolein mixture by syringe to the illuminated mixture. The final concentration of fixative was 1% glutaraldehyde and 1% acrolein. The chloroplasts were approximately 20 min older when fixed for electron microscopy than when their phosphorylating activity was terminated.

Additional data[8] (not reported here) were obtained using a rapid-mixing flow apparatus constructed to run the photophosphorylation and fixation experiments simultaneously. Chloroplasts fixed in this apparatus showed approximately one-half the light-dependent decrease in interlamellar distance shown by chloroplasts fixed as described above.

The light-dependent increase in 90° light scattering was measured in a Brice-Phoenix light scattering photometer, as described by DILLEY and VERNON[12]. Chloroplasts were fixed for electron microscopy by addition of a glutaraldehyde–acrolein mixture (1% final concentration of each) to the chloroplasts in the photometer cuvette, either at the peak of the light scattering response (after about 2 min illumination) or after turning off the light and allowing the signal to return to essentially the dark scattering level. The light to dark difference in signal decreased by approx. 50% of its initial value during the 50-min period required to fix sufficient chloroplast material for electron microscopy. The light-induced percent increase in scattering was calculated on the basis of the dark level of scattering as 100%.

The light intensity in photophosphorylation experiments (and in corresponding experiments in which chloroplasts were fixed for electron microscopy) was 6500 ± 200 ft-candles, which corresponded to a total radiant energy of $5 \cdot 10^5$ erg/cm^2·sec. In light scattering experiments, the intensity of the red, actinic beam was $5 \cdot 10^4$ erg/cm^2·sec.

Chloroplasts were prepared for electron microscopy by standard procedures, as modified by GREEN *et al.*[13], and thin sections were examined in a Siemens I electron microscope. The magnification, which ranged from 34000 ± 800 to 48200 ± 1300, was calibrated using a carbon replica of a diffraction grating with crossed lines (2160 lines/mm, E. F. Fullam).

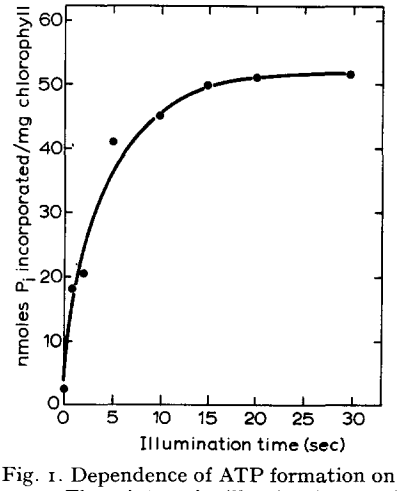
162 Groups of lamellar membranes were photographed and measured during the study reported here. Whole or partially fragmented grana (containing from 3 to 30 lamellar membranes per granum or granum fragment) were photographed for measurement. Occasional grana showing obvious signs of damage were excluded from measurement. Measurements obtained by microcomparator from the original negatives were converted to center-to-center distances between lamellar membranes, in Ångstroms ("interlamellar distance").

The *t* test was applied to determine whether the observed differences in mean interlamellar distances were statistically significant.

RESULTS

Photophosphorylation

With each experiment in which chloroplasts were fixed for electron microscopy, a corresponding two-stage (preillumination and dark stages) photophosphorylation experiment was conducted on the same chloroplasts. The results of a representative two-stage photophosphorylation are shown in Fig. 1. The experiment of Fig. 1 was carried out as described by HIND AND JAGENDORF[7], whereas the photophosphorylation experiments corresponding to the electron microscope data reported below were conducted using the flow apparatus. The phosphorylation data obtained using this apparatus seldom followed such smooth curves as shown in Fig. 1; however, the shape of the curve was essentially the same in all experiments, in that phosphoryla-



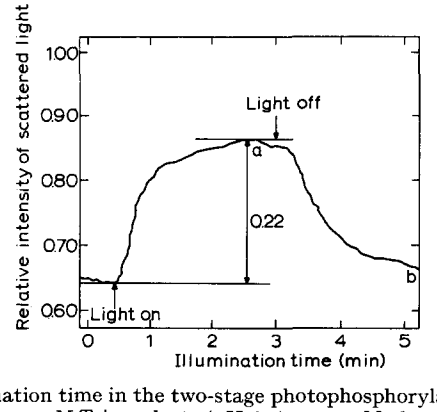


Fig. 1. Dependence of ATP formation on illumination time in the two-stage photophosphorylation assay. The mixture for illumination consisted of 1.7 mM Tris maleate (pH 6.0), 0.03 mM phenazine methosulfate, 67 mM NaCl and chloroplasts containing 74 μg chlorophyll/ml in 1.5 ml. The dark mixture contained 4.0 mM Tris chloride (pH 8.0), 67 mM NaCl, 4.0 mM $MgCl_2$, and 1.34 mM ADP and 1.34 mM P_i containing $1.3 \cdot 10^7$ counts/min ^{32}P per ml in 1.5 ml. The mixture containing chloroplasts and phenazine methosulfate was suspended in a syringe, illuminated, and injected into the dark mixture, as described by HIND AND JAGENDORF[7]. The pH of the combined mixture was 7.6.

Fig. 2. Light-induced increase in light scattered at 90° by isolated spinach chloroplasts. The suspension consisted of 0.5 mM Tris maleate (pH 6.0), 0.3 mM phenazine methosulfate, 64 mM NaCl and chloroplasts containing 15 μg chlorophyll per ml in 3.0 ml. The pH of the suspension was 6.3. In experiments for electron microscopy, fixative was added at a or b (see METHODS).

tion reached its half-maximal value during 3–5 sec illumination and its maximal value after 15–20 sec illumination.

Chloroplasts illuminated 20 sec in the presence of $1 \cdot 10^{-4}$ M CCCP showed 97% inhibition of photophosphorylation.

Light scattering

The dependence of the light-induced increase in 90° light scattering on illumination time is shown in Fig. 2. The 31.5% light-induced increase shown in Fig. 2 was obtained at the beginning of an experiment in which chloroplasts were fixed for electron microscopy; corresponding interlamellar distances are given later.

Data from electron microscopy

The data from electron microscopy are summarized in Table I. Part I gives the interlamellar distances found in samples fixed under two-stage photophosphorylation conditions. To obtain a direct comparison between the level of energized intermediate(s) formed in the photophosphorylation assay and the structural state of the membrane, samples were fixed for electron microscopy after illumination for periods corresponding to the abscissa in Fig. 1. Therefore, the distribution of membrane dimensions reported in Table I (part I) for each illumination time corresponds to the level of X_e formation at the same illumination time in Fig. 1.

As shown in Table I, samples illuminated for 5 sec had a smaller mean interlamellar distance (202 Å) than the dark control (215 Å). Thus, illumination for 5 sec caused a 6.0% decrease in interlammellar distance. Samples illuminated under the same conditions for 15, 20, 25 or 30 sec had significantly smaller interlamellar distances than samples illuminated only 5 sec but showed no systematic decrease in interlamel-

TABLE I

INTERLAMELLAR DISTANCES IN DARK AND ILLUMINATED CHLOROPLASTS: DATA FROM ELECTRON MICROSCOPY

Experiment	*Illumination time before fixation (sec)*	*Number of measurements*	*Interlamellar distance (Å)*	*Light-dependent decrease (%)*
I. Two-stage photophosphorylation	0	33	215 ± 19*	0.0
	5	23	202 ± 15	6.0
	15	28	175 ± 22**	
	20	12	190 ± 20	
	20 (+CCCP)	12	202 ± 13	6.0
	25	20	160 ± 23	
	30	11	190 ± 17	
	15–30 (av.)	71	175 ± 24*	18.6
II. Light scattering	0	21	164 ± 23	0.0
	120	36	146 ± 18	11.0

* Average of data from two experiments.
** Data from Expt. A; data not marked by an asterisk are from Expt. B.

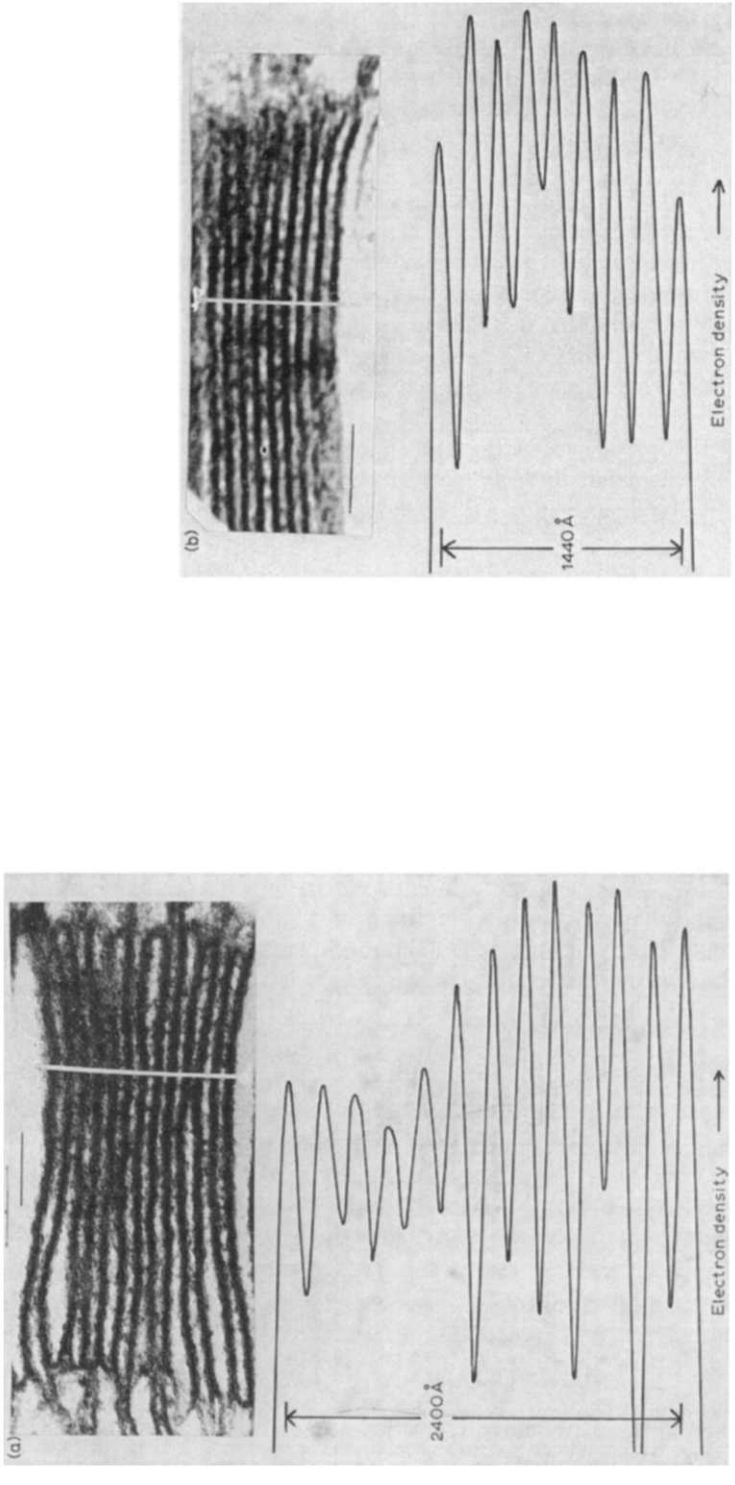


Fig. 3. Electron micrographs of dark (a) and illuminated (b) chloroplasts and corresponding microdensitometer traces. The microdensitometer traces were obtained by scanning the original negatives along the white line, which also marks the position at which the microcomparator measurements were made. Interlamellar distances in the samples were (a) 200 Å (dark control from Expt. A in Table I, part I) and (b) 180 Å (sample illuminated 15 sec in the same experiment). The bar on the print = 1000 Å.

lar distance after 15–20 sec illumination. Therefore, interlamellar distances in samples illuminated 15–30 sec were averaged to give a mean interlamellar distance of 175 Å, which represents a light-dependent decrease of 18.6%. Samples illuminated under the same conditions for 20 sec in the presence of $1 \cdot 10^{-4}$ M CCCP had a mean interlamellar distance of 202 Å, which represents a light-dependent decrease of 6.0%. This 6.0% decrease obtained with CCCP represents a 68% inhibition of the average decrease in interlamellar distance obtained during 15–30 sec illumination in the absence of CCCP.

By the *t* test, the light-dependent decreases were found to be significant at the 5% level. The mean interlamellar distance in the dark control is statistically different from the mean interlamellar distances in samples illuminated either 5 sec or 15–30 sec. The percent inhibition by CCCP is also statistically significant, since the three mean values used in the calculation of this percentage inhibition are, by the *t* test, derived from different populations of means (175 Å, 202 Å, 215 Å).

Under the conditions of the light-scattering experiment, the light-dependent decrease in interlamellar distance was 11.0% (Table I, part II). By the *t* test, the difference between mean values for the dark and illuminated samples was significant at the 5% level.

Representative electron micrographs of samples fixed under photophosphorylation conditions are shown in Fig. 3. The corresponding microdensitometer traces give a quantitative indication of the characteristically regular pattern of the membrane system.

DISCUSSION

Correlation between structure and biochemical state

The mean interlamellar distances in chloroplasts fixed under the conditions of the two-stage photophosphorylation assay (Table I, part I) are plotted against illumination time in Fig. 4, to allow direct comparison with the photophosphorylation curve in Fig. 1. Comparison of Figs. 1 and 4 shows that the two curves are similar in shape. Photophosphorylation reached about one half its maximal value during 5 sec illumination, whereas structural change reached about one third its maximum

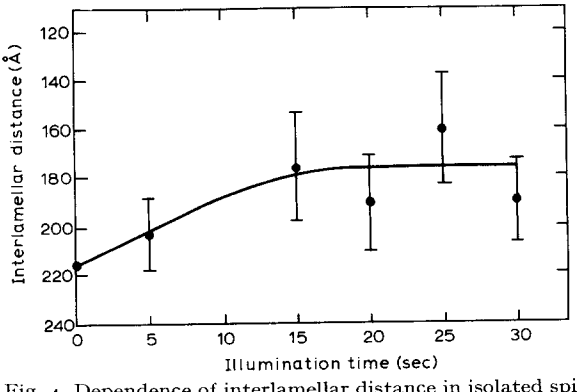


Fig. 4. Dependence of interlamellar distance in isolated spinach chloroplasts on illumination time. The data from Table I, part I, are plotted as a function of illumination time. The vertical bars represent standard deviations.

value during this period. However, both curves reached their maxima after 15–20 sec illumination.

The formation of ATP in the two-stage photophosphorylation assay depends on the light-dependent generation of high energy intermediate(s) (X_e). The similarity in illumination time dependence of photophosphorylation in this assay and structural change suggests, therefore, that the structural change also depends on the generation of high energy intermediates in photophosphorylation, hence ultimately on the light-driven electron flow leading to the formation of X_e.

The 68% inhibition of structural change by $1 \cdot 10^{-4}$ M CCCP is consistent with the interpretation that structural change depends on electron flow, since data in the literature[14] indicate that electron flow (as measured by photoreduction of dichlorophenolindophenol) is 60% inhibited by comparable levels of CCCP. Phosphorylation itself is apparently more sensitive to $1 \cdot 10^{-4}$ M CCCP (97% inhibition observed in this study).

Light scattering

Comparison of Figs. 4 and 2 shows that the illumination time dependence of structural change is different from the illumination time dependence of light scattering. The structural change occurs completely within 15–20 sec after turning on the light, whereas the light scattering curve requires 2–2.5 min to reach its maximum value. However, the light scattering level reaches about 80% of its maximum value during 15–20 sec illumination, suggesting that the initial, rapid change in light scattering might reflect the lamellar structural changes occurring during this period.

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