PROTONATION AND CHLOROPLAST

MEMBRANE STRUCTURE

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ABSTRACT

Light changes the structure of chloroplasts. This effect was investigated by high resolution electron microscopy, photometric methods, and chemical modification. (*a*) A reversible contraction of chloroplast membrane occurs upon illumination, dark titration with H^+, or increasing osmolarity. These gross structural changes arise from a flattening of the thylakoids, with a corresponding decrease in the spacing between membranes. Microdensitometry showed that illumination or dark addition of H^+ resulted in a 13–23% decrease in membrane thickness. Osmotically contracted chloroplasts do not show this effect. (*b*) Rapid glutaraldehyde fixation during actual experiments revealed that transmission changes are closely correlated with the spacing changes and therefore reflect an osmotic mechanism, whereas the light scattering changes have kinetics most similar to changes in membrane thickness or conformation. (*c*) Kinetic analysis of light scattering and transmission changes with the changes in fluorescence of anilinonaphthalene sulfonic acid bound to membranes revealed that fluorescence preceded light scattering or transmission changes. (*d*) It is concluded that the temporal sequence of events following illumination probably are protonation, changes in the environment within the membrane, change in membrane thickness, change in internal osmolarity accompanying ion movements with consequent collapse and flattening of thylakoid, change in the gross morphology of the inner chloroplast membrane system, and change in the gross morphology of whole chloroplasts.

INTRODUCTION

Ample evidence has been brought forward in recent years to demonstrate, both in vitro (1–9) and in vivo (10–13), the conditions under which light-induced proton and ion transport across the chloroplast membrane leads to reversible changes in the volume and configuration of these organelles. Dilley and Vernon (2), Deamer, Crofts, and Packer (4), and Crofts, Deamer, and Packer (5) have suggested that changes in distribution of hydrogen ions generated by light reactions of chloroplasts play a central role in controlling the morphological changes following illumination, although the precise mechanism whereby changes in hydrogen ions bring about the morphological

changes in chloroplast membranes is still undetermined.

This investigation was undertaken to elucidate the sequence of steps by which changes in hydrogen gradients alter the gross morphology of the chloroplast membrane system. Our preliminary investigations (14) have revealed that changes in the thickness of the thylakoid membrane are caused by the action of light and accompany the establishment of hydrogen gradients. This contraction of the membrane is distinct from the more commonly recognized "flattening" of the chloroplasts. In order to clarify the nature of these microstructural changes, we initiated the present studies

using such techniques as high resolution electron microscopy, microdensitometry, light-scattering and transmission measurements, and chemical probes, i.e., hydrophobic-seeking fluorochrome (8-anilinonaphthalene-1-sulfonic acid [ANS]) and a sulfhydryl-seeking probe (phenylmercuric acetate [PMA]).

MATERIALS AND METHODS

Preparation of Chloroplasts

Spinach (*Spinacia oleracea*) leaves were homogenized in 50 mM Tris-HCl buffer, pH 8.0, containing 175 mM NaCl. The homogenate was filtered through four layers of cheesecloth and centrifuged for 2 min at 500 g to remove debris. The supernatant was decanted and centrifuged again at 1500 g for 4 min. The chloroplast fraction was then washed once in an isolation medium by centrifugation at 1500 g for 6 min, resuspended in a small amount of the same medium, and stored in an ice bath in the dark until used in experiments performed at 25°C.

For the study of photoshrinkage, isolated chloroplasts were incubated in either sodium chloride-PMA medium (50 mM Tris-HCl, pH 8.0, 175 mM NaCl, 15 μM phenazine methosulfate [PMS] and 20 μM PMA) or sodium acetate (150 mM sodium acetate, pH 6.7, plus 15 μM PMS) in the dark and then illuminated by red light. For osmotic studies, chloroplasts were incubated in the dark in either 0.05 or 0.5 M sucrose solution containing 44 mM NaCl and 12.5 mM Tris-HCl, pH 7.9.

90° Light Scattering and Transmission Studies

Experiments were carried out in a cuvette in a Brice-Phoenix light-scattering photometer (Phoenix Precision Instrument Co., Philadelphia, Pa.) connected to a dual channel recorder (recti/riter, Texas Instruments, Inc., Houston, Texas). The reaction mixture was continuously stirred by a small magnetic bar. Light-induced changes in 90° light scattering and transmission were recorded simultaneously. To prevent a concentration-dependent interference by multiscattering of light by pigmented particles, the chlorophyll concentration was kept less than 15 μg per ml incubation medium. Within this range the responses are proportional to concentration. The measuring light was filtered at 546 nm with an interference filter, and the intensity in the dark before illumination or acidification of the suspension was adjusted to read 100% on the chart paper. Chloroplasts were illuminated by red light (600–700 nm, 900 ft-c) from the side of the cuvette opposite to the light-scattering measurement.

pH Studies

For pH studies, chloroplasts were suspended in 100 mM NaCl solution at pH 7.8 (adjusted with 0.05 N NaOH) and then titrated with 0.05 N HCl. Changes in pH of the incubation were measured with a glass electrode inserted into the cuvette and connected to a Radiometer pH meter 22, the output of which was continuously recorded.

PMA Binding

Chloroplasts (13–15 μg chlorophyll/ml) were incubated for 4 min in 7.5 ml of 50 mM Tris-HCl, pH 8.0, containing 175 mM NaCl, 15 μM PMS, and 20 μM PMA in the dark, in the light, and in the dark following 5 min illumination. Neutral glutaraldehyde (N₂ sealed, Polyscience Inc., Warrington, Pa.) was added at a concentration of 0.1 M, and after 10 min the suspension was removed from the cuvette and centrifuged for 10 min at 20,000 g. The amount of unreacted PMA in the supernatant was then assayed by dithizone (6 μg per ml chloroform) according to the procedure described by Miller et al. (15) from readings at 620 nm, and the amount of PMA taken up by the chloroplast membranes was calculated by difference from controls.

ANS Fluorescence

To avoid artifacts from actinic light, the difference in ANS fluorescence between unfixed and fixed chloroplasts was taken as a measure of the light-induced structural change. Chloroplasts were treated for 10 min with 0.1 M glutaraldehyde, and then washed three times by centrifuging for 10 min at 20,000 g. Chloroplasts thus "fixed" do not exhibit any light scattering and transmission responses upon illumination. The reaction mixture contained chloroplasts, 100 μg ANS and 15 μM PMS in the dark, the fluorescence level was adjusted to read 100%. Then the reaction mixture was illuminated or titrated with HCl in the dark. For experiments in a sodium chloride-PMA medium, PMA (20 μM) was added before illumination. Identical procedures were used with fixed chloroplasts. Fluorescence was detected between 455 and 578 nm at low angle (20°) to the exciting beam (290–404 nm) to prevent artifacts from scattering samples.

Electron Microscopy

After 0.1 M glutaraldehyde treatment in the cuvette, reaction mixtures were transferred to test tubes and fixation was continued for 1 hr under continuous light or dark. Samples were then treated with 1%

osmium tetroxide in 50 mM phosphate buffer, pH 7.4, and then washed with this buffer, dehydrated with ethanol, and embedded in Epon-Araldite mixture (16) as modified by Johnson and Porter (17). Sections were observed in an EMU-3H (RCA) or Elmiskop I (Siemens) electron microscope without poststaining by heavy metal compounds.

Dimension of Membranes

Thickness and spacing of membranes were estimated by two methods: (a) direct measurement on enlarged photographs at a final magnification of 200,000 by using a magnifier equipped with microscale and (b) microdensitometry tracing of electron microscope negatives. In order to obtain measurements closest to real dimensions, certain precautions were taken. The grana portion of the membrane system was selected for measurement, since the grana membrane is extremely flat, and is stacked with membranes at constant spacing. Under such circumstances, the orientation of the membrane with respect to the direction of electron beam can be defined. Since this is very critical, especially for tightly packed grana structure, stacked membranes separated by 30–40 A clear zone were examined. If the orientation of the membrane within the section tilted by more than a few degrees, it would not be possible to clearly resolve individual membranes. Densitometric curves were obtained by tracings on

electron microscope negatives (X 20,000) by microbeam on the double-beam Microdensitometer MK IIIC (Joyce Loeble & Co., Inc., Burlington, Mass.) and recorded on the chart paper at 50 times expansion. The size of the microbeam used was about 50 μ, which is small enough to resolve the thickness and spacing of the membranes even when they become tightly packed, because (a) the width of the grana on the electron microscope negative is 200–300 μ and (b) the interspace or clear zone between two adjacent membranes is more than 80 μ at 50 times expansion. Measurement of half-width of the peak was done by extrapolating the slope of the tracing to the background level as shown in Figs. 6, 10, 12, and 15.

RESULTS

Kinetics of Light Scattering and Transmission Changes

Photometric methods permit evaluation of the dynamic aspects of light-induced changes in chloroplast structure. Fig. 1 shows that when chloroplasts are suspended in sodium acetate medium, reversible photoshrinkage of chloroplasts can be readily monitored by reversible changes in either light scattering or transmission. Such changes have been observed previously (5).
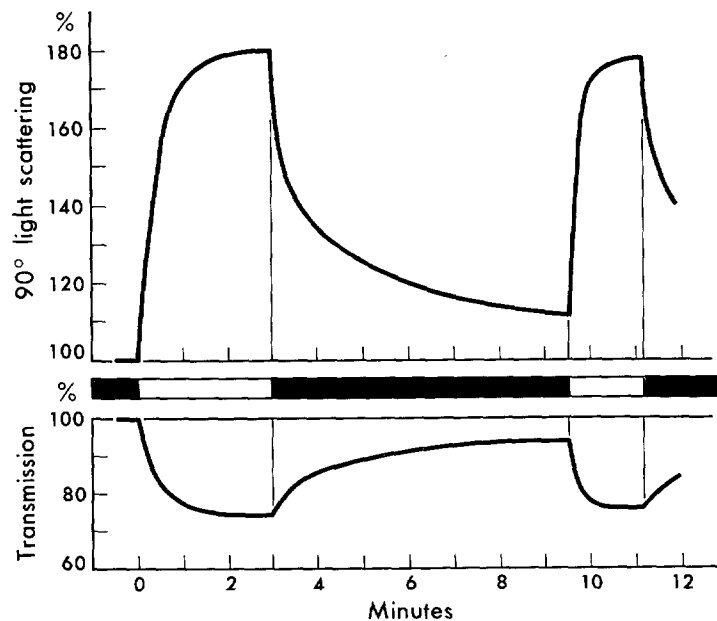

FIGURE 1 Kinetics of light-induced 90° light scattering and transmission changes of isolated spinach chloroplasts accompanying photoshrinkage in a sodium acetate medium. Other conditions as in Methods.
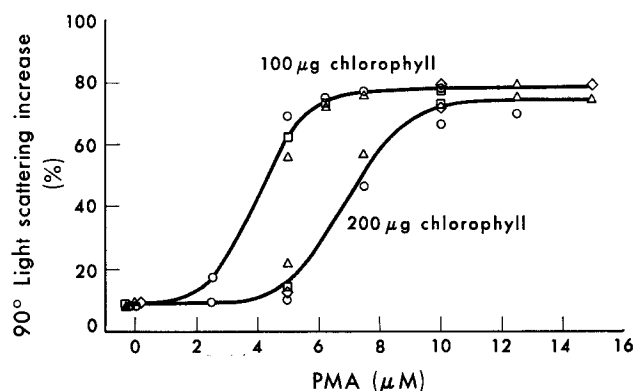
FIGURE 2   Effect of PMA concentration on the light scattering response of illuminated chloroplasts. Chloroplasts suspended in 7.0 ml of 50 mM Tris-HCl, pH 8.0, containing 175 mM NaCl and 15 μM PMS were illuminated by actinic red light and, after 1 min, PMA was added to the incubation at varied concentrations.

TABLE I

PMA Uptake by Chloroplast Membranes

	PMA bound			
	Experiments			
Incubation	1	2	3	Average
	mμmoles/mg chlorophyll			
Dark, 4 min (before illumination)	478	478	461	472
Light, 4 min	494	537	566	532
Dark, 4 min (after 4 min illumination)	500	516	500	505

Chloroplasts (140 μg chlorophyll) were incubated in a sodium chloride-PMA medium. PMA was assayed as described in Methods. The difference between the amount of PMA added initially and the amount detected in the supernatant was taken as the amount of PMA bound. Values are for experiments with chloroplasts from same preparation.

Light-induced chloroplast shrinkage is enhanced by organic mercurial PMA, which has been studied by Siegenthaler and Packer (18) and Siegenthaler (19). It provides a useful system for examining light-induced changes in chloroplast structure. Fig. 2 shows the relation between PMA concentration and the magnitude of the light-induced light scattering increments observed in chloroplasts suspended in sodium chloride-PMA medium. Maximal photoshrinkage is induced by 10 μM PMA or less, under usual conditions of chlorophyll concentration in in vitro experiments. The enhanced photoshrinkage can be correlated with the amount of membrane-bound PMA as seen in Table I. Upon illumination, an increase in the extent of PMA binding occurs which falls by about 50% when the light is removed.

Light scattering and transmission changes were examined simultaneously in chloroplasts suspended in the sodium chloride-PMA medium and were found to be parallel to one another after illumination. When the dithiol dithioerythritol (DTE) is added to chloroplasts in an amount which exceeds, on a stoichiometric basis (see Table II and Fig. 3), the quantity of PMA present, then the light-induced changes are reversed, even under the conditions of continuous illumination. Changes occur immediately upon the addition of DTE in both light scattering (decrease) and transmission (increase). However, light scattering decrease reaches a steady state but the transmission change continues. This difference in kinetics of light-scattering and transmission is also seen upon addition of PMA to chloroplasts under continued

TABLE II

Reversal of Light-Induced Light Scattering and Transmission Changes of Chloroplasts by DTE

Concentration		90° Light scattering			Transmission		
PMA	DTE	Increase	$t_{1/2}$	Inhibition by DTE	Decrease	$t_{1/2}$	Inhibition by DTE
μM	μM	%	sec		%	sec	
0	0	12			3		
0	20	11			3		
10	0	84	48		21	40	
10	2.5	84	60	None	22	45	None
10	5	10		Complete	3		Complete
20	0	103	40		25	35	
20	5	102	55	None	24	45	None
20	10	11		Complete	4		Complete
20	20	13		Complete	4		Complete
40	10	95		None	24		None
40	20	10		Complete	3		Complete

Chloroplasts were incubated in 50 mM Tris-HCl, pH 8.0, containing 175 mM NaCl and 15 μM PMS in the presence of PMA and DTE at the concentrations indicated. Illumination was by red light (600–700 nm). Chlorophyll concentration was 120 μg in 7.5 ml of reaction mixture. Half-maximum time ($t_{1/2}$) is presented to show the effect of DTE on the kinetics of both light scattering and transmission changes.
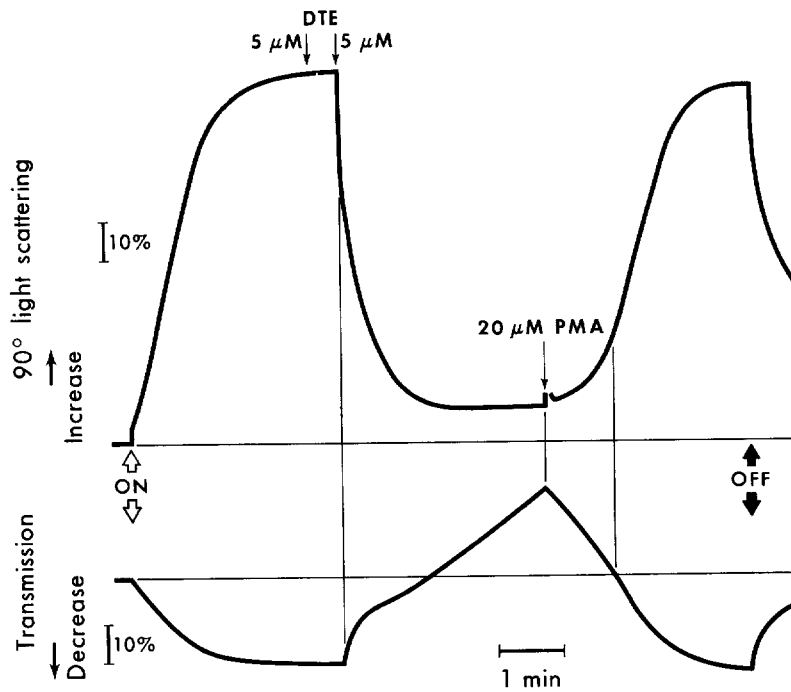

FIGURE 3   Effect of DTE on light-induced 90° light scattering and transmission changes of chloroplasts. Conditions: sodium chloride-PMA medium; at the points indicated, 10 μM DTE and 20 μM PMA were added to the incubation during illumination.
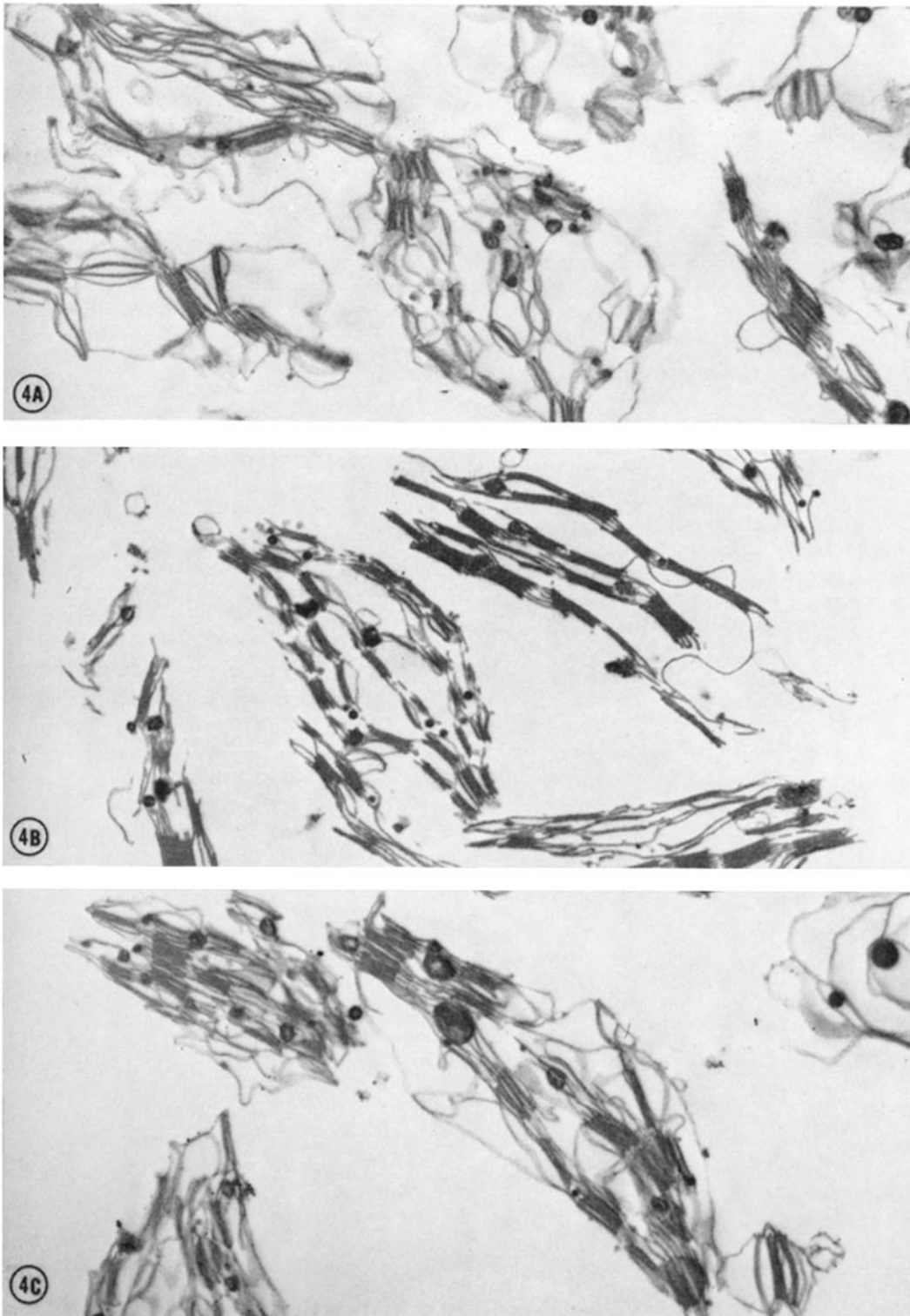
FIGURE 4 Ultrastructure of chloroplasts suspended in a sodium chloride-PMA medium under different light conditions. Chloroplasts were incubated and fixed as described in Methods. (A) Dark, before illumination. (B) 3 min illumination, (C) 4 min in the dark following illumination as in (B). ×16,500.

337

illumination. Hence the parameters which are being measured by light scattering and transmission changes are not identical. Various theoretical (20, 21) and experimental (14, 21, 23) studies, especially from Shibata's laboratory (22, 36), suggested that light scattering changes appear to measure not only volume changes, but also microstructural changes occurring within the membrane itself.

Electron Microscopy and Microdensitometry

The ultrastructural changes observed upon illuminating chloroplasts in sodium chloride-PMA medium are shown in Fig. 4. Chloroplasts are devoid of outer membranes but have a relatively intact inner membrane system where grana membranes are relatively loosely stacked in the dark (Fig. 4 A). After illumination in the presence of PMA, these membranes become very tightly packed together (Fig. 4 B), making it quite difficult to discern clear space between individual membranes at low magnification. Cessation of illumination in the presence of PMA results in a restoration of the characteristic appearance of dark chloroplasts. These changes are seen more clearly when the results are subjected to the scrutiny of higher resolution electron microscopy as shown in Fig. 5, where grana regions are shown in a typical ap-

pearance. Although the organization of the chloroplasts seems unchanged by photoshrinkage, a tighter ordering of the membranes in the grana region occurs. These samples were subjected, as shown in Fig. 6, to analysis by microdensitometry which revealed that a decreased thickness of the membranes had occurred upon illumination. This surprising finding led us to undertake a more detailed study.

The changes which occur in grana membranes under different light conditions are shown in Fig. 6. The microdensitometric tracings clearly reveal the reversible decrease in the spacing of 32% and thickness of 21% upon illumination, when the dimensions are taken as 100% in the dark.

The presence of repetitive structures in grana affords a means to obtain accurate information on dimensions if the profile of the membranes on an electron micrograph is in very sharp focus throughout the entire grana region. When, however, the dimensions of single intergranal membranes are considered, the exact orientation of membranes within the section are more difficult to ascertain. Therefore, numerous photodensitometric traces of single thylakoid membranes in the intergrana region were made, one of which is illustrated in Fig. 7. Precise values of the dimensions are purposely omitted here because of the difficulty
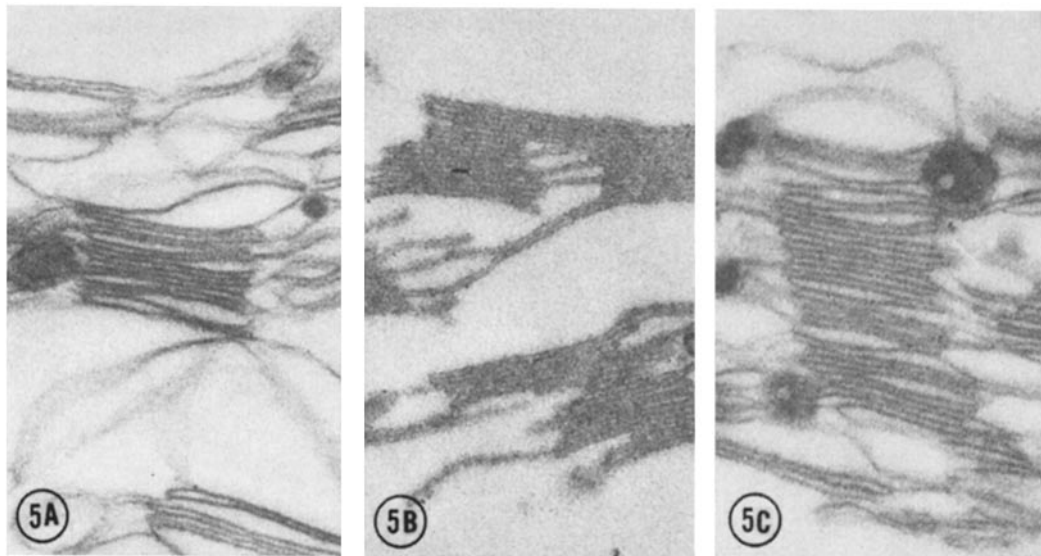

Figure 5 Light-dependent reversible ultrastructural changes of chloroplast membrane system, in a sodium chloride-PMA medium. Other conditions as in Methods. (A) Dark, before illumination; (B) 3 min illumination; (C) 4 min following illumnination as in (B). × 46,800.

DARK
(Before illumination)

131 ±10 A
Thickness

212 ±8 A
Spacing

LIGHT

104 ±6 A

144 ±9 A

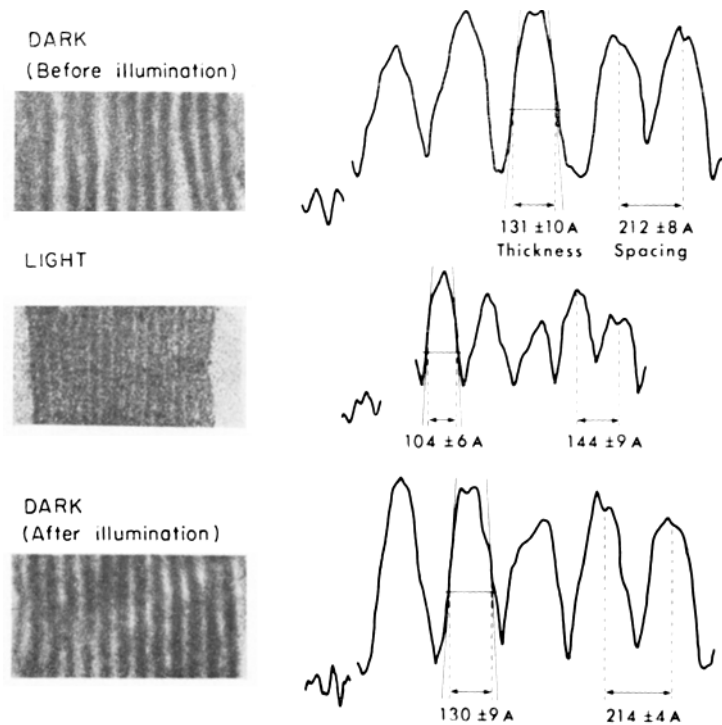DARK
(After illumination)

130 ±9 A

214 ±4 A

FIGURE 6 Dimensions of the grana membranes in a sodium chloride-PMA medium under the different light conditions. Other conditions as in Fig. 5 and Methods.

in assigning these accurately. The data, nevertheless, indicate that all thylakoid membranes within the chloroplasts probably undergo the same changes upon illumination.

It was next decided to investigate whether the kinetics of light scattering and transmission changes could be correlated with the thickness and spacing changes. The results are presented in Fig. 8. These studies were made possible by the use of the glutaraldehyde technique (6) to rapidly stop changes in structure during the actual course of the experiment. The thickness and spacing of the grana membranes, using microdensitometry and measurements on enlarged photographs, gave similar results. Hence within the limits of error of the measurements, thickness and spacing changes follow closely upon the corresponding photometric tracings. The results further show that the thickness changes more nearly correlate with the kinetics of light scattering responses and that the spacing changes are more nearly reflected by kinetics of transmission changes.

The question was now asked whether shrinkage of chloroplast, regardless of the mechanism by

which it was brought about, could cause similar changes? Chloroplasts suspended in the presence of weak acid anion solutions like sodium acetate and illuminated (as in Fig. 1) show the spacing and thickness changes as shown in Fig. 9. The thickness decreased by 13% and the spacing changes by 27% when the chloroplasts were illuminated (Fig. 10).

PH TITRATION STUDIES: Examination of the ultrastructural changes at different pH's was of interest since it is known that light scattering of chloroplasts is enhanced by lowering the pH of the medium (2, 4, 25). When chloroplasts in 100 mM NaCl solution were titrated in the dark with 0.05 N HCl, light scattering increased on lowering the pH below 6, reaching a maximal level at pH 4, and then it decreased abruptly beyond pH 3.8. Light scattering increment induced by acidification of the suspension was reversed by realkalinization with 0.05 N NaOH, though the backtitration curve did not exactly follow the forward-acid titration curve and shifted slightly towards the higher pH side. Samples for electron microscopy were taken from the suspension at pH 7.7 (before

acidification), pH 4.7 (acidification, recorded 80% light scattering increment), and pH 7.3 (after realkalinization). Chloroplasts at pH 7.7 exhibit, in cross-section, swollen configuration due to the

Dark
(Before illumination)

Light

Dark
(After illumination)

FIGURE 7 Photodensitometric traces of a single thylakoid membrane at the intergrana region of chloroplasts in a sodium chloride-PMA medium.

slightly hypotonic condition of the medium. Acidification to pH 4.7 and realkalinization to pH 7.3 did not induce notable changes in gross morphology. Similar results of gross morphology of the chloroplasts in acid medium was reported by Deamer, Crofts, and Packer (4).

Striking changes were also found with grana at higher magnification as shown in Fig. 11. Acidification from pH 7.7 to 4.7 results in a decrease in the thickness and spacing of the grana membranes, which is reversed by back titration of the pH from 4.7 to 7.3. Fig. 12 shows the corresponding microdensitometry traces which verify that the thickness and spacing changes at different pH values selected for comparison of structure are the same as those observed upon illumination. Decreases in thickness and spacing induced upon acidification are, respectively, 23 and 31%. The pH differential chosen for these experiments is more or less equivalent to that calculated as the magnitude of the light-induced proton uptake by chloroplasts, i.e., external pH was adjusted to simulate the magnitude of the light-induced pH gradient that would be established under conditions where chloroplasts are suspended in lightly buffered media in the presence of strongly dissociated ions.
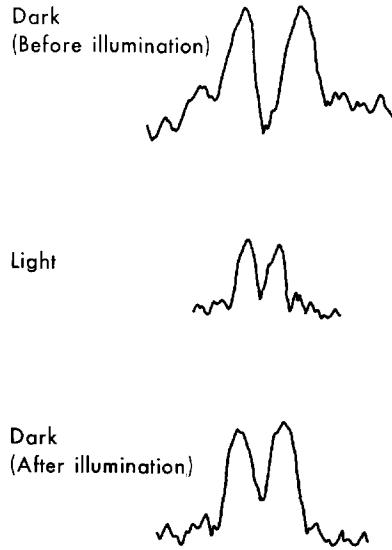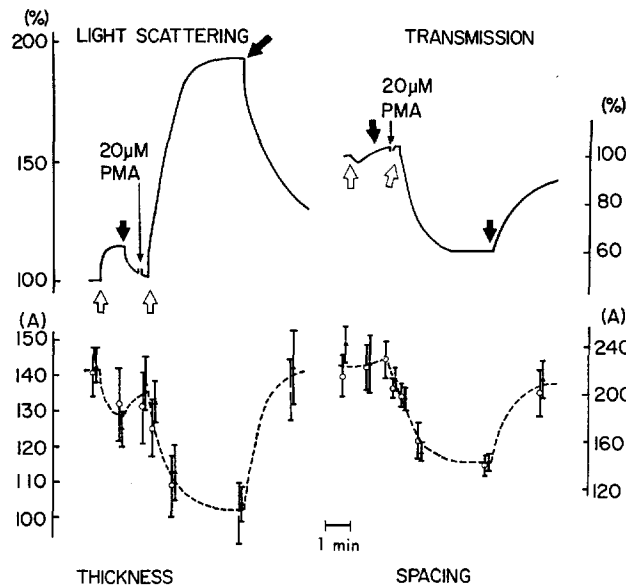

FIGURE 8 Comparison of kinetics of photometric and ultrastructural changes of chloroplast membrane upon illumination. Chloroplasts were incubated in 50 mM Tris-HCl, pH 8.0, containing 175 mM NaCl and 15 μM PMS and, as indicated, 20 μM PMA was added to the incubation. At the points indicated by vertical lines chloroplasts were fixed with 0.1 M glutaraldehyde.
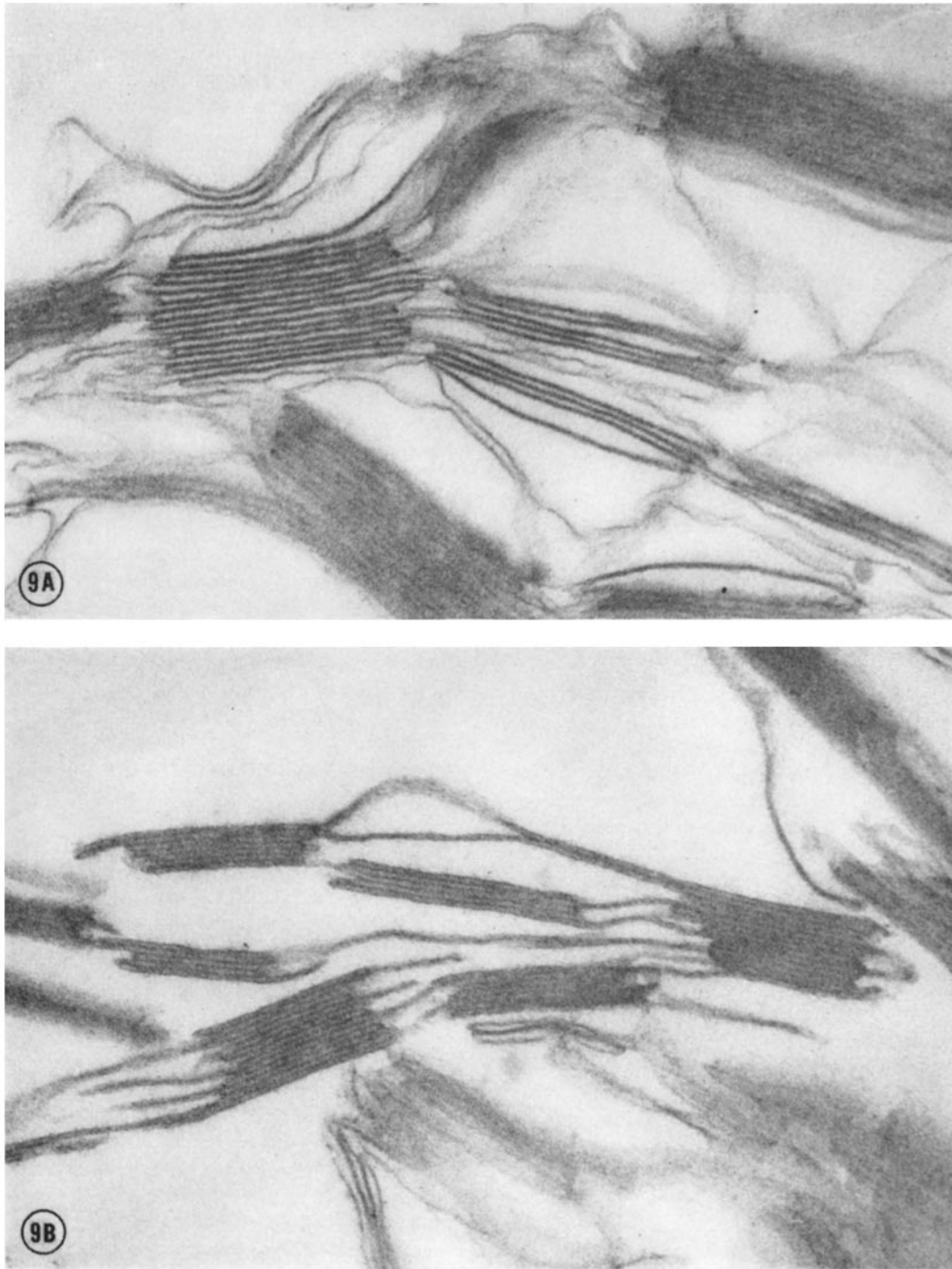
FIGURE 9   Ultrastructure of chloroplast membrane system in a sodium acetate medium under different light conditions. (A) Chloroplasts in the dark, before illumination. Both grana and intergrana thylakoids exhibit a slightly swollen configuration. (B) Chloroplasts illuminated for 3 min. Flattening of the thylakoids and contraction of thylakoid membranes have occurred. × 62,800.

Dark
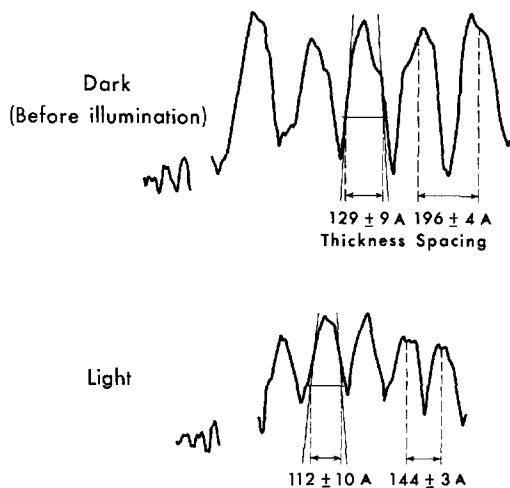(Before illumination)

129 ± 9 A 196 ± 4 A
Thickness Spacing

Light

112 ± 10 A 144 ± 3 A

FIGURE 10 Comparison of dimensions of grana thylakoid membranes in sodium acetate medium under different light conditions. Conditions as in Fig. 9 and Methods.

OSMOTIC STUDIES:—Shrinkage of chloroplasts can also be brought about by increasing the external osmotic pressure of the medium in which they are suspended. To test this effect, chloroplasts were suspended in a hypotonic (0.05 M) or hypertonic (0.5M) sucrose solution containing sodium chloride. Fig. 13 shows that increasing the concentration of sucrose in a suspension leads the chloroplasts to contract, which results in the characteristic flattening of the membrane system. This effect was caused by the osmotic gradient corresponding to a 10-fold change in the concentration of sucrose, which was about equivalent to the conditions chosen above for examining the transition between darkness and illumination. Fig. 14 shows the ultrastructural changes in greater detail. A quantitative analysis of the dimension of thylakoid membranes under the different osmotic conditions was provided by microdensitometry (Fig. 15). It is evident that the expected spacing changes (32%) are observed, but that no statistically significant changes occur in thickness of the membrane under these conditions.

Table III, based on statistical analysis of microdensitometric tracings, summarizes the changes of dimension in the thickness and spacing of thylakoid membrane under various light, pH, and osmotic conditions employed to bring about reversible shrinkage of chloroplasts. It is evident from the data that all conditions employed to bring about

reversible shrinkage of chloroplast result in both spacing and thickness changes, with the singular exception of osmotic changes which lead only to spacing changes but not to membrane thickness changes.

Studies on Refractive Index

The observed changes in membrane thickness upon illumination are indicative of changes in internal structure. Related studies were undertaken to verify if this view was correct because it was possible that the differences observed by microdensitometric analysis of electron micrographs were brought about by peculiarities in the way in which chemical fixation affected structure of the membranes.

Chloroplasts were incubated in the sodium chloride-PMA system and fixed with glutaraldehyde before, during, and following illumination. The samples were then washed twice and resuspended in sucrose solutions of differing refractive index (24). The light scattering level (as a parameter of refractive index difference between the chloroplast membrane and the medium) was plotted against the refractive index of the sucrose solution (Fig. 16). Over a wide range of refractive index (1.333–1.485), the light scattering level was always higher in illuminated chloroplasts than in those in the dark.

Membrane Protonation Studies

Since it has been disclosed by our observations that pH titration can lead to changes in microstructure, this effect was studied further. Fig. 17 shows that as the pH is lowered the light scattering increases, reaching a maximum (100–120% increment) around pH 3.8–4.0. Further lowering of the pH results in a decrease of the light scattering. It is known from earlier studies by Deamer et al. (4) and Mukohata, Mitsudo and Isemura (25) that pH titration causes enhancement of chloroplast light scattering and that this change is reversible by back titration. pH-dependent light scattering is associated with changes in internal membrane structure. Fig. 17 also shows that protonation occurs during acidification since the membrane system has considerable buffer capacity in the pH range between 7 and 4.5. The amount of hydrogen ions taken up was 4.5 μeq/mg chlorophyll; this value compares favorably with earlier reports (4).
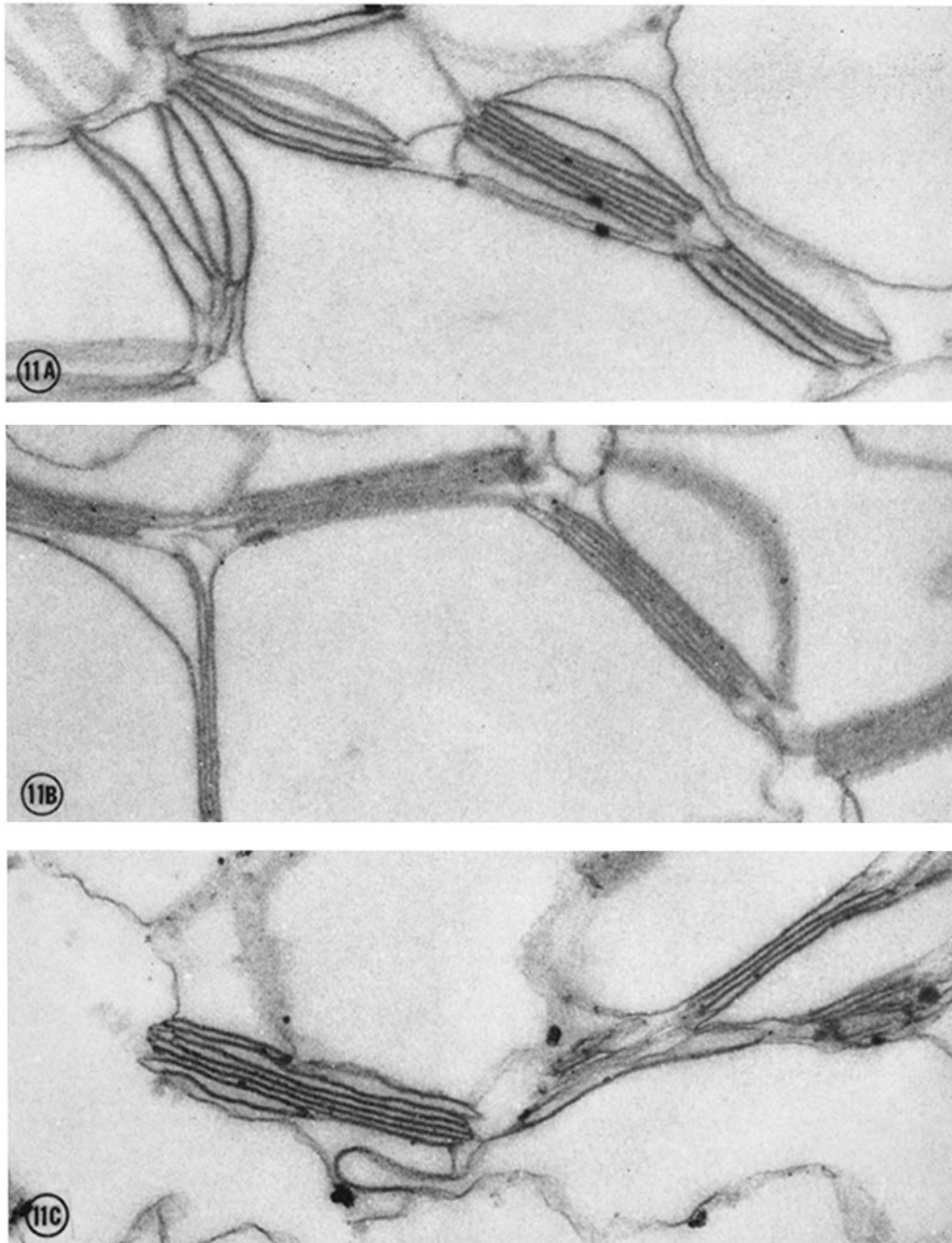
Figure 11 Effect of pH on ultrastructure of chloroplast membrane. Chloroplasts were incubated in a 100 mM sodium chloride solution at different pH's in the dark in a weakly alkaline medium. (A) pH 7.7. Before acidification; the thylakoid membrane system exhibits swollen configuration due to slightly hypotonic condition. (B) pH 4.7. Upon acidification, flattening of grana thylakoids and contraction (decrease in thickness) of thylakoid membranes are induced. (C) pH 7.3. Reversal after medium is back-titrated with alkali. × 67,600.

pH 7.7

136 ± 8 A   206 ± 15 A
Thickness   Spacing

pH 4.7

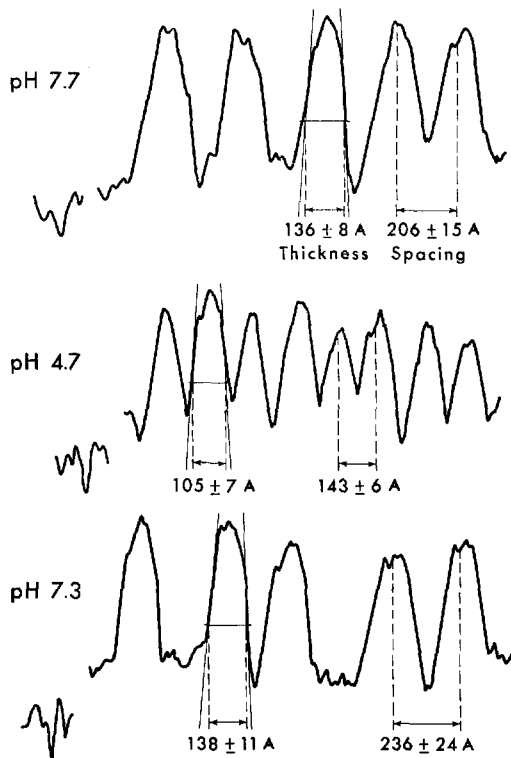105 ± 7 A   143 ± 6 A

pH 7.3

138 ± 11 A   236 ± 24 A

FIGURE 12   pH dependence of dimensions of grana membranes. Chloroplasts were incubated in 100 mM sodium chloride at the pH indicated in the dark.

ANS Fluorescence Studies

ANS is known to enhance its ultraviolet-excited fluorescence when it becomes associated with hydrophobic environments such as occur when it is bound to biological membranes or placed in solutions of low dielectric constant (26). When ANS is added to the chloroplasts, fluorescence is enhanced by accompanying binding to the membrane (Fig. 18). Upon illumination a further increase in this fluorescence intensity is observed. Chloroplasts treated with glutaraldehyde are no longer capable of undergoing changes in volume as measured by light scattering, transmission, or electron microscopy. Nevertheless, it was found that the light-dependent increases in the ANS fluorescence still occurs. This finding suggests that changes in microstructure of the membrane resist chemical fixation sufficient to prevent gross morphological changes. The difference in ANS fluorescence observed between fixed and unfixed chloroplasts probably represents changes in

binding sites or occupancy of ANS in the membrane as a consequence of illumination.

Light-induced ANS fluorescence increases in illuminated chloroplasts in the presence of weak acid anions (sodium acetate) or strongly dissociated ions (NaCl-PMA) show similar kinetics as shown in Fig. 19. In both systems the speed of the ANS fluorescence changes upon illumination are faster than the corresponding light scattering changes. Furthermore, the rate of the ANS fluorescence responses are similar in the two conditions, but the light scattering kinetics are dissimilar. The half times for ANS fluorescence changes taken from these first order plots indicate that they are 14 sec in the NaCl-PMA system and 15 sec in sodium acetate system. These rates are considerably faster than light scattering changes, where the half time for changes are 60 and 33 sec for the sodium chloride-PMA and sodium acetate systems, respectively.

Since kinetic analysis of the ANS fluorescence changes indicated that they occurred more rapidly than the light scattering responses, it was of considerable interest to determine how ANS fluorescence changes are related to structural changes in thylakoid membrane. Since it is known that illumination of chloroplasts leads to an uptake of hydrogen ions, the effect of protonation of chloroplasts on ANS fluorescence was studied. Fig. 20 shows experiments in which pH titrations of the light scattering and ANS fluorescence changes are compared. It is clear that the fluorescence changes induced by protonation of the membrane are more sensitive to pH change than light scattering. When a similar titration was made with potassium (as chloride), no increases in either ANS fluorescence or light scattering are observed. Similar negative results have been obtained with other monovalent ions.

DISCUSSION

From the findings in this investigation, it may be concluded that the temporal sequence of events involved in the hierarchy of changes in structure in chloroplast membranes following illumination probably is: (a) protonation, (b) change in the environment within the membrane, (c) change in membrane thickness, (d) change in internal osmolarity accompanying ion movements with consequent collapse and flattening of thylakoids, (e) change in the gross morphology of the inner
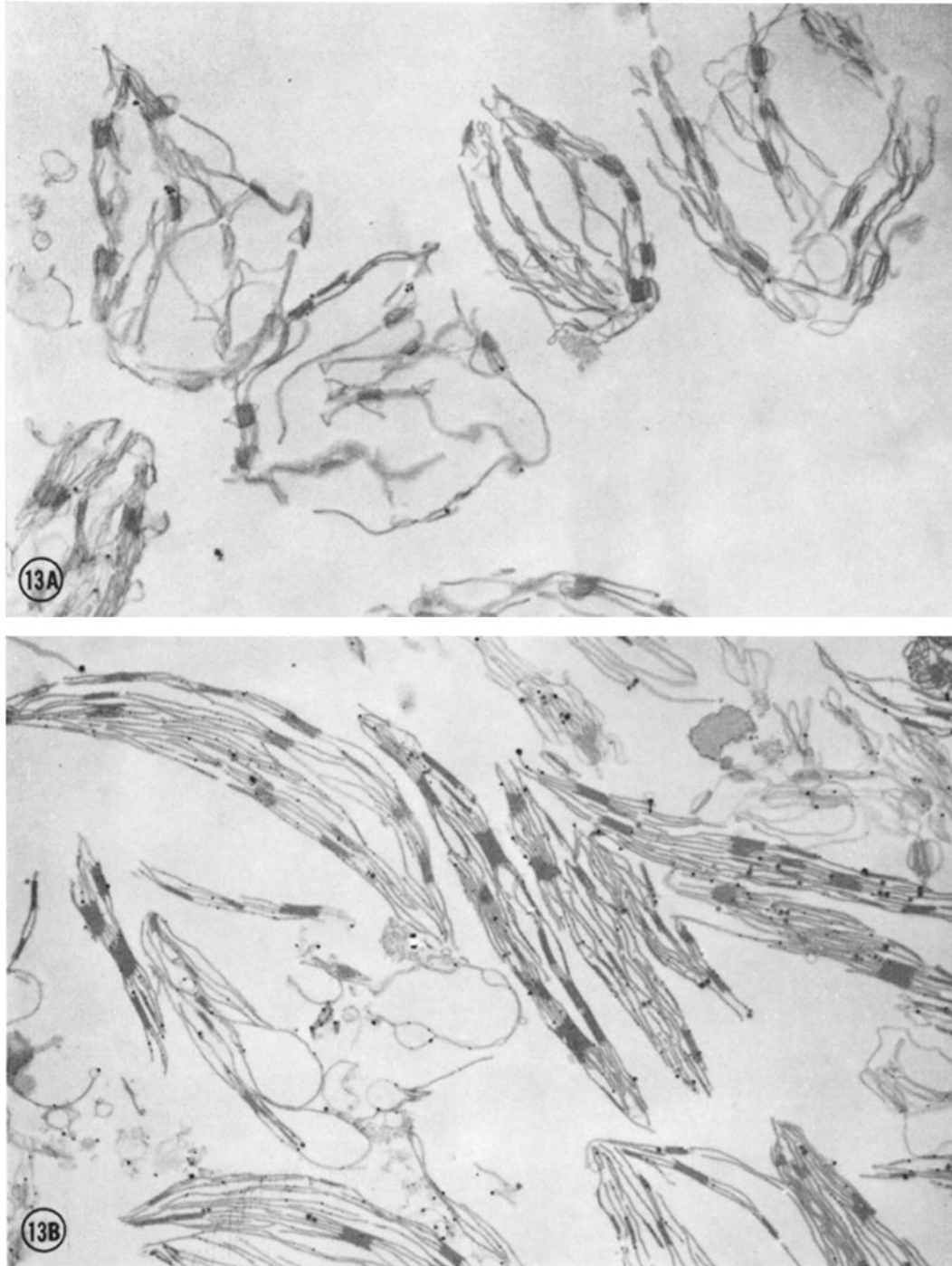
FIGURE 13 Ultrastructure of osmotically contracted chloroplasts. (A) Hypotonic (0.05 M) sucrose-sodium chloride medium in the dark. Chloroplasts appear round, and grana are easily resolved. (B) Hypertonic (0.5 M) sucrose-sodium chloride medium in the dark. Osmotically-induced flattening of the membrane system is evident. × 8800.
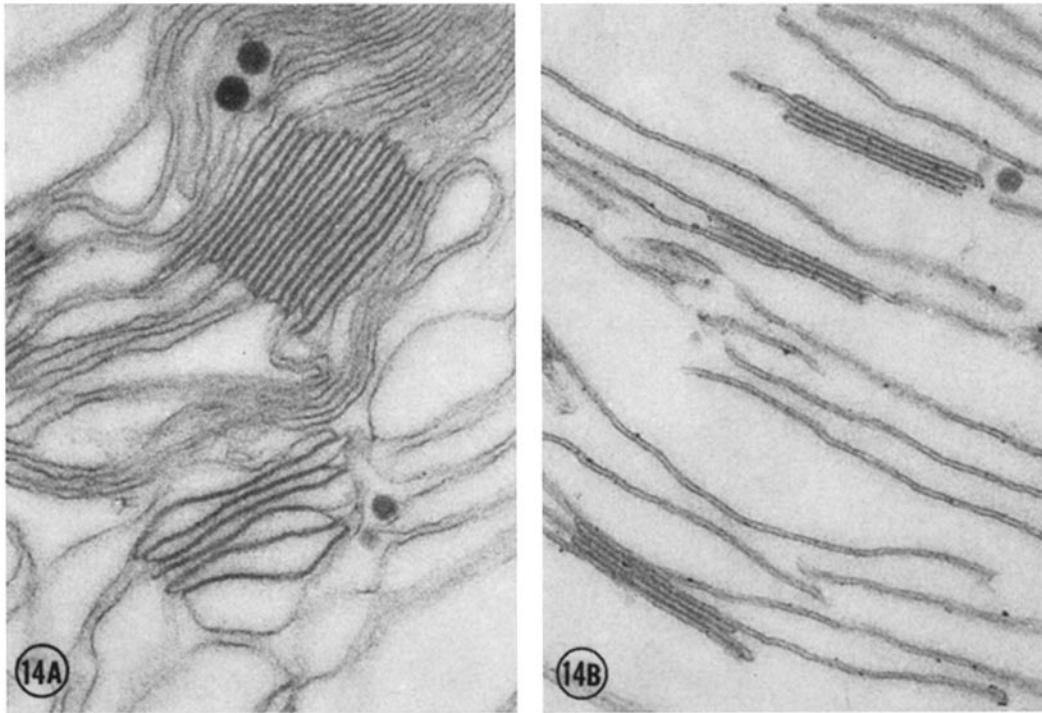
Figure 14 Ultrastructure of chloroplast membranes under hypotonic and hypertonic conditions. (A) 0.05 M sucrose-sodium chloride medium in the dark. (B) 0.5 sucrose-sodium chloride medium in the dark. × 67,200.
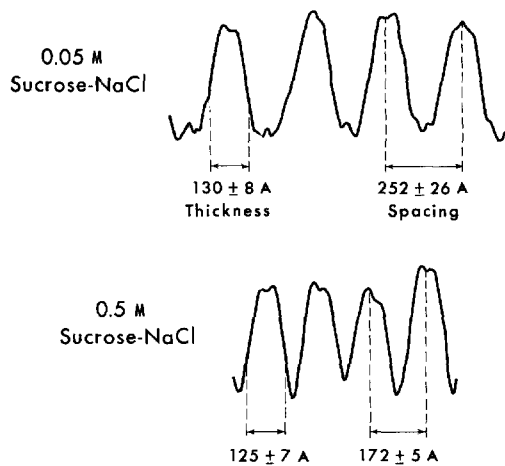

Figure 15 Effect of osmolarity upon dimensions of grana thylakoid membrane. Chloroplasts were incubated in either 0.05 M or 0.5 M sucrose solution containing 44 mM NaCl and 12.5 mM Tris-HCl, pH 7.9 in the dark.

chloroplast membrane system, and (*f*) change in the gross morphology of intact chloroplasts.

A mechanism which relates membrane "protonation" to this hierarchy of changes in structure in chloroplast membrane is presented in Fig. 21. The evidence in support of this hierarchy of changes in structure is presented below.

Protonation and Microstructural Changes

The evidence in support of the idea that protonation is involved in the decrease in thickness can be summarized as follows: (*a*) Several treatments (light, H^+, hypertonicity) have been found to decrease membrane spacing; all of them cause a decrease in membrane thickness, except an osmotic force. Those treatments, again except for osmotic changes, have in common a protonation resulting from illumination or acidification in the dark. (*b*) By using artificial means (titration with HCl), in the dark the acidification of the membrane causes an increase in light scattering and a decrease in

TABLE III

Effect of Incubation Conditions upon Dimensions of the Grana Thylakoid Membrane

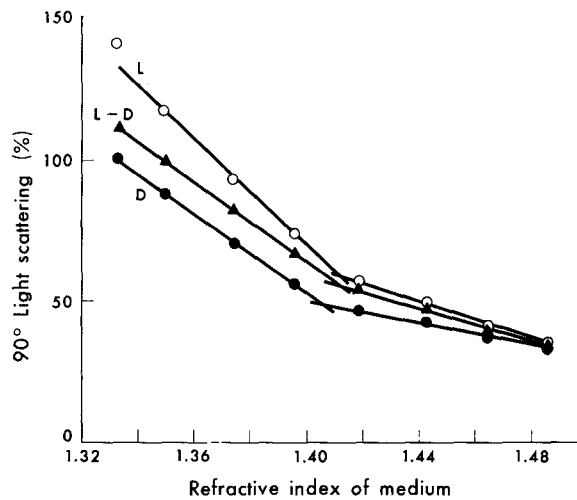| Condition	Thickness (SD)			Spacing (SD)		
	(A)			(A)		
Light-induced shrinkage	Dark → light → dark			Dark → light → dark		
NaCl-PMA	131 ± 10	104 ± 6	130 ± 9	212 ± 8	144 ± 9	214 ± 4
Sodium acetate	129 ± 9	112 ± 10		196 ± 4	144 ± 3	
pH-induced shrinkage (dark)	pH 7.7 → 4.7 → 7.3			pH 7.7 → 4.7 → 7.3		
NaCl	136 ± 8	105 ± 7	138 ± 11	206 ± 15	143 ± 6	236 ± 24
Osmotic shrinkage						
0.05 M sucrose-NaCl	130 ± 8			252 ± 26		
0.5 M sucrose-NaCl	125 ± 7			172 ± 5		

FIGURE 16   Influence of refractive index of the suspending medium upon 90° light scattering of chloroplast membranes. Chloroplasts were incubated in a sodium chloride-PMA medium in the dark, light (4 min) or dark (10 min) after 4 min illumination, and then fixed with 0.1 M glutaraldehyde and washed thoroughly by centrifugation. The fixed chloroplasts, containing 10 μg chlorophyll/ml, were suspended in 3.5 ml sucrose solution of different concentrations (0–85%). The light scattering level of the chloroplasts, which were fixed in the dark and suspended in distilled water, was adjusted at 100%. Refractive index of suspending medium was estimated from refractive index of aqueous solution of sucrose (23). Chloroplasts in the dark (before illumination) (D), —●—; light (4 min) (L), —○—; dark (10 min) after 4 min illumination (L-D), —▲—.

the thickness of the membrane in a reversible fashion if back titration is instituted. Changes in internal structure of the membranes monitored by light scattering are correlated closely with the protonation of the membranes between pH 7.0 and 4.5. (c) Changes in ANS fluorescence by chloroplast membranes have been shown to occur at a faster rate than light scattering changes and to be pH dependent. In both of these instances the

changes can be satisfactorily accounted for by a prior protonation.

Three considerations suggest that a study of fixed charges on the chloroplast membrane warrants further investigation.

The first is that light-induced ANS fluorescence intensity changes are retained in glutaraldehyde-fixed chloroplasts. Hence, changes in microstructure of the membrane can occur independent of
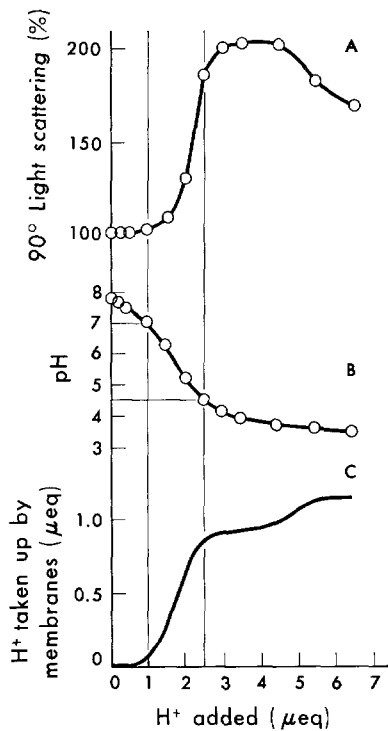
FIGURE 18 Light-induced ANS fluorescence changes of chloroplasts. 100 μg ANS was added to the chloroplasts equivalent to 14 μg chlorophyll per ml in 50 mM Tris-HCl, pH 8.0, containing 175 mM NaCl and 15 μM PMS. When fluorescence yield in the dark reached the maximal level (100%), 20 μM PMA was added and then chloroplasts were illuminated by red light. For the experiment with fixed chloroplasts, chloroplasts were fixed with 0.1 M glutaraldehyde in the dark, thoroughly washed, and incubated.

FIGURE 17 Comparison of 90° light scattering with pH and protonation of the chloroplast membrane. Chloroplasts containing 200 μg chlorophyll were suspended in 7.5 ml of 100 mM sodium chloride and titrated with 0.05 N HCl in the dark. (A) 90° light scattering. (B) pH changes. (C) protonation. Hydrogen ion concentrations were calculated from pH values measured for both chloroplast suspension and suspending medium, and the difference between these two values was taken as the amount of proton uptake.

changes in gross morphology and configuration, which do not occur under these conditions of fixation.

Possible explanations for ANS fluorescence changes are: (a) Protonation of negative charges in the membrane would lead to an expulsion of attractive water dipoles localized in the vicinity of the negatively charged groups. This would also have the effect of increasing the attractive forces between the molecules, resulting in a decrease in membrane thickness as a consequence of the closer apposition of unit structures within the membrane. This type of effect would also increase the "hydrophobicity" of the membrane. (b) Increase in the hydrophobicity of the thylakoid membrane might be brought about by a combination of effects, among which are polarization of fixed negative
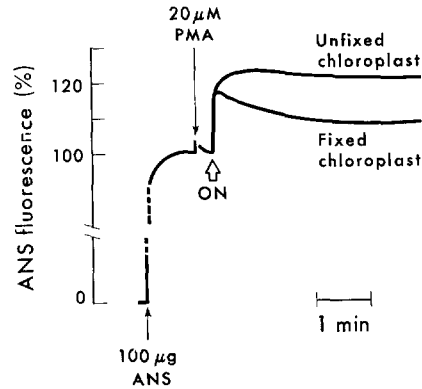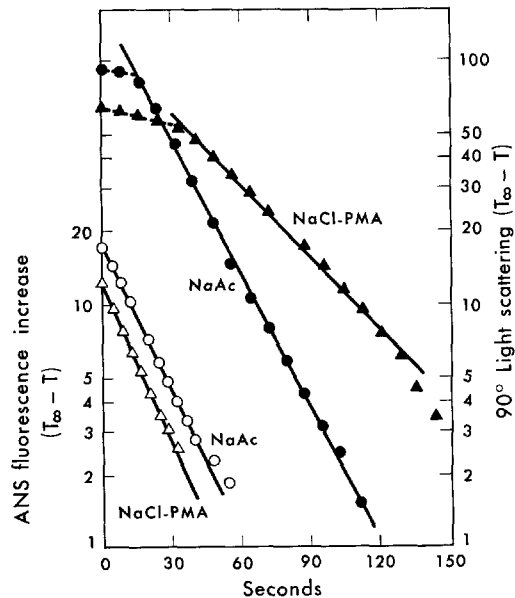

FIGURE 19 Kinetics of light-induced 90° light scattering and ANS fluorescence changes in chloroplasts. Chloroplasts equivalent to 14 μg chlorophyll per ml were suspended in either a sodium chloride-PMA or sodium acetate medium. Light scattering and ANS fluorescence increase upon illumination were expressed as the difference between the value at steady state and that at various times on a semilog scale.
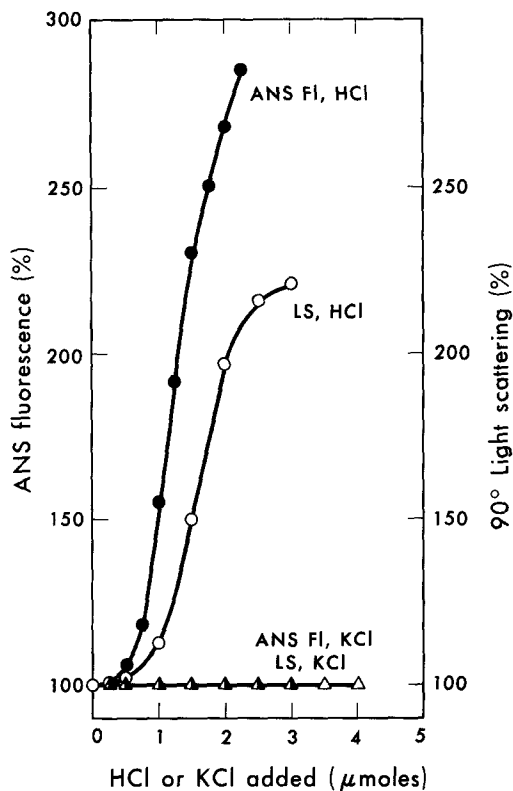
FIGURE 20 Effect of H^+ and K^+ on 90° light scattering and ANS fluorescence intensity of chloroplasts. Spinach chloroplasts were incubated in 100 mM sodium chloride, and 90° light scattering and ANS fluorescence were titrated with 0.05 N HCl or 0.05 N KCl as described in Methods.

charges, expulsion of water, and neutralization of the phospholipids.

The second reason is that pH titration studies suggest that protonation is affecting only certain fixed charges, such as carboxyl groups, on the membrane. In support of this, it is found that the buffer capacity of the membrane between pH 4.5 and 7 is roughly equivalent to 4.5 μeq protons per mg of chlorophyll. A similar value was reported by Deamer et al. (3). This value for the buffer capacity of the membrane would most nearly correlate with the neutralization of β and γ carboxyl and imidazole groups of protein and secondary phosphate groups of lipids as being the most likely negative charges neutralized by the protonation process, since their pK values (30, 31) are in the pH range at which protonation occurred. Partici-

pation of these negatively charged groups in protonation of the membrane seems to be plausible from the fact that aspartic and glutamic acids, which have side carboxyl groups, are the most predominant amino acids in the protein of the chloroplast membrane (32, 33).

The third reason is that charge changes of the membrane as a result of protonation would alter the attractive and repulsive forces causing changes in quaternary protein structure. These could lead to a 20% decrease in membrane dimensions observed. Cassim and Yang (34) examined the pH dependence of conformation of poly-L-glutamic acid by circular dichroism and optical rotatory dispersion techniques. Their results suggest that protonation of carboxyl groups causes aggregation in acid solution. Also, Sun (35) has shown that the conformation of bovine serum albumin is a function of electrostatic charge of the molecule and, hence, in this case it exists in a compact form between pH 4.3 and 10.5 where negatively charged groups are protonated.

Configurational and Gross Morphological Changes

Illumination of chloroplasts in the presence of weak acid anions (5, 27, 28) or the NaCl-PMA system (14, 18, 19) results in their flattening or decrease of volume. This change decreases the spacing between grana membranes, as is particularly clearly revealed by microdensitometry studies reported herein and by related low angle X-ray scattering studies. Comparison of the kinetics of changes in membrane structure and photometric parameters (Figs. 3, 8) further shows that these spacing changes are best correlated with changes in transmission, which is known to most closely correlate with volume changes. Therefore, it is reasonable to assume an osmotic basis for the observed spacing changes. Hence, the initial osmolarity of the medium in which chloroplasts are suspended would be expected to play a significant role in determining the magnitude of spacing changes. Deamer and Packer (29), by employing a special rapid centrifugation technique, have demonstrated by isotopic analysis some quantitative and qualitative aspects of anion transport that are commensurate with explaining volume (size) and configuration (shape) changes upon an osmotic basis.

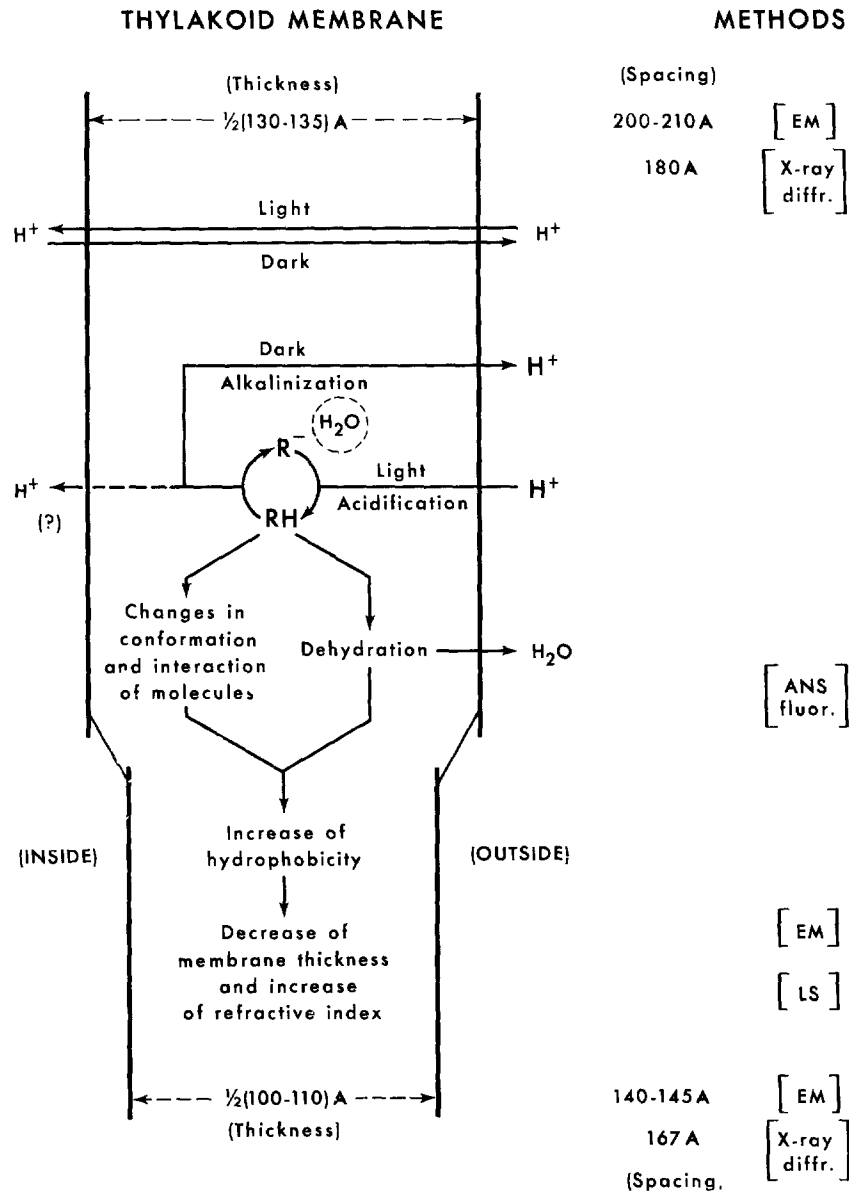THYLAKOID MEMBRANE   METHODS

Figure 21   Proposed mechanism for structural changes in chloroplast membrane by protonation.

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In collaboration with Doctors V. Luzzati and T. Gulik-Krzywicki (unpublished results), we have studied in glutaraldehyde-fixed chloroplasts in the

dark (expanded) and in illuminated (contracted) conditions the spacing changes as observed by low angle X-ray scattering. Preliminary results indicate that a change from 180 to 167 A occurs after illumination of chloroplasts in a sodium chloride-PMA medium.

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