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MECHANISMS OF LIGHT-INDUCED STRUCTURAL CHANGES IN CHLOROPLASTS

I. LIGHT-SCATTERING INCREMENTS AND ULTRASTRUCTURAL CHANGES MEDIATED BY PROTON TRANSPORT

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SUMMARY

Chloroplasts suspended in NaCl solutions undergo structural modifications when illuminated by red actinic light in the presence of electron carriers. These changes are manifested by increments in light scattering and volume, and disruptive alterations of grana structure as demonstrated by electron microscopy. Simultaneously with these modifications protons are transported into the chloroplasts in a light-dependent process. It was postulated that the decrease in internal pH which would result from the proton transport can cause many of the changes observed in chloroplasts during illumination. The following experimental results support this argument:

(a) The kinetics of proton transport and light-scattering increments resemble each other both in onset and decay.

(b) The pH activation curves are similar for both processes, with optima lying between 5.5 and 6.5.

(c) Lowering external pH in the dark to levels which reproduce theoretical internal hydrogen ion concentrations causes alterations in light scattering, volume, and ultrastructure similar to those induced by illumination.

Hence light-induced modifications of chloroplast structure in NaCl solutions are satisfactorily explained by a decreased internal pH resulting from proton transport into the chloroplast.

INTRODUCTION

Several years ago, PACKER[1] reported that chloroplasts undergo pronounced structural changes when illuminated by red actinic light. These modifications can readily be followed by measurements of light scattering and have since been extensively studied by a variety of techniques both *in vitro* and *in vivo*[2–4]. Although light-induced structural modifications have now been well established, there is still no consensus on the basic processes and the literature has many seemingly conflicting observations. For instance, ITOH, IZAWA AND SHIBATA[2] have clearly shown that

Abbreviation: PMS, *N*-methyl phenazonium methosulphate.

illumination of chloroplasts in phosphate buffer results in a decrease in volume which can be measured by Coulter counter, packed volume and electron microscopy, confirming PACKER's original report of light-scattering increments under phosphorylating conditions. In contrast, PACKER, SIEGENTHALER AND NOBEL[5] have also demonstrated marked light-dependent swelling when chloroplasts are suspended in NaCl solutions. This situation has been somewhat clarified by PACKER AND SIEGENTHALER's observation[6] that certain anions, including phosphate, inhibit swelling and enhance shrinking processes, but the underlying mechanism of light-induced structural modifications is still unclear.

Another light-dependent process in isolated spinach chloroplasts, reported at about the same time as the original observation of structural modifications, was JAGENDORF AND HIND's demonstration[7] of light-induced pH increases in chloroplast suspensions. This would presumably result in a decrease in the internal pH of chloroplasts, and the resulting gradient may serve as an energy source for driving biochemical reactions and ion transport. Assuming no internal buffering capacity, JAGENDORF AND NEUMANN[8] have calculated that enough protons are pumped into chloroplasts to lower the internal pH to 2.5. It seems reasonable to refer to this process as proton transport even though the actual mechanism is still unknown. It occurred to us that such a decrease of internal pH might contribute to structural changes in chloroplasts by a reversible charge modification of either soluble or membrane proteins. This hypothesis was tested by varying the pH of chloroplast suspensions and comparing the effects with those produced by light, *i.e.* 90° light-scattering increments and changes in chloroplast volume and ultrastructure. An isoelectric 'precipitation' of chloroplast membranes as proton transport occurs apparently does account for certain changes observed in chloroplast structure during illumination. In a companion study of the action of weak acid anions by CROFTS, DEAMER AND PACKER[9], evidence will be presented for a proton pump which drives anion transport and results in pronounced structural modifications of chloroplasts by an osmotic mechanism.

METHODS

Chloroplast preparation

Chloroplasts were isolated from commercially obtained spinach (*Spinacea oleracea*) in the cold by homogenizing for 20 sec in 0.35 M NaCl buffered at pH 8.0 by 0.04 M Tris–HCl. The homogenate was filtered through 8 layers of cheesecloth and then centrifuged at 300 × *g* for 1 min to remove debris. The supernatant was carefully decanted and centrifuged at 300 × *g* for 5 min. The resulting pellet was washed once, followed by further centrifugation at 300 × *g* for 5 min, and resuspended to a concentration of 1 mg chlorophyll/ml. This yielded 'naked' chloroplasts lacking outer membranes[10].

pH and light-scattering measurements

In experiments chloroplasts were suspended in 3 ml of 0.1 M NaCl at a final concentration of 10 μg chlorophyll/ml in the presence of 20 μM phenazine methosulfate (PMS). The chloroplasts themselves were the main buffering element present, although about 0.4 mM Tris–HCl came over with the chloroplasts.

pH was varied by addition of HCl or NaOH to vigorously stirred suspensions

either from micropipets or continuously with Harvard infusion pumps. With the latter the rate of change of pH was adjusted so that a drop from 8 to 3 took about 5 min. pH was monitored with a Radiometer pH meter and 90° light scattering at 546 mμ was recorded simultaneously in a cuvette located in a light-scattering photometer[1]. Changes in pH and light scattering induced by broad-band red actinic light (1000 foot candles) were measured in the same apparatus.

Electron microscopy

Chloroplasts in suspension were fixed for electron microscopy by addition of 1 ml of 10 % glutaraldehyde to 3 ml of reaction mixture. Glutaraldehyde was distilled at 100° from 50 % glutaraldehyde solution (Fisher Chemical Company). Since the glutaraldehyde was added directly to the cuvette, changes of pH and light scattering occurring during fixation could be followed. After 5 min in the cuvette 0.5-ml aliquots were transferred to Beem capsules* and kept at 0° for 1 h. The chloroplasts were then centrifuged at low speed to the bottom of the capsule and fixed at 0° for 1 h in 1 % osmium tetroxide buffered with 0.1 M sodium phosphate (pH 7.2) followed by dehydration in acetone and embedding in Epon 812. Specimens were sectioned, stained with REYNOLDS' lead citrate[11] and photographed with a Tronscope TRS 50 electron microscope.

RESULTS

pH and light scattering

The simultaneous occurrence of light-scattering increments and proton transport in chloroplasts led us to considering a cause–effect relationship between them. Fig. 1

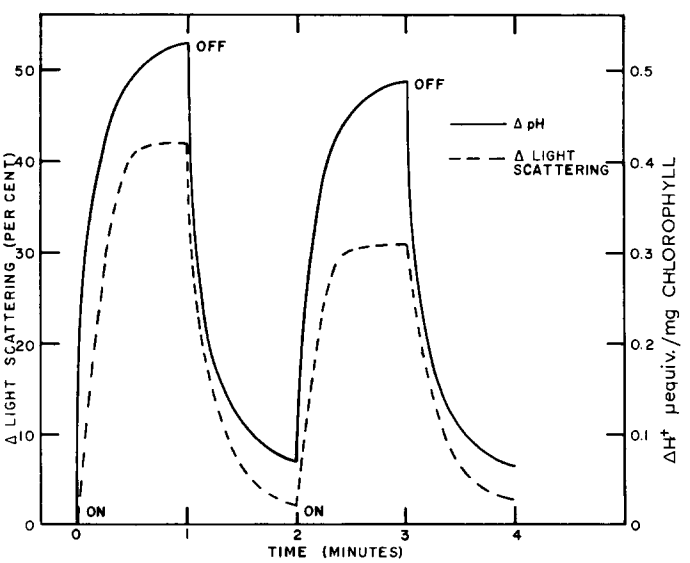

Fig. 1. Kinetics of light-induced pH and scattering increments in chloroplasts. Chloroplasts were suspended in 3 ml of 0.1 M NaCl at a concn. of 10 μg chlorophyll/ml in the presence of 20 μM PMS. Light scattering is expressed as per cent of the dark scattering level.

* Beem Inc., Bronx, N.Y.

shows light-induced proton movements at pH 6.5 and light-scattering changes plotted together. Our results of 0.5 μequiv for proton transport per mg chlorophyll agrees with those of NEUMANN AND JAGENDORF[12] who found 0.65 μequiv with osmotically-shocked or 'broken' chloroplasts. Although 0.5 μequiv was an average value, some of our unbroken chloroplast preparations gave values as high as 0.8 μequiv. This would result in a pH drop to about 2.0 (for the average value) if there were no internal buffering. Light-scattering changes had nearly the same kinetics as the pH change, both for the onset and the decay of the responses. The total light-scattering increment varied from 40 to 90 % with different preparations.

If a decrease in internal pH of chloroplasts causes light-scattering increments, and if no spaces are impermeable to externally added hydrogen ions, then lowering external pH should mimic light-induced scattering responses. Light scattering of chloroplast suspensions as a function of external pH is given in Fig. 2. Scattering begins to increase at pH 6, rises to a maximum at about 4.5, then decreases abruptly below pH 4.0. By back-titrating to pH 8, scattering increments are reversible to the same extent as light-induced scattering changes.

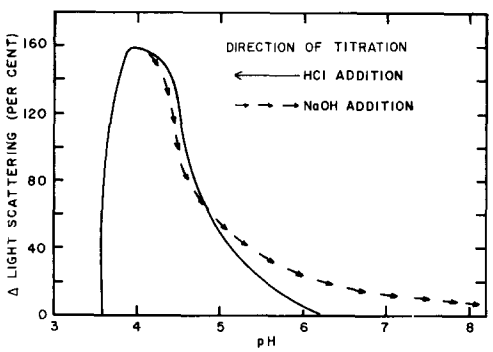

Fig. 2. Light-scattering increments induced in chloroplasts by lowered external pH. Chloroplasts were suspended in 3 ml of 0.1 M NaCl at a concn. of 10 μg chlorophyll/ml. pH was varied continuously by addition of 0.05 M HCl or NaOH. Dilution factors were negligible at this concentration of HCl.

Since hydrogen ions were being added to chloroplast suspensions to reproduce hypothetical light-induced internal proton concentrations, it was necessary to know if there were any internal spaces which were closed to externally added hydrogen ions. An estimate of chloroplast buffer capacity would also be useful in determining internal pH during illumination. Therefore, concentrated chloroplast suspensions isolated in 0.35 M NaCl were titrated with HCl, monitoring H^+ concentration with the pH meter. For these experiments buffers were not present during isolation of chloroplasts since they would interfere with subsequent titrations. The pH of the homogenate in 0.35 M NaCl was about 6.0 during the isolation procedure. The pH of the chloroplast suspension in the cuvette was brought to 7.0 with NaOH before beginning the titration. Another titration was performed on aliquots of the chloroplast preparation as described above, except that 0.01 % Triton X-100 was present. As a control for the presence of buffering capacity external to the chloroplasts, the suspension was centrifuged at 11000 $\times$ g for 20 min and the supernatant also titrated as described above.

It is clear from Fig. 3 that NaCl solutions had no buffering capacity and therefore did not contribute to the titration curves. However, a slight deviation from the theoretical curve was observed. Since this error was constant in all titrations no correction was necessary. A small amount of buffering material was present external to the chloroplasts, but the major buffering contribution was by chloroplasts. Moreover, there was no discernible difference between chloroplasts and Triton-treated chloroplasts, indicating externally added hydrogen ions equilibrated between all compartments.

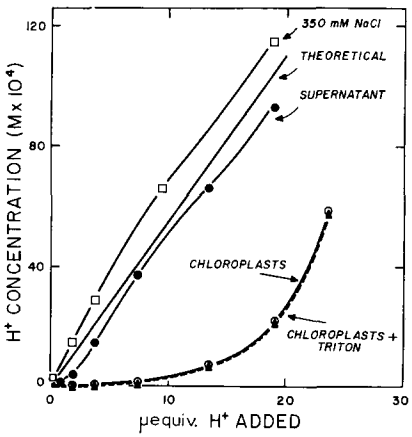

Fig. 3. Determination of chloroplast-buffering capacity and permeability to protons. Chloroplasts were suspended in 5 ml of 0.35 M NaCl at a concn. of 0.58 mg chlorophyll/ml and titrated by addition of 0.05 M HCl. H^+ addition is expressed as μequiv/mg chlorophyll, and equivalent additions were made to the NaCl and supernatant. Hydrogen ion concentration was calculated from the pH.

TABLE I

DETERMINATION OF THE BUFFER CAPACITY OF SPINACH CHLOROPLASTS

Chloroplasts were suspended in 5 ml of 0.35 M NaCl at a concn. of 0.58 mg chlorophyll/ml and titrated by addition of 0.05 M HCl. H^+ addition is expressed as μequiv /mg chlorophyll, and equivalent additions were made to the NaCl and supernatant. Hydrogen ion concentration was calculated from the pH.

μequiv H^+ added	*Hydrogen ion concentration (moles/l) × 10^5*			
	Chloroplasts		*Controls for non-chloroplast buffering capacity*	
	No addition	*0.01% Triton*	*0.35 M NaCl*	*Supernatant*
0.38	0.08	0.07	26	1
0.76	0.32	0.30	56	6
1.14	0.79	0.76	85	14
1.52	1.41	1.38	115	25
1.90	2.00	1.90	148	43
2.86	2.95	2.82	219	89
3.80	4.46	4.46	289	145
5.70	6.30	6.03	417	252
7.60	11.20	10.50	—	372
9.50	22.40	21.40	662	469

Since the main biological interest of the titration lies in the portions of the curve above pH 3.5, additional data are presented in Table I. Chloroplasts had buffering capacity throughout the pH range examined and bound 6.4 μequiv H^+/mg chlorophyll. This was calculated from Table I as follows: at pH 3.65, 9.5 μequiv H^+ had been added per mg chlorophyll, and the concentration, calculated from the pH, was $22.4 \cdot 10^{-5}$ moles/l. In the supernatant, an equivalent addition lowered the pH to 2.33, or $469 \cdot 10^{-5}$ moles/l, and in 0.35 M NaCl the pH decreased to 2.18 or $662 \cdot 10^{-5}$ moles/l. The μequiv bound per mg chlorophyll is then:

$$\frac{469 \cdot 10^{-5} \text{ moles/l} - 22.4 \cdot 10^{-5} \text{ moles/l}}{662 \cdot 10^{-5} \text{ moles/l}} \times 9.5 \ \mu\text{equiv/mg chlorophyll} =$$

6.4 μequiv. bound per mg chlorophyll, or 1.8 μequiv./mg chlorophyll per pH unit (1)

If light-induced scattering increments of chloroplasts suspended in NaCl solutions are due to decreased internal pH, then levels of pH- and light-induced scattering should be similar in magnitude and at some point be competitive. Fig. 4 is a typical result showing pH-induced scattering to be somewhat greater than light-induced increments, usually about twice the magnitude. As pH-induced scattering begins to increase, light-induced scattering declines, reaching zero as the pH approaches 4.5.

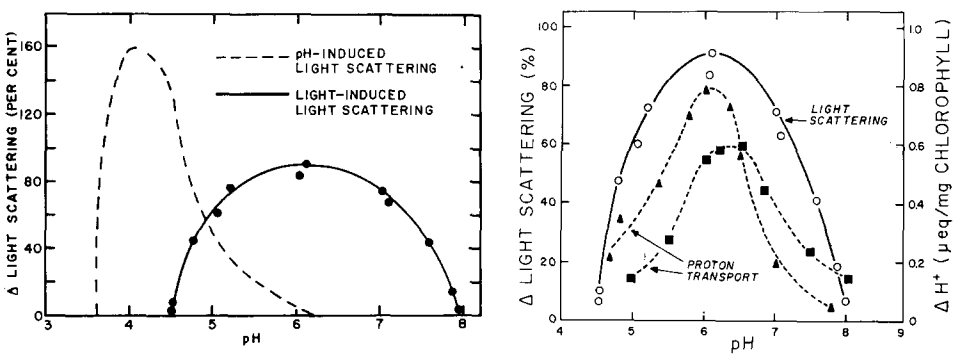

Fig. 4. pH dependence of pH- and light-induced scattering increments in chloroplasts. Chloroplasts were suspended in 3 ml of 0.1 M NaCl at a concn. of 10 μg/mg chlorophyll in the presence of 20 μM PMS. pH was varied by addition of 0.05 M HCl.

Fig. 5. pH activation curves of light-induced proton transport and scattering increments in chloroplasts. Chloroplasts were suspended in 3 ml of 0.1 M NaCl at a concn. of 10 μg chlorophyll/ml in the presence of 20 μM PMS. Whole chloroplasts (▲---▲); NEUMANN AND JAGENDORF'S values[12] for 'broken' chloroplasts (■---■).

This result could be interpreted either as competition between internal and external protons for the same chloroplast component, or as an inhibition of proton transport at low pH. The latter possibility seems more likely, since light-induced light-scattering increments and proton transport have similar pH activation curves, as seen in Fig. 5. Both curves have maxima between pH 5.5 and 6.5 and decline sharply below pH 5. As shown in Fig. 5 these results are similar to those of NEUMANN AND JAGENDORF[12] who found a maximum for proton transport at pH 6.0–6.5.

Investigation of chloroplast component causing light scattering

A pH-induced increase in light scattering by chloroplast substructure could

arise in several ways. For example, a soluble or loosely bound protein might be undergoing an isoelectric precipitation. Alternatively, modification of internal membrane systems by changes in refractive index or packing of grana lamellae as they approached their isoelectric point could also influence light scattering. To choose between these possibilities, a number of experiments were performed in which chloroplasts were osmotically disrupted in distilled water, then centrifuged at 11000 × *g* for 30 min. The supernatant was filtered through a 0.45-μ millipore filter which removed remaining chlorophyll-containing material. The filtered supernatant so obtained contained soluble protein which accounted for about 10% of the original chloroplast protein. (A similar yield of soluble protein was obtained if the chloroplasts were first acetone extracted.) Both the supernatant and the green pellet were then made up to 0.1 M with NaCl. As a control, chloroplasts were disrupted in distilled water and immediately brought back to 0.1 M with NaCl. Fig. 6 shows typical results of pH-induced light-scattering curves run on each of these solutions at equivalent concentrations. About 10% of the original light scattering could be accounted for in the soluble material. Another 50–60% was present in the membrane fragment fraction. About 10–20% seemed due to a modification of the integrated chloroplast structure, since osmotic shock typically lowered pH-induced light scattering by this amount. This result, *i.e.*, that the major scattering increment at low pH lies in membrane fragments, is in accord with the observations of GROSS AND PACKER[13] who found that chloroplast membrane fragments undergo marked volume and light-scattering changes in response to actinic light.

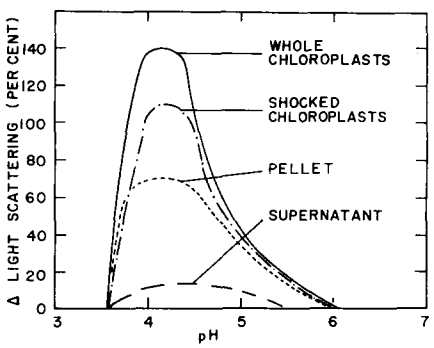

Fig. 6. pH-induced scattering increments for chloroplast fractions. Scattering increments are expressed as per cent of base level set for whole chloroplasts. Fractions were suspended in 3 ml 0.1 M NaCl and pH was varied continuously by addition of 0.05 M HCl.

Chloroplast volume determination

The volume of chloroplasts was measured by 2 methods, chlorocrit or packed volume[5], and by the use of a Coulter counter[14]. Determinations of chloroplast volume as a function of pH is given in Table II. Packed volume of dark chloroplasts decreased by about 35% as pH was lowered from 7.8 to 5.0. However, this result is probably an artifact (perhaps due to decreased surface charge) since no decrease in Coulter counter volume could be found in the same pH range. Coulter counter volume in dark chloroplasts varied from 23 μ^3 in 0.35 M NaCl to 57 μ^3 in 0.1 M NaCl, a result consistent with the known osmotic effect of the salt[15]. Sodium acetate in the dark

TABLE II

CHLOROPLAST VOLUME DETERMINATIONS

For Coulter counter volume chloroplasts were suspended at concentrations giving 30000–50000 particle count/50 μl, using a 50-μ orifice. The reaction mixture contained 20 μM PMS and the concentration of salt indicated in the table. Illumination was 500 foot candles of broad-band red actinic light for 1 min before and during volume determinations. Packed volume was measured according to PACKER, SIEGENTHALER AND NOBEL[5] in a reaction medium containing 20 μM PMS, the concentration of salt indicated in the table and about 2 mg chlorophyll/ml.

Medium	*Coulter counter volume* (μ^3)		
	pH	*Dark*	*Light*
0.35 M sodium chloride	8.0	27	55
0.35 M sodium chloride	6.0	23	35
0.10 M sodium chloride	7.0	45	80
0.10 M sodium chloride	5.0	57	75
0.20 M sodium acetate	8.0	22	7
0.10 M sodium acetate	8.0	45	15
0.10 M sodium acetate	5.5	57	15
Packed volumes (ml/mg chlorophyll)			
0.10 M sodium acetate	7.8	0.17	—
0.10 M sodium acetate	5.0	0.11	—

also appeared to act primarily in a simple osmotic fashion since chloroplast volume varied inversely with concentration.

The effect of light on Coulter volume in the absence and presence of a weak acid anion (sodium acetate) was quite marked. After 1 min of illumination the volume increased to twice the original value in NaCl, whereas a marked shrinkage to a third of the original volume took place in sodium acetate. These findings compare with the results of ITOH, IZAWA AND SHIBATA[2] who noted a shrinkage in phosphate buffer, and PACKER, SIEGENTHALER AND NOBEL[5], who found that chloroplasts swell in NaCl upon illumination. Phosphate, with a pK_a greater than 1, is a weak acid anion.

Electron microscopy

ITOH, IZAWA AND SHIBATA[2] found a pronounced ultrastructural 'shrinkage' in chloroplasts illuminated in phosphate buffer which corresponded to a decrease in volume. However, the ultrastructural changes in light-swollen chloroplasts in NaCl have not been described. It was reasoned that the effects of light and low pH on chloroplast structure should be similar if the main effect of illumination is to lower the internal pH. To test this possibility chloroplasts were fixed in the absence and presence of sodium acetate before and during illumination. The same preparations were also fixed after dark incubation at pH 4.5. In the dark or at low pH, addition of glutaraldehyde to 2.5 % produced a dilution effect, decreasing light scattering by about 20 %. Little further change in scattering was then observed. Glutaraldehyde added during illumination at the maximum scattering level caused a small decrease in scattering beyond that of dilution. However, final scattering was at least half of the original maximum scattering and did not reverse in the dark.

Fig. 7 is a micrograph showing several chloroplasts in the dark in NaCl. Loosely stacked grana are typical of dark chloroplasts either in sodium acetate or NaCl.

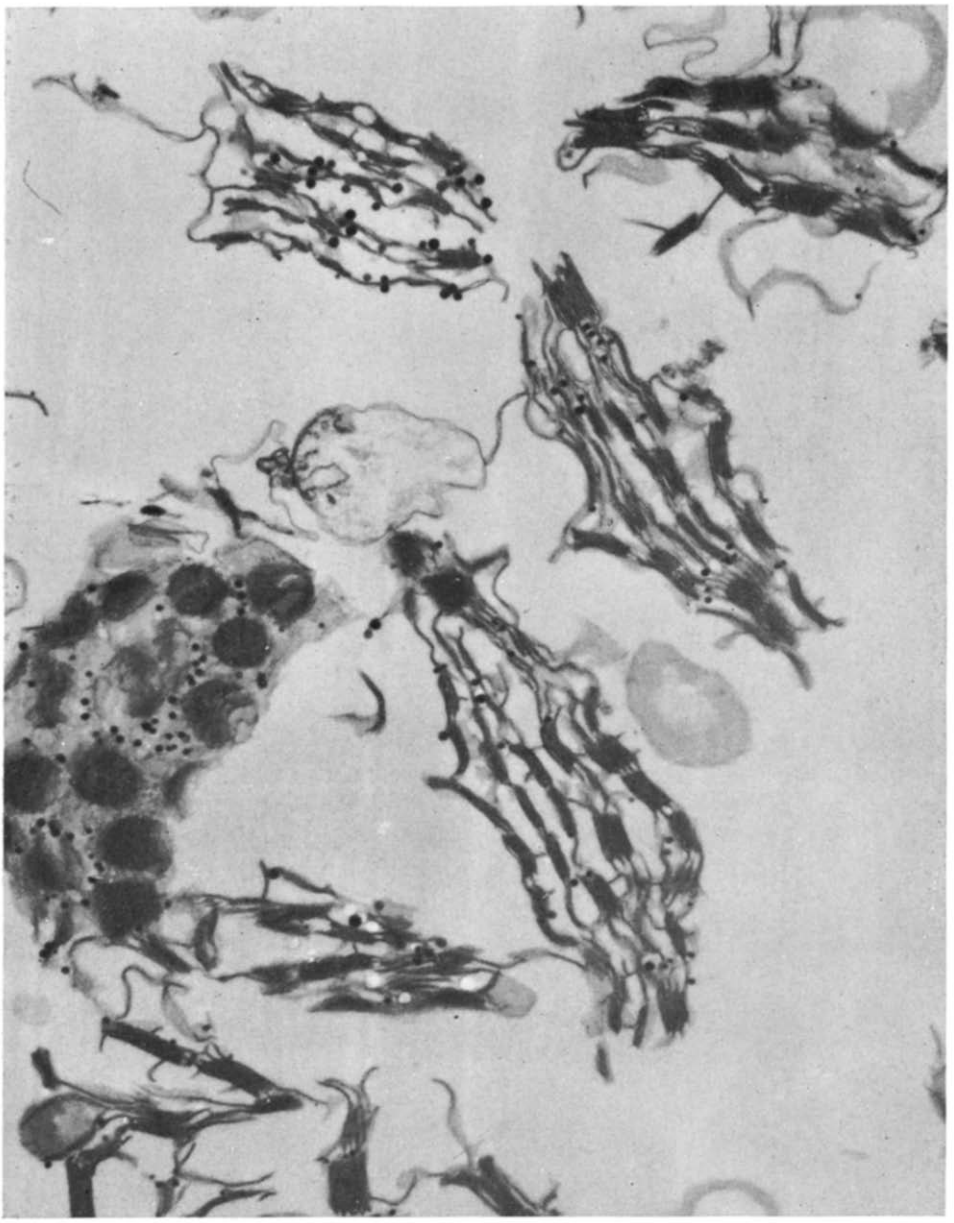

Fig. 7. Dark control chloroplasts fixed in 0.1 M NaCl at pH 6. Similar results were obtained at pH 6.0–8.0 in NaCl or sodium acetate. Magnification 8000 ×.

Fig. 8 shows the same preparation during illumination in NaCl. The chloroplasts are badly disrupted, confirming earlier reports from this laboratory of a light-induced degradative type of swelling in NaCl (ref. 5). In spite of the apparent disruption, a 80% increment in scattering had occurred at the time of fixation. Light-induced swelling has also been observed by IZAWA AND GOOD[16] for chloroplasts illuminated

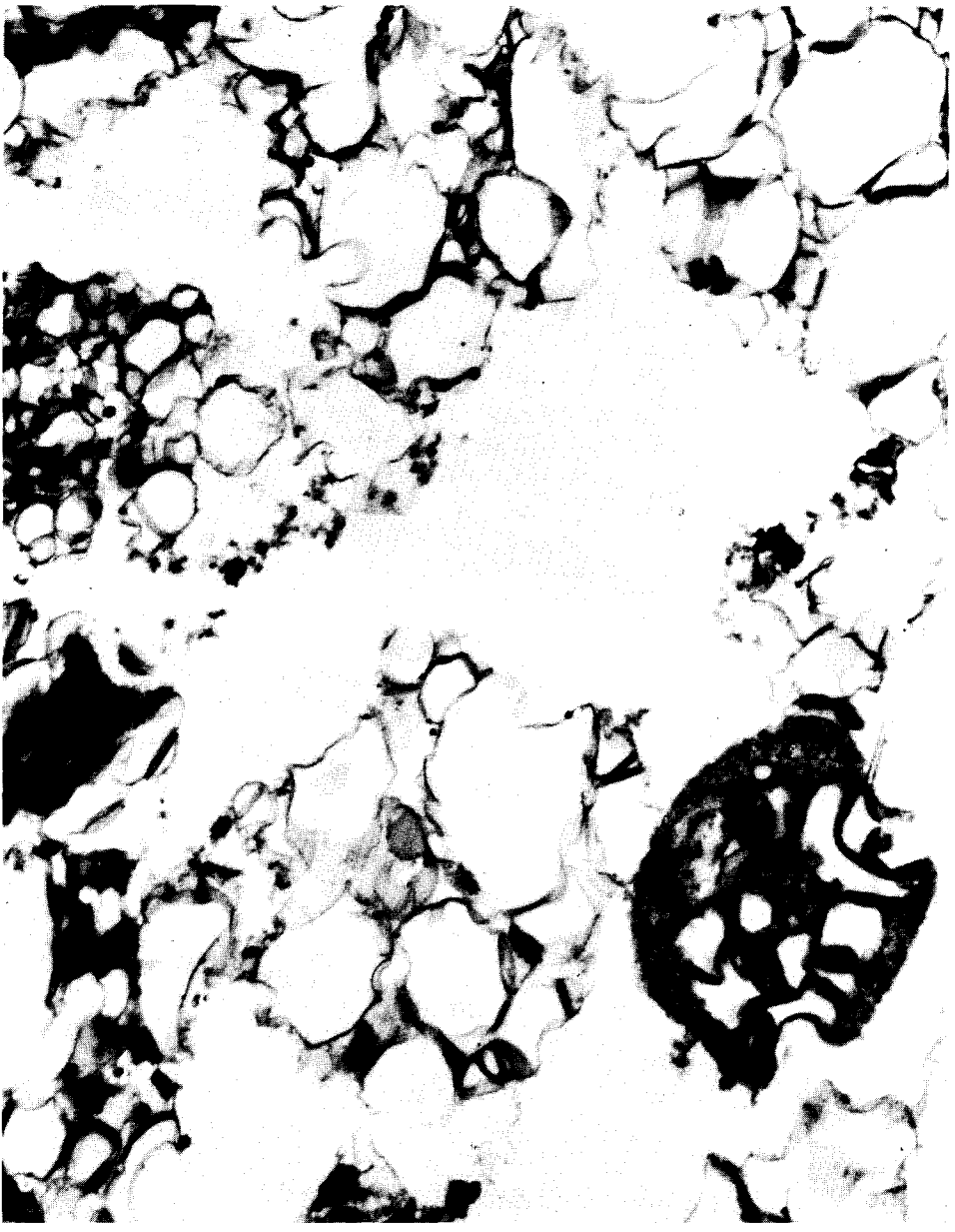

Fig. 8. Chloroplasts illuminated for 1 min with 1000 foot candles red actinic light. Medium was 0.1 M NaCl (pH 6.0) with 20 μM PMS present. Magnification 8000 ×.

in methylamine hydrochloride. For comparison, Fig. 9 shows the same preparation illuminated in 0.1 M sodium acetate. Here, the chloroplasts have shrunk, structure is well preserved and grana lamellae are tightly packed. The light-scattering increment under these conditions is twice that in NaCl, and about equivalent to the maximum pH-induced scattering increment. ITOH, IZAWA AND SHIBATA[2] have found a similar

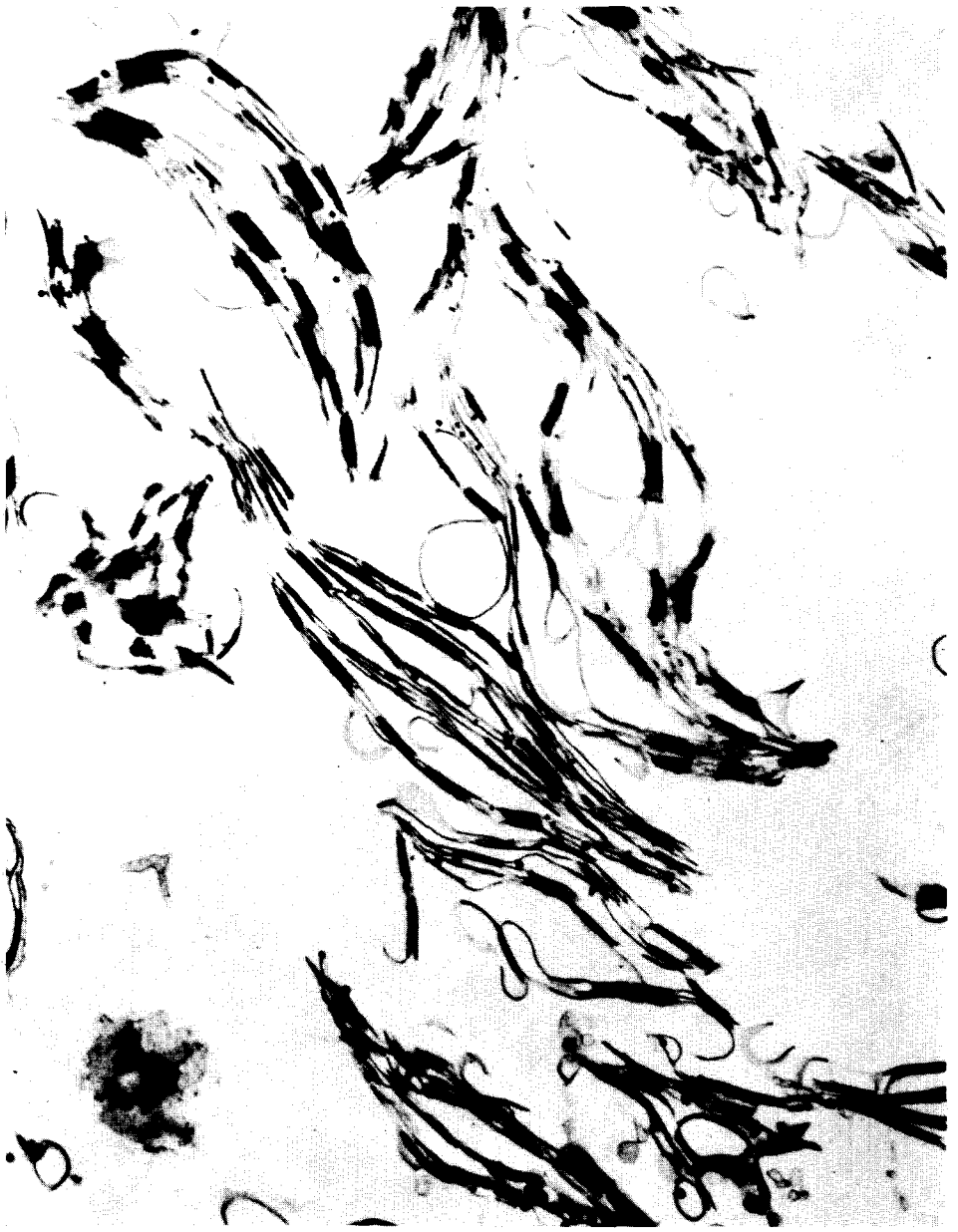

Fig. 9. Chloroplasts illuminated for 1 min in 0.1 M sodium acetate (pH 6.0) with 20 μM PMS present. Similar results were obtained in the pH range 6.0–8.0. Magnification 8000 ×.

shrinkage when chloroplasts were illuminated in the presence of phosphate. Fig. 10 shows the corresponding morphological condition of dark chloroplasts brought to pH 4.5 in 0.1 M NaCl. The chloroplasts are for the most part disrupted, although a few whole ones may be found. Similar disruption of structure occurs in 0.1 M sodium acetate at pH 4.5 (not shown). In general, chloroplasts illuminated in NaCl and chloro-

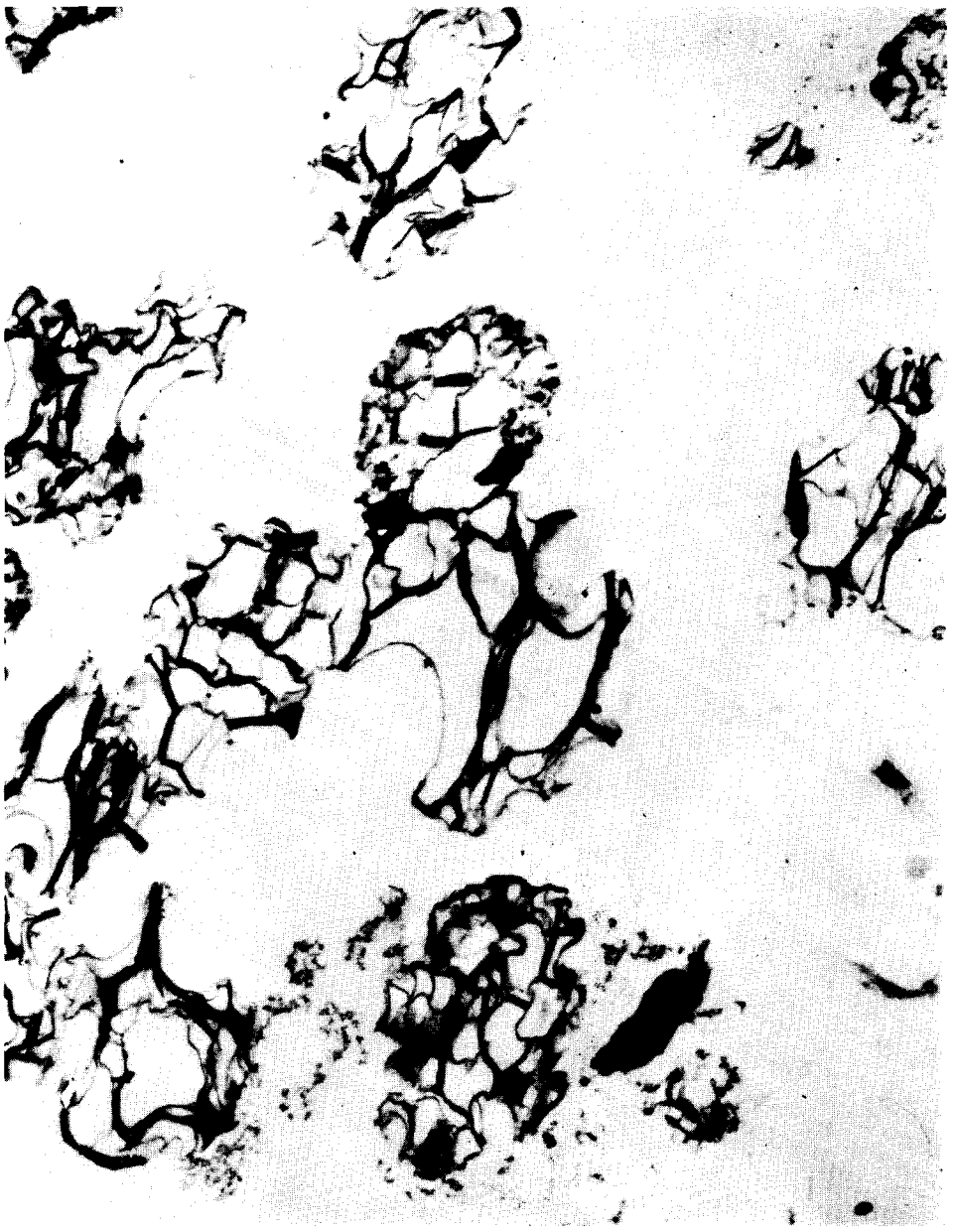

Fig. 10. Chloroplasts fixed in the dark in 0.1 M NaCl (pH 4.7). Similar results were obtained at low pH ($4.0 < pH < 5$) in sodium acetate. Magnification 8000 ×.

plasts brought to pH 4.5 show similar modes of structural disruption. The normal shape of the chloroplasts has disappeared for the most part, although membranes seem to remain intact. Large vesicles appear in both, with deformed grana stacks loosely connected by the vesicular structure. The main difference between the 2 preparations is a tendency toward formation of larger vesicles in the illuminated chloroplasts.

DISCUSSION

Mechanism of light-scattering increments in chloroplasts during illumination

The hypothesis that light-induced scattering increments of chloroplasts in NaCl solutions are caused by lowered internal pH is supported by the following results:

(1) The kinetics of the light-induced proton transport and light-scattering increments are nearly the same (Fig. 1). Furthermore, compounds capable of inhibiting the pH change are also known to inhibit scattering increments as seen in Table III. The action of most of these compounds can be interpreted in terms of either producing physical 'leaks' in the membrane (detergents) or transporting hydrogen ion in such a way as to discharge the pH gradient. MITCHELL[17] has suggested similar mechanisms to account for the uncoupling action of these and related compounds; the uncoupling action of amines will be discussed in the accompanying paper[9].

TABLE III

COMPARISON OF INHIBITORS OF pH CHANGES AND LIGHT-SCATTERING INCREMENTS

Compound	*mM*	*Per cent inhibition*	
		Light scattering	*pH*
3-Chloro-carbonyl cyanide phenylhydrazone	0.08	100 (ref. 21)	—
	0.004	—	37 (ref. 8)
Pentachlorophenol	0.02	100 (ref. 21)	—
	0.15	—	38 (ref. 8)
3-(3,4-dichlorophenyl)-1-1-dimethyl urea	0.001	100	100
NH_4Cl	5.0	100 (ref. 21)	—
	1.0	—	42 (ref. 8)
Triton X-100	0.01 %	100 (ref. 22)	42 (ref. 8)

(2) The pH activation curves for both proton transport and scattering changes are similar and have similar optima.

(3) Lowering the external pH causes light-scattering increments similar in magnitude to light-induced changes. Furthermore, they are reversible, as are light-induced increments in the decay or off cycle. The fact that pH-induced increments are always somewhat larger may be due to the involvement of the whole chloroplast structure, rather than only the grana structure which would be involved in a light-induced pH change.

There are several factors which might contribute to pH-induced light-scattering increments. Two of these were of no biological interest and had to be ruled out as major contributions. First, there may have been a soluble material external to the chloroplasts which was precipitating as pH was lowered. This was shown to be insignificant by centrifuging down the chloroplasts in the test medium prior to lowering the pH. Only 5 % of the original light-scattering increment could be demonstrated when pH of the supernatant was lowered to 4.5. A more serious objection was that chloroplasts tend to aggregate at low pH, which could conceivably contribute to

90° light scattering. However, a number of tests indicated that this was not a contributing factor:

(1) The light-scattering increment began at pH 6, long before aggregates became visible to the eye.

(2) As pH was increased again from 4.5 to 8, the aggregates remained even though 90° scattering was reversed.

(3) Rates of aggregation in particulate systems are proportional to particle concentration. However, there was no discernible increment in the rate of pH-induced scattering change as concentration of chloroplasts was increased from 1 to 20 μg chlorophyll/ml.

(4) Finally, and most convincing, pH-induced and light-induced scattering were competitive. If a maximum light-induced change was produced under optimum conditions, lowering the pH to 4.5 produced only a small further increment in light scattering.

We would view light-scattering increments at low pH as primarily due to dehydration of membranes. As pH is lowered and membranes lose their net charge, less water would be bound and refractive index of the membrane would increase. That chloroplast membranes do have an isoelectric point in this region is supported by the fact that whole chloroplasts from a number of species have isoelectric points between 4.4 and 5.0 (ref. 18). A similar effect should be induced by binding of polyvalent cations, since this would also reduce membrane charge. Light-scattering increments in the presence of magnesium, calcium and barium have been observed by NISHIDA AND KOSHII[15]. Similar views of cationic dehydration of membranes have been advanced by TOBIAS[19].

Internal pH of chloroplasts during illumination

Our calculation of 2.0 for the internal pH of chloroplasts during illumination (assuming no internal buffering) is similar to the estimate of 2.5 of JAGENDORF AND NEUMANN[8], who based their value on packed volume of chloroplasts. Our calculation is based upon the Coulter counter volume and particle count:

$$\mathrm{pH} = -\log \frac{(\text{moles } H^+_{\text{initial}}) + (\text{moles } H^+_{\text{transported}})}{pV} \qquad (2)$$

where p equals 10^9 particles/mg chlorophyll, V equals $45 \cdot 10^{-15}$ l per chloroplast, and moles $H^+_{\text{transported}}$ equals $0.5 \cdot 10^{-6}$/mg chlorophyll. (Initial moles H^+ is very small and can be ignored.) The values for p and V derived from our data are substantially in agreement with literature values[2,5,20]. The value of 2.0 is probably conservative, since the volume into which protons are transported is not that of the whole chloroplast but only that of the inter-lamellar spaces of the grana. However, the figure of pH 2.0 is instructive in emphasizing that chloroplasts are potentially capable of drastically lowering the pH of certain internal compartments.

Titrations of chloroplast suspensions with acid were done in an attempt to arrive at a more meaningful figure for internal pH. Over the pH range between 6 and 3, calculations similar to those of Eqn. 1 showed that chloroplasts bound 95 % of the hydrogen ion available (*i.e.*, protons not bound by the supernatant buffering material). This figure varied from 92 % at pH 6.1 and 3.0, to 97.5 % at pH 4.2.

Assuming 95 % as the maximum binding capacity of chloroplasts, the internal pH can be recalculated to be 3.6 from Eqn. 2:

$$\mathrm{pH} = -\log \frac{0.05 \times 0.5 \times 10^{-6} \text{ moles } H^+}{10^9 \times 45 \times 10^{-15}} = 3.6$$

Even if 99 % of the transported protons were bound, the internal pH would still drop to 4.0.

If the hypothesis that light-induced light-scattering increments are directly caused by decreased internal pH is correct, then scattering increments should provide an indicator of internal pH. The difference in pH units between the lower portions of the curves in Fig. 4 would then be a measure of the trans-membrane pH gradient, since both increments are due to binding of hydrogen ions to chloroplast membranes. This turns out to be a maximum of 2.4 pH units. At a starting pH of 6.0, this would be equivalent to an internal pH of 3.6, which agrees with the estimate above derived from the proton movement, chloroplast volume, and buffering capacity.

Light-induced swelling and structural degradation

A simple decrease in internal pH would not be expected to cause a decrease in chloroplast volume without an accompanying osmotic effect. Indeed no decrement in Coulter counter volume was observed either in chloroplasts illuminated in NaCl solutions or when the external pH was lowered in the dark. These findings are confirmed by the results of electron microscopy. A decrease in the volume of illuminated chloroplasts was seen only when a weak acid anion, like acetate, was present. No evidence for volume decrease was apparent in chloroplasts illuminated in the presence of a dissociated anion like chloride (as NaCl).

The pronounced light-dependent swelling which is evident in chloroplasts suspended in NaCl is still unexplained. IZAWA AND GOOD[16] have suggested that such swelling results from the uncoupling action of Tris buffer which has been extensively used in chloroplast systems. However, this is not a satisfactory explanation since light-dependent swelling occurs when Tris buffer is absent. The fact that lowering the external pH affects chloroplast ultrastructure in a manner similar to illumination in NaCl suggests that structural disruption is partially caused by a drastic reduction in the internal pH. Compensating ionic movements, for instance an influx of chloride, probably contributes to swelling. This is further substantiated by the protective action of permeant anions and photophosphorylation conditions[9]. It has been suggested that the free energy of the pH gradient may be used to drive ion transport or chemical reactions in these instances[3,17]. In the absence of energy-requiring reactions, high concentrations of hydrogen ion could accumulate within chloroplasts and result in random disruption of membrane structure.

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