

# Plants under Climatic Stress

## II. LOW TEMPERATURE, HIGH LIGHT EFFECTS ON CHLOROPLAST ULTRASTRUCTURE

Received for publication September 24, 1970

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### ABSTRACT

Mesophyll chloroplasts of the C<sub>3</sub>-pathway grasses *Sorghum* and *Paspalum* and of the C<sub>4</sub>-pathway legume soybean undergo ultrastructural changes under moderate light intensities (170 w·m<sup>-2</sup>, 400–700 nanometers) at a time when photosynthesis is much reduced by low temperature (10 C). The pattern of ultrastructural change was similar in these species, despite some differences in the initial sites of low temperature action on photosynthesis and differences in their mechanisms of CO<sub>2</sub> fixation. Starch grains in the chloroplasts rapidly reduce in size when chilling stress is applied. At or before the time starch grains completely disappear the membranes of the individual stromal thylakoids close together, reducing the intraspace between them while the chloroplast as a whole begins to swell. Extensive granal stacking appears to hold the thylakoids in position for some time, causing initial swelling to occur in the zone of the peripheral reticulum, when present. At more advanced stages of swelling the thylakoid system unravels while the thylakoid intraspaces dilate markedly. Initial thylakoid intraspace contraction is tentatively ascribed to an increase in the transmembrane hydrogen ion gradient causing movement of cations and undissociated organic acids from the thylakoid intraspace to the stroma. Chloroplast swelling may be caused by a hold-up of some osmotically active photosynthetic product in the chloroplast stroma. After granal unraveling and redilation of the thylakoid intraspaces, chloroplasts appear similar to those isolated in low salt hypotonic media. At the initial stages of stress-induced ultrastructural change, a marked gradient in degree of chloroplast swelling is seen within and between cells, being most pronounced near the surface of the leaf directly exposed to light.

synthetic disruption. Chloroplasts of the C<sub>3</sub>-pathway tropical grasses *Paspalum* and *Sorghum* and of the warm temperature C<sub>4</sub>-pathway legume soybean were investigated.

### MATERIALS AND METHODS

**Plants and Plant Conditioning.** Plants used were *Sorghum* hybrid NK 145, *Paspalum dilatatum* Poir, and *Glycine max* (L) Merr. cv. Merit. Potting media, plant nutrients, and controlled environment cabinets were as described previously (18).

Plants were given three phases of treatment, usually at 170 w·m<sup>-2</sup> (400–700 nm) with 12-hr photoperiods. They were pre-conditioned at a constant day-night temperature of 25 C for 11 to 14 days. The temperature was then lowered to 10 C day and night for several days and then returned to 25 C. Temperatures were lowered at the commencement of a dark period, unlike previous work (18), and took 10 to 15 min to reach and stabilize at 10 ± 1 C. In some investigations involving *Sorghum*, only the day or only the night temperature was reduced to 10 C and some plants were shaded to 50 w·m<sup>-2</sup> during the low temperature treatment.

**Electron Microscopy.** Samples were taken from the middle region of youngest-mature leaves 9 hr after the commencement of 12-hr photoperiods. Leaf sections 1 × 5 mm were cut under cold 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2), vacuum-infiltrated for 10 min, and fixed in fresh fixative at 4 C for 3 hr in darkness. Specimens were subsequently buffer-washed, postfixed in 1% OsO<sub>4</sub> (0.1 M phosphate; 3 hr), buffer-washed, and dehydrated in a graded ethanol series. They were then infiltrated via a propylene oxide series and embedded in Araldite. Sections were post-stained for 5 min in saturated aqueous uranyl nitrate, 5 min in lead citrate, and examined with a Philips EM-200 electron microscope.

Electron microscope negatives were traversed with a Joyce Loebel Mk III C recording microdensitometer to measure thylakoid membrane and thylakoid intraspace widths.

### RESULTS

Effects of light and temperature on chloroplast ultrastructure and development have been investigated in various pigment-deficient mutants (6, 17), in detached leaves of etiolated plants (9), and in normal mature leaves (2). Rapidly occurring changes in the ultrastructure or size of isolated chloroplasts caused by light (3) and divalent cations (7) have also been described. Some ultrastructural changes are often very characteristic and relate to specific changes in functional parameters such as electron transport (8).

In this paper changes in chloroplast ultrastructure are investigated in relation to photosynthetic damage under low temperature, high light stress (18). This is an attempt to describe more fully the nature of and reasons behind photo-

*Sorghum*. Changes in chloroplast ultrastructure in *Sorghum* leaves under low temperature, high light stress are shown in Figures 1 to 10. The extent of ultrastructural change after various periods of stress was reasonably consistent, and generally micrographs have been chosen of chloroplasts typical of the day in question. Ultrastructural changes occurred first, however, near the surface of the leaf directly exposed to the light (Fig. 8), so that a marked gradient in change was seen both within and between cells. The gradient could start from either the ad- or abaxial surfaces of the leaf, depending on leaf orientation. Since light intensity has been demonstrated to regulate the onset of photosynthetic disruption (18), it seems

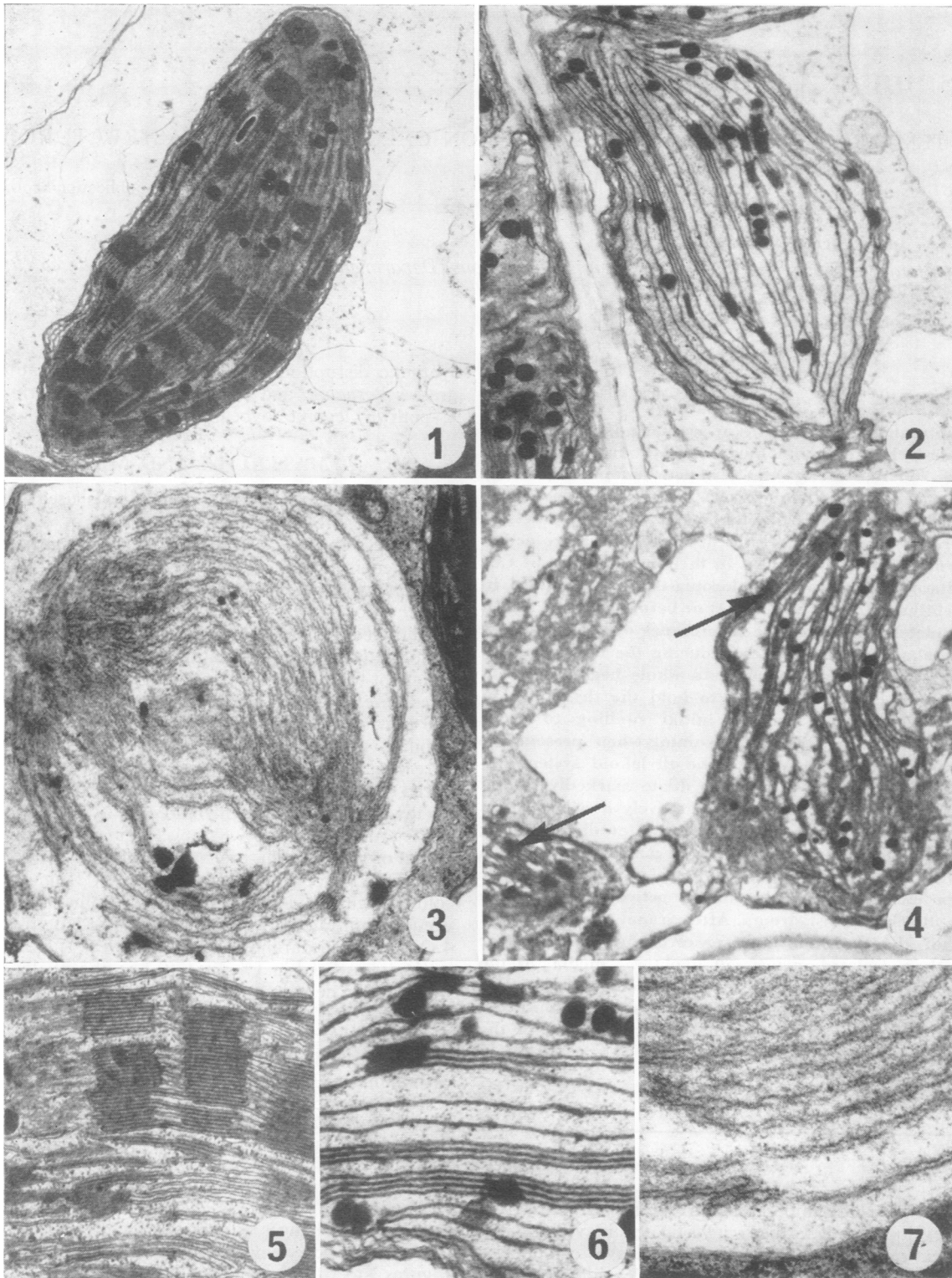


FIG. 1. *Sorghum* upper mesophyll chloroplast developed at 25 C ( $170 \text{ w} \cdot \text{m}^{-2}$ ).  $\times 15,000$ .

FIG. 2. *Sorghum* upper mesophyll chloroplast developed at 25 C, then given 1.5 days of chilling treatment. Stromal swelling, decrease in thylakoid intraspaces widths, parallel runs of thylakoids, and reduction in granal stacking can be seen.  $\times 15,000$ .

FIG. 3. *Sorghum* upper mesophyll chloroplast after 2.5 days of chilling treatment. No grana remain in this chloroplast, and thylakoid intraspaces show marked redilation.  $\times 12,000$ .

FIG. 4. *Sorghum* upper mesophyll chloroplast after 2.5 days of chilling treatment. Stromal material has precipitated. Some grana (arrows) remain in this chloroplast.  $\times 8000$ .

FIGS. 5, 6, AND 7. Portions of *Sorghum* chloroplasts from Figures 1, 2, and 3 showing thylakoid detail at higher magnification.  $\times 30,000$ .

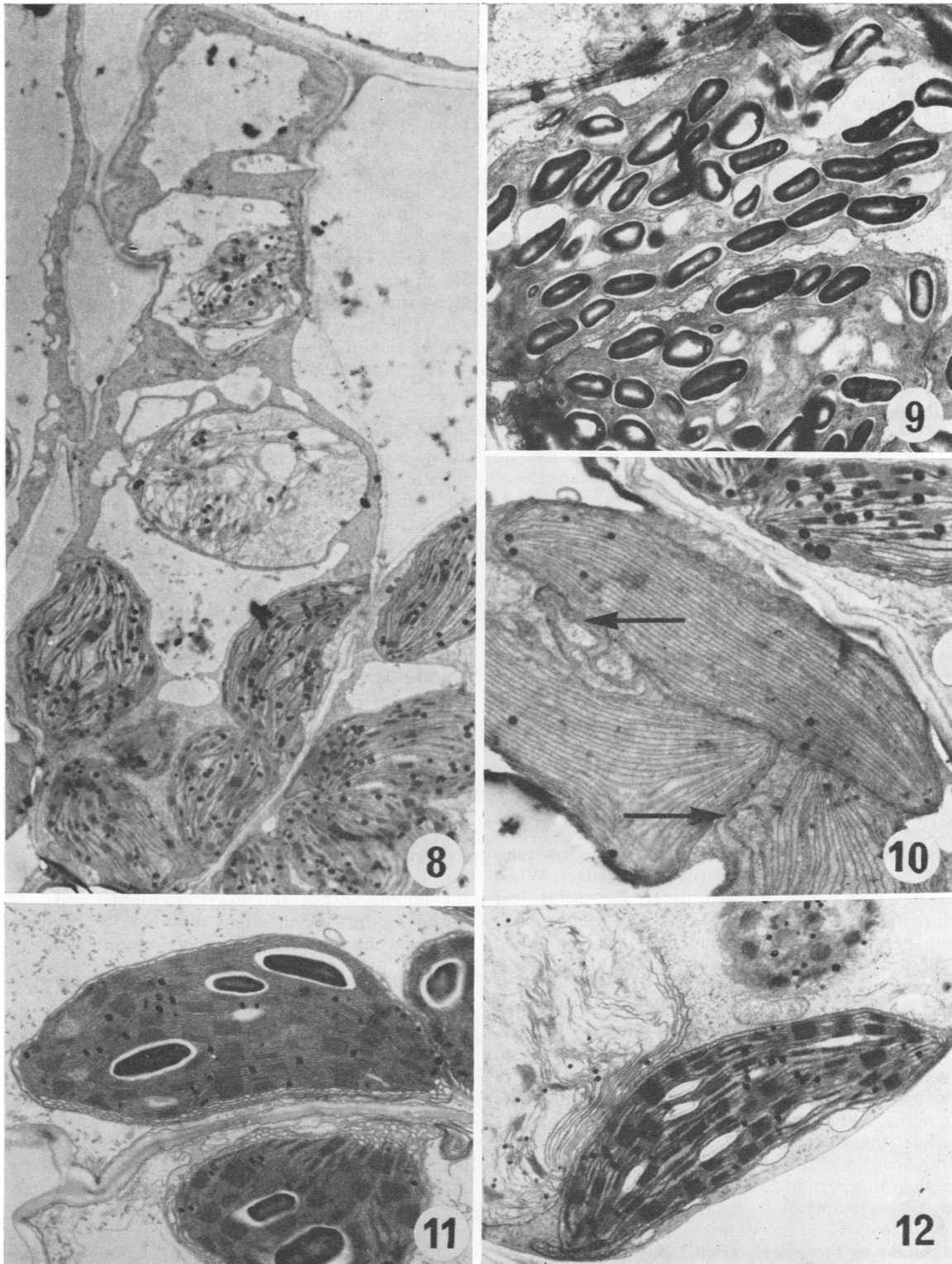


FIG. 8. *Sorghum* upper mesophyll cell after 1.5 days of chilling treatment. A marked gradient in chloroplast ultrastructural change is seen from the top (adaxial leaf surface) to the bottom of the cell.  $\times 5500$ .

FIG. 9. *Sorghum* bundle sheath chloroplasts developed at 25 C ( $170 \text{ w}\cdot\text{m}^{-2}$ ).  $\times 8000$ .

FIG. 10. *Sorghum* bundle sheath chloroplasts developed at 25 C, then given 1.5 days of chilling treatment. Starch has disappeared, and irregular protuberances (arrows) have developed.  $\times 8000$ .

FIG. 11. *Paspalum* upper mesophyll chloroplasts developed at 25 C ( $170 \text{ w}\cdot\text{m}^{-2}$ ). Extensive peripheral reticulum is seen adjacent to cell wall.  $\times 12,000$ .

FIG. 12. *Paspalum* upper mesophyll chloroplasts developed at 25 C, then given 3.5 days of chilling treatment. Several dome-shaped ruptures in the zone of the peripheral reticulum and starch grains of reduced size can be seen in the less damaged chloroplast. The other chloroplast is grossly swollen with much reduced granal stacking.  $\times 12,000$ .

likely that light intensity gradients within the leaf are responsible for these gradients in ultrastructural change.

**Upper mesophyll.** When the temperature was lowered, swelling of the chloroplast stroma occurred (Figs. 1 and 2) while the membranes of the individual thylakoids came together, eliminating the intraspaces (Figs. 5 and 6), and the thylakoids were seen with more contrast against a lighter staining stroma. As swelling became more pronounced, the thylakoids moved apart from one another, and the size and number of grana became reduced. Granal stacking can eventually disappear entirely, at which stage the thylakoid membranes become separated, showing a grossly dilated intraspaces (Figs. 3 and 7). At or before the stage at which all grana disappear, material from the stroma condenses about the thylakoids (Fig. 4), and ultimately the chloroplasts become almost unrecognizable, densely staining inclusions in an apparently desiccated cytoplasm.

Osmiophilic droplets were observed in these chloroplasts at all stages of damage and did not obviously change in number or size.

**Bundle sheath.** The first and most obvious change seen in the bundle sheath was the almost complete disappearance of starch grains following only 2 days of low temperature treatment (Figs. 9 and 10). The granaless character of these chloroplasts could then be seen very clearly, and unusual irregular protuberances of the outer chloroplast membrane also occurred. Light intensity gradients caused the whole palisade to become seriously damaged before more pronounced changes could be seen in the bundle sheath, and by this time the leaf has become very flaccid and difficult to fix, embed, and section.

**Lower mesophyll.** Large and numerous starch grains originally present in the chloroplasts disappeared almost completely after 2 days at 10 C. Ultrastructural change in chloroplasts of the lower mesophyll was slight with the leaf in normal orientation. They will swell in a similar manner to those of the upper mesophyll, however, if the leaves are turned upside down.

**General.** Sorghum plants were also used to see whether high light needs to be applied concurrently with the low temperature to produce chloroplast ultrastructural changes. When day temperatures only were dropped to 10 C, visible leaf necrosis, chloroplast swelling, and rate of starch loss were all somewhat enhanced over the 10 C day/night control. When only the night temperature was dropped to 10 C, the most obvious feature was a pronounced increase in starch. After 3 days of treatment the bundle sheath and lower mesophyll became packed to the stage where about two-thirds of the sectional plane through the chloroplast appeared as starch grain, and some moderate-sized grains also developed in chloroplasts of the upper mesophyll. The number of osmiophilic droplets also appeared to increase in chloroplasts of the upper mesophyll while the degree of granal stacking decreased. Thylakoid intraspaces width was somewhat variable but generally greater than that seen in 25 C day/night controls. After 7 days of low night temperature treatment of these mature leaves, some 10 to 20% of upper mesophyll cells contained chloroplasts similar to those seen after 2 days of 10 C day/night treatment (Fig. 2). Intraspace widths were again reduced and chloroplasts were swollen. These effects as well as starch build-up (5) may reduce photosynthesis of tropical grasses held under low night temperatures.

Under shaded conditions ( $50 \text{ w}\cdot\text{m}^{-2}$ ), some chloroplast swelling was observed after 3 days and nights at 10 C. This swelling commonly appeared first in the peripheral reticulum (10, 16). Initial swelling in this region was also frequently observed in *Paspalum*. In *Sorghum* under high light conditions initial swelling in the zone of the reticulum was not seen. It

may not be readily observed because of the rapidity with which it occurs and is subsequently effaced by more general swelling.

**Paspalum.** Photosynthesis of *Paspalum* was less severely disrupted by low temperature, high light stress than that of *Sorghum* (18). It was therefore no surprise to find that chloroplast ultrastructural changes, although similar in their general pattern, occurred more slowly and with less severity in *Paspalum* than in *Sorghum*.

Chloroplasts in the upper mesophyll of *Paspalum* resemble those of *Sorghum* except that they possess a more extensive peripheral reticulum, especially on the side adjacent to the cell wall (Fig. 11), and they contain several moderate-sized starch grains. After 2 days in low temperature, high light stress some swelling could be seen in a few chloroplasts at the top of the mesophyll, and after 3 days this became reasonably pronounced in about half of the upper mesophyll chloroplasts.

The first stress-induced ultrastructural changes seen were small bulges along the outer margins of the chloroplasts (Fig. 12), arising in the zone of the peripheral reticulum. As swelling progressed, the whole outer chloroplast membrane pulled away, leaving the peripheral reticulum as a scattered array of tubules (Fig. 13), some remaining with outer fret membranes and some with the outer chloroplast membrane. How much breakage of the tubules occurred during the stress-induced swelling is difficult to assess in sectional plane, but many remain intact despite pronounced chloroplast swelling, and they do not appear to swell or otherwise deform. As the outer chloroplast membrane swelled to produce a chloroplast several times its original volume, remaining grana appeared to hold the internal membrane system at least partially together. Eventually, however, chloroplasts can appear very similar to the almost granaless state seen in *Sorghum* (cf. Figs. 3 and 12). Advanced stages of chloroplast swelling are commonly associated with rupture of the tonoplast membrane, which then fragments further and rounds up into vesicles (Figs. 13 and 14). Essentially undamaged cells opposing those with a ruptured tonoplast may show bulges commencing in the tonoplast opposite plasmodesmata connecting the two cells.

Large amounts of starch remained in *Paspalum* chloroplasts during initial stages of this chilling stress, especially in those of the bundle sheath and lower mesophyll cells. Although reduced in size, starch grains may also be present in severely damaged chloroplasts of the upper mesophyll after 3 days at 10 C.

**Soybean.** Micrographs in this series were taken from youngest-mature leaves of plants exposed only to low day temperatures. Night temperatures were kept at 25 C in attempt to maintain the relative water content of the leaves (1).

Chloroplasts from the palisade region of soybean plants grown at 25 C under moderate light intensity (Fig. 15) were usually long and thin and contained several quite large starch grains and very few grana, although some overlapping of thylakoids was seen (2). An extensive, apparently ordered array of tubules or peripheral reticulum (10, 16) within the outer chloroplast membrane has only been reported in plants possessing the  $C_4$ -photosynthetic pathway. A considerable number of visibly similar tubules do, however, occur in the same region of soybean chloroplasts (Fig. 16).

After lowering the day temperature, the first stress effect noted was a rapid disappearance of starch, which was virtually complete after 2 days. As in the tropical grasses, swelling rapidly followed, although there appeared to be no specific center for this and the thylakoids moved apart rather evenly, presumably because of limited granal stacking. In a number of cases, membranes could become realigned in close parallel runs (Fig. 17), a feature occasionally seen in this work at intermediate stages of stress damage in *Sorghum* and reported in

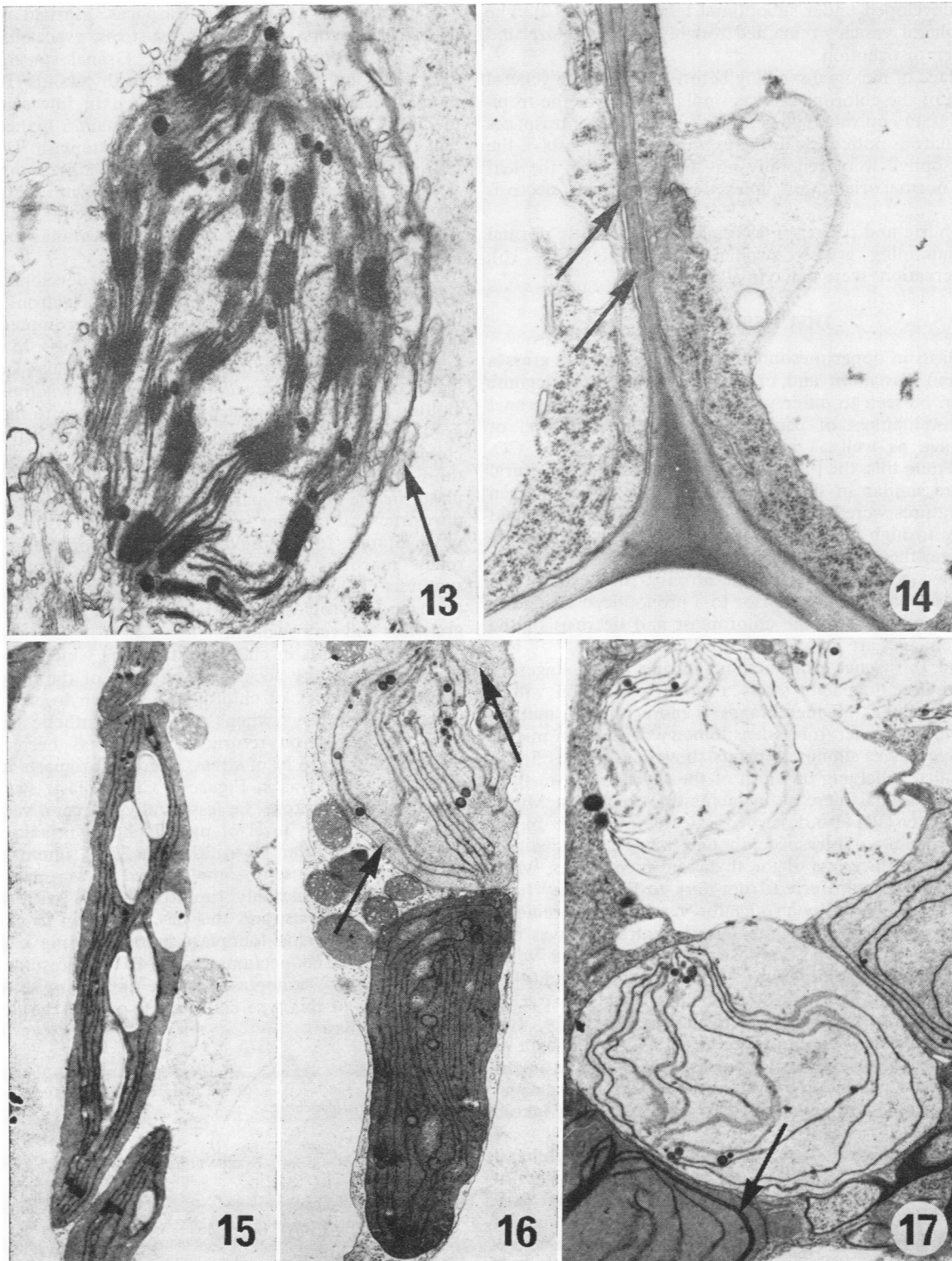


FIG. 13. *Paspalum* upper mesophyll chloroplast after 3.5 days of chilling treatment. Massive swelling in the zone of the peripheral reticulum, essentially undilated reticulum tubules and vesicular remains of the ruptured tonoplast (arrow) can be seen.  $\times 18,000$ .

FIG. 14. Portions of adjacent upper mesophyll cells of *Paspalum* after 2.5 days of chilling treatment. Tonoplast in left-hand cell has ruptured and rounded into vesicles while the tonoplast in the neighboring cell shows initial stages of disruption opposite plasmodesmata (arrows).  $\times 18,000$ .

FIG. 15. Soybean palisade chloroplasts developed at 25°C ( $170 \text{ w} \cdot \text{m}^{-2}$ ).  $\times 8000$ .

FIG. 16. Soybean palisade chloroplasts developed at 25°C, then given 1.5 days of chilling treatment. Stromal swelling, peripheral tubules (arrows), and absence of starch can be seen.  $\times 8000$ .

FIG. 17. Soybean palisade chloroplasts after 2.5 days of chilling treatment. Pronounced stromal swelling and some membrane realignment in parallel array (arrow) can be seen.  $\times 8000$ .

*Zea mays* developed under suboptimal temperatures (12). The small peripheral vesicles remained roughly the same size and retained their distribution.

At the stage of maximal swelling before necrosis, the general appearance of the chloroplasts was similar to that of the tropical grasses at an equivalent stage, but the thylakoid intraspaces did not redilate as noticeably as in *Sorghum*. Chloroplasts of the spongy mesophyll were generally not damaged when the leaf was held in normal orientation, unless a whole sphere of necrosis developed.

Mitochondria and peroxisomes remained apparently normal in cells containing grossly swollen chloroplasts (Fig. 16); similar observations were made in all species.

## DISCUSSION

Chloroplasts in upper mesophyll cells of the tropical grasses *Sorghum* and *Paspalum* and in palisade cells of the legume soybean can be seen to differ widely in their degree of granal stacking, development of peripheral reticulum, and level of starch storage as well as in their mechanisms (4) of CO<sub>2</sub> fixation. Despite this, the patterns of chloroplast ultrastructural change were similar in these chilling-sensitive species, when leaf temperatures were lowered from 25 C to 10 C while held at moderate to high light intensities. Starch grains rapidly reduced in size; there was a contraction of the thylakoid intraspaces coupled with swelling of the stroma, and usually the thylakoid intraspaces redilated prior to a pronounced change in the staining properties of the chloroplast and necrosis of the cell. These changes are interpreted as follows.

**Thylakoid Intraspace Contraction.** During initial stages of low temperature, high light stress, osmium deposition within the individual thylakoid membranes is more intense, making them apparently thicker; microdensitometry of electron micrograph transparencies similar to prints shown as Figures 5 and 6 demonstrated a halving in width of the thylakoid intraspaces with only a marginal increase in membrane thickness. Murakami and Packer (13) also described a decrease of 50% in the width of the thylakoid intraspaces, though this is coupled with a 23% decrease in thickness of the thylakoid membranes, when *Porphyra* thalli are transferred from dark to light. They have duplicated these effects by artificially varying pH gradients (14), and they concluded that membrane contraction was due to protonation of charged groups in the membrane while thylakoid intraspaces contraction was assigned to acidity-induced loss of organic acids and subsequent osmotic collapse.

Thylakoid intraspaces contraction under high light, low temperature stress may therefore be caused by development of an abnormally low pH within the intraspaces. Changes in the oxidation state of xanthophylls described in a previous paper (18) were also consistent with a decrease in pH of the thylakoid intraspaces.

**Stromal Swelling.** Initial sites of swelling in the peripheral reticulum seen especially in *Paspalum*, but also in *Sorghum* at lower light intensities, could suggest that the motivating force for swelling arises in the reticulum. Tubules of the reticulum did not dilate, however, even at quite advanced stages of stress-induced swelling (Fig. 13), and swelling in this region was soon followed by more general stromal swelling. The reticulum is probably mechanically weaker than the internal thylakoid network, which may be held together for some time by the co-directional helical arrangement of the stromal thylakoids (15) and intermembrane attractions within the grana.

The swelling has all the outward appearances of an osmotic effect, and, if this is so, whatever causes the increase in osmolarity of the stromal matrix must be a general feature of both C<sub>3</sub>- and C<sub>4</sub>-pathway (4) chloroplasts.

**Redilation of Thylakoid Intraspaces.** Marked redilation of thylakoids occurs after prolonged stress, especially in chloroplasts of *Sorghum* and *Paspalum*. Granal stacking virtually disappears and the unravelled dilated thylakoids (Fig. 3) come to look very similar to those isolated in low salt hypotonic media (8). Chloroplasts normally maintain a higher ionic content than the vacuole or surrounding cytoplasm (11), presumably through some active ion pump. If this pump was damaged during chilling, then this coupled with dilution effects caused by stromal swelling might lower the cation concentration sufficiently to cause the observed granal reduction and thylakoid dilation.

**Damage Gradients.** Gradients in the development of low temperature stress lesions observed by electron microscopy in the leaves of a maize mutant (12) have prompted the postulation of a translocatable retardant of this stress. Marked gradients in the onset of ultrastructural change also occur in the leaves of *Sorghum*, *Paspalum*, and soybean when placed under chilling stress (Fig. 8), but we attribute these to light intensity gradients within the leaf. At comparable levels within the leaf, chloroplasts close to a bundle sheath swell at similar rates to those more distant from the bundle sheath. Moreover, chloroplasts in the phloem parenchyma of *Paspalum* can undergo ultrastructural changes before chloroplasts in the lower mesophyll. Starch, for example, could be important in chloroplast repair reactions under these conditions of reduced photosynthesis (18), but some starch grains were often seen to remain in chloroplasts at a stage when they were virtually granaless and very swollen.

These gradients in chloroplast change which develop under stress preclude any accurate evaluation of the *in vitro* photosynthetic efficiency of chloroplasts swollen to any defined extent. Nevertheless, when the photosynthetic capacity of *Sorghum* leaves (on return to 25 C) has been reduced to roughly half by 36 hr of stress, many chloroplasts in the upper mesophyll appear as in Figure 2. Chloroplasts swollen to this extent may therefore be essentially inoperative in carbon fixation. Even this level of partial photosynthetic recovery is not stable, and after 15 to 20 hr at 25 C, photosynthesis decreases again because of some delayed stress sensitization (18). The tonoplast commonly ruptures in cells with significantly swollen chloroplasts, and this has been seen to cause inward facing bulges of the tonoplast in neighboring cells opposite plasmodesmata connections (Fig. 14). We presume this could ultimately cause tonoplast rupture in otherwise undamaged cells. Effects of this type could cause part of the delayed drop in photosynthesis.

*Acknowledgments*—Thanks are due K. I. Williamson and D. H. Hoppercroft for contributing much to the electron microscopy, and to Director Ken Mitchell for continued encouragement.

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