Light and Electron Microscopy of Plant Leaf Cells After Short-Term Exposure to Bromine Gas

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ABSTRACT

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Immediate and delayed (24 hr) effects of a 20-min exposure of cowpea primary leaf tissue to 1, 3, or 5 ppm bromine gas were studied with light and electron microscopy. The formation of circular, adaxial, and abaxial surface lesions and leaf compression were the most visible changes. Major cytological modifications included cytoplasmic vacuolation, disruption of cellular membranes, plasmolysis, alteration of nucleolar structure, anticlinal cell wall interdigitation, and collapse of the protoplast and/or cell wall. Although degree and type of injury varied, all epidermal and mesophyll cells were affected by the treatments. Spongy mesophyll cells surrounded by large air spaces were more severely affected by the bromine gas than were the closely packed palisade mesophyll cells. At 1 ppm bromine, certain cellular changes such as adaxial depression and anticlinal cell wall interdigitation were reversed when the exposed plants were returned to a normal environment; at 5 ppm bromine, however, the entire cell population immediately became irreversibly necrotic.

Additional key words: air pollutant, cytological effects.

After its discovery in 1825, bromine found immediate application in photography and medicine. More recently, the use of bromine has greatly increased as in the commercial production of antiknock fuels, soil fumigants, flame retardants, and dyes (26). Problems of bromine as an air pollutant arose when the pine forests near bromine production sites in southwestern Arkansas were affected presumably by the uptake of high concentrations of bromine. Results of further study indicated that bromine accumulated in pine needles and that the quantity decreased proportionally as the distance from the source increased (22).

The effects of bromine gas on vegetation were reported as early as 1899 by Sorauer and Raymann (18). They noted macroscopic effects on spruce needles which included needle banding, needle-tip burn, and premature casting. Later, Haselhoff and Lindau (9) studied the effects of varying concentrations of bromine vapor on a variety of plants and found that pea plants were the most sensitive to the gas, developing dry leaf margins and interveinal translucent spots. Bean leaves were, however, less affected and exhibited some distortion occasionally accompanied by a narrow band of necrosis along the margin. Histological changes were most noticeable in the cells near the stomata and included tannin accumulation, plasmolysis, bleaching of chloroplasts, and disintegration of other cell organelles. No other anatomical or histological studies describing effects of bromine on plants have been reported.

The purpose of this study was to determine the effects of varying concentrations of bromine gas on plant leaves at both the tissue and cellular levels and to compare the results with those of other air pollutants which have been well documented.

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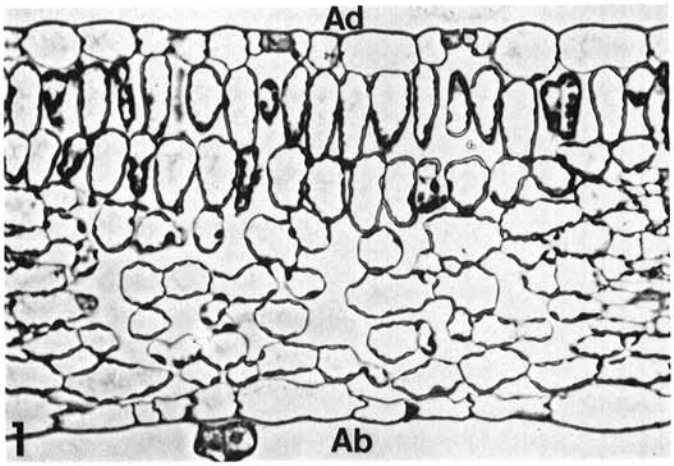
MATERIALS AND METHODS

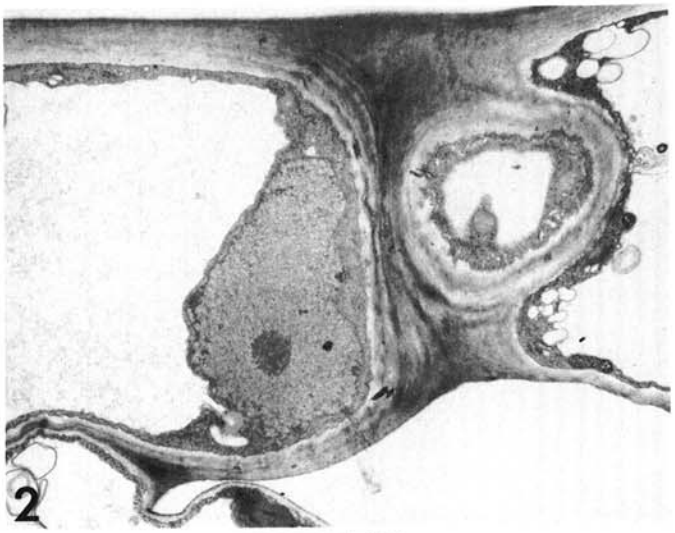
Fully expanded cowpea (*Vigna unguiculata* (L.) Walp. 'Monarch') primary leaves were used as the experimental tissue. Cowpea was chosen as a model plant for this study because it is a common agricultural crop plant that can be handled easily in the greenhouse and the laboratory. Single plants were grown in 7.5-cm-diameter clay pots in Terralite Rediearth Peat-Lite Mix. Plants were fertilized once with 20-20-20 Peters Soluble Fertilizer (60 gm/L) diluted 1:50 and were maintained during July and August in a sunlit greenhouse.

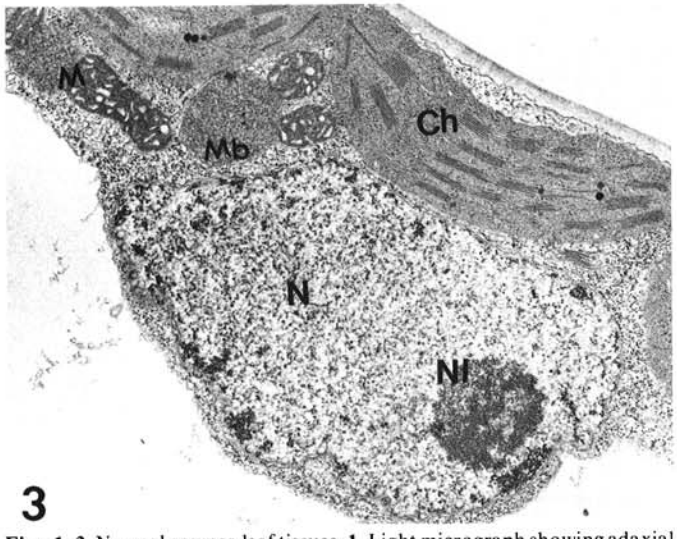
The cowpeas were fumigated with bromine at 25 C and atmospheric pressure for 20 min. The fumigation chamber consisted of a 9.5 L-glass desiccator sealed with a rubber stopper that had both an inlet and an outlet, each fitted with glass tubing. This tubing was connected to a vacuum pump that circulated the bromine gas throughout the chamber. Plants were exposed, in room light, to three different concentrations of bromine gas. Liquid bromine, 10, 30, and 50 μl, was allowed to vaporize in a ground glass weighing bottle sealed with inert plumber's putty. The bottle was placed in the fumigation chamber, the cap was removed so that the gas could expand to approximately 1, 3, or 5 ppm, and the vacuum pump was started.

Tissue samples were taken from the primary leaves immediately after fumigation and 24 hr later. For electron microscopy several 1-mm^2 pieces of interveinal leaf tissue from bromine-treated and untreated plants were fixed for 2 hr in 4% glutaraldehyde in 0.05 M cacodylate buffer. They were rinsed three times with buffer for 10 min each and postfixed for 2 hr in 1% osmium tetroxide followed by staining overnight in 0.5% aqueous uranyl acetate. Tissues were dehydrated in a graded ethanol series and embedded in Spurr's epoxy resin (19). Thin sections (60–80 nm) were cut with a diamond knife, stained in 2% uranyl acetate followed by Reynold's lead citrate (16), and viewed with either a Siemens Elmiskop 1A or JEOL 100CX transmission electron microscope.

For light microscopy, thick sections (1.5–2.0 μm) were cut with a glass knife, collected and flattened on glass slides coated with Haupt's adhesive, and stained for epoxy sections (8). This material was examined and photographed on an Olympus Vanox microscope.







Figs. 1–3. Normal cowpea leaf tissues. **1,** Light micrograph showing adaxial (Ad) and abaxial (Ab) epidermal surfaces, palisade and spongy mesophyll, and stomatal apparatus (×264). **2,** Junctions of three epidermal cells. The cell at the left shows the nucleus and nucleolus. Bleblike small vacuoles along the tonoplast are shown in the cell at the right (×3,400). **3,** Mesophyll cell with nucleus (N), nucleolus (Nl), microbody (Mb), chloroplasts (Ch), and mitochondria (M) (×12,000).

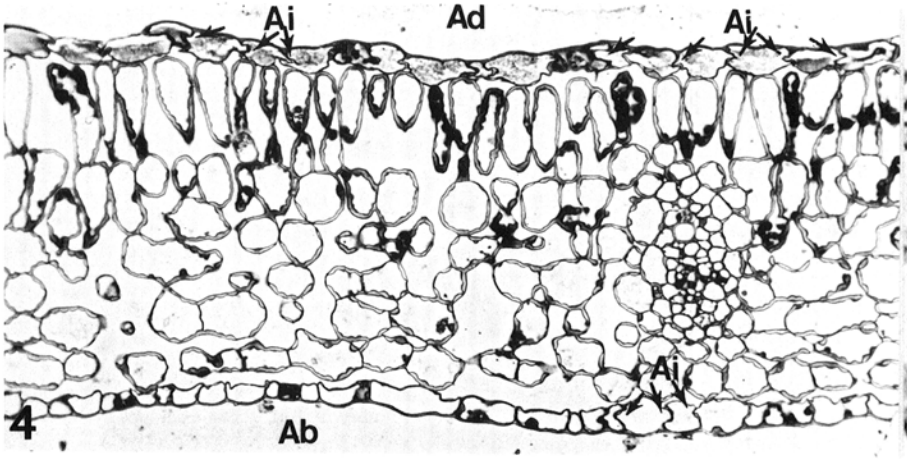
RESULTS

Untreated plants. *Light microscopy.* Cross sections of untreated leaves exhibited well-differentiated adaxial and abaxial epidermal surfaces and palisade and spongy mesophyll (Fig. 1). A slight degree of anticlinal wall folding especially in the abaxial epidermis was occasionally observed. Stomata occurred on both surfaces of the leaf, and large intercellular spaces were evident in the mesophyll.

Electron microscopy. Figures 2 and 3 are electron micrographs representing normal epidermal and mesophyll cells, respectively. They were characterized by a large central vacuole surrounded by cytoplasm containing typically arranged chloroplasts, mitochondria, nuclei, and nucleoli. The fibrillar nucleolonema occurred as a closely packed network in the granular portion of the nucleolus (Fig. 3). The tonoplast separated the deeply stained cytoplasm and unstained central vacuole.

Immediate effects of 1 ppm bromine. *Light microscopy.* When plants were treated with 1 ppm bromine for 20 min, epidermal cells exhibited more changes than cells of any other tissue. Adaxial

Figs. 4–10. Cowpea tissues sampled immediately after 20 min of exposure to 1 ppm bromine gas. **4,** Light micrograph of initial injury to the adaxial (Ad) and abaxial (Ab) epidermis. Note anticlinal cell wall interdigitation (Ai) and increased staining (×264). **5,** Light micrograph of collapsed palisade mesophyll cells (arrows) beneath a stoma (×660). **6,** Electron micrograph showing severely injured abaxial epidermal cells (Ab) with disrupted organelles, anticlinal cell wall interdigitation (Ai) and fibrillar material (F) scattered in the cells (×6,000). **7,** Adaxial epidermal cells (Ad) showing anticlinal cell wall interdigitation (Ai), vacuole formation (V) and fibrillar material (F) in the central vacuole. P = palisade cells (×2,500). **8,** Mesophyll cell with cytoplasmic vacuolation (V) (×14,000). **9,** Mesophyll cell nucleolus with distinct granular (G) and fibrillar (F) regions (×24,000). **10,** Necrotic adaxial epidermal (E) and palisade (P) cells associated with the stomata. Note apparently less-affected adjacent cells (unlabeled double arrows) (×3,000).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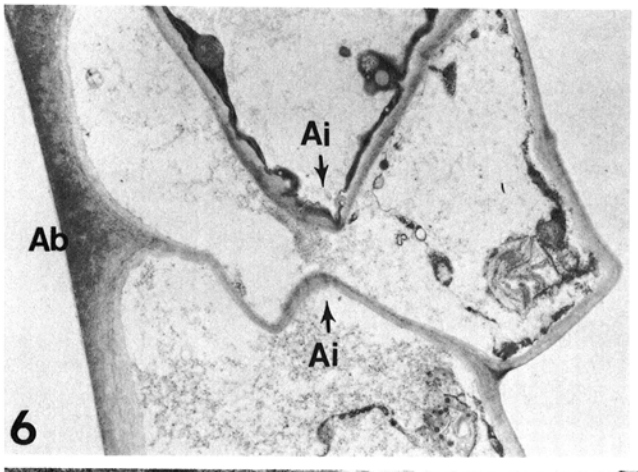
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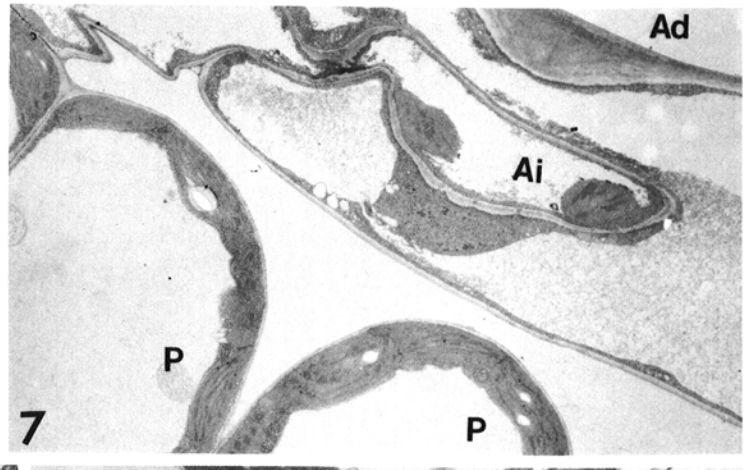
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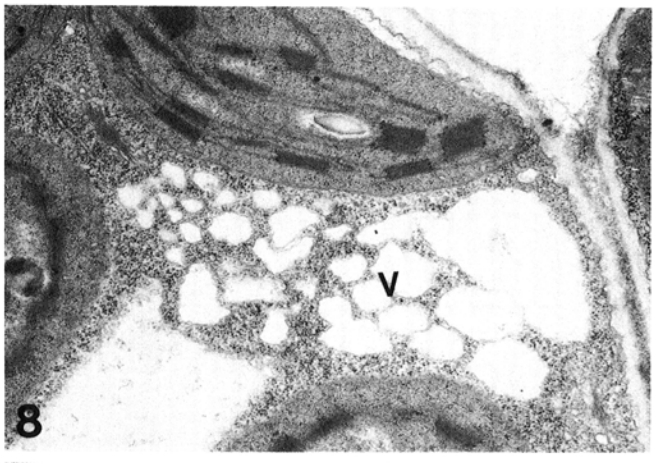
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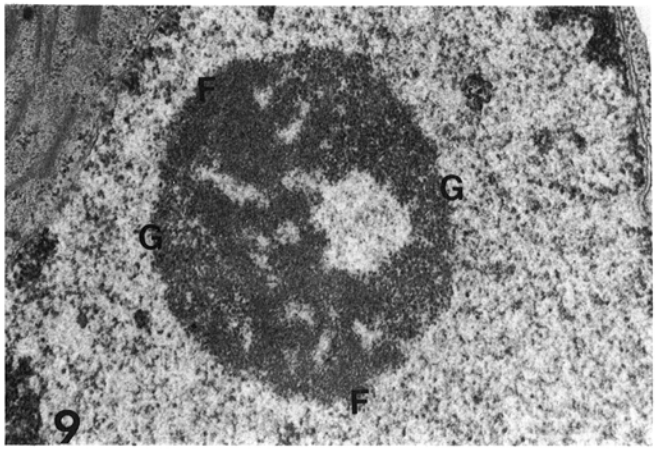
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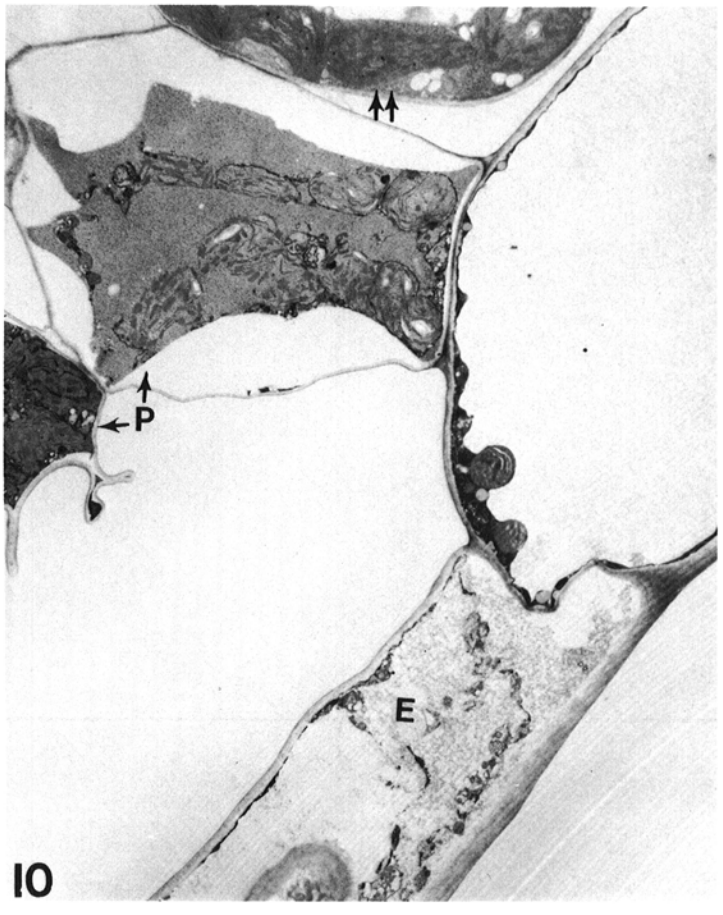
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epidermal cells contained large amounts of darkly staining material in their central vacuoles and displayed prevalent surface depressions (Fig. 4). The latter apparently resulted from periclinal wall collapse and the interdigitation of the anticlinal walls of adjacent epidermal cells. Cell length increased concomitantly (Figs. 1 and 4). The cytoplasm of these cells was no longer peripheral but filled the cell volume, suggesting rupture of the tonoplast. The abaxial epidermis showed less surface depression and less anticlinal wall interdigitation, and the vacuoles lacked darkly staining material (Fig. 4). The majority of mesophyll cells appeared unaffected; however, a few cells located immediately beneath the stomata had collapsed. These cells appeared as thick, darkly stained strands tightly appressed to the adjacent cell wall resulting in the formation of enlarged intercellular spaces (Fig. 5).

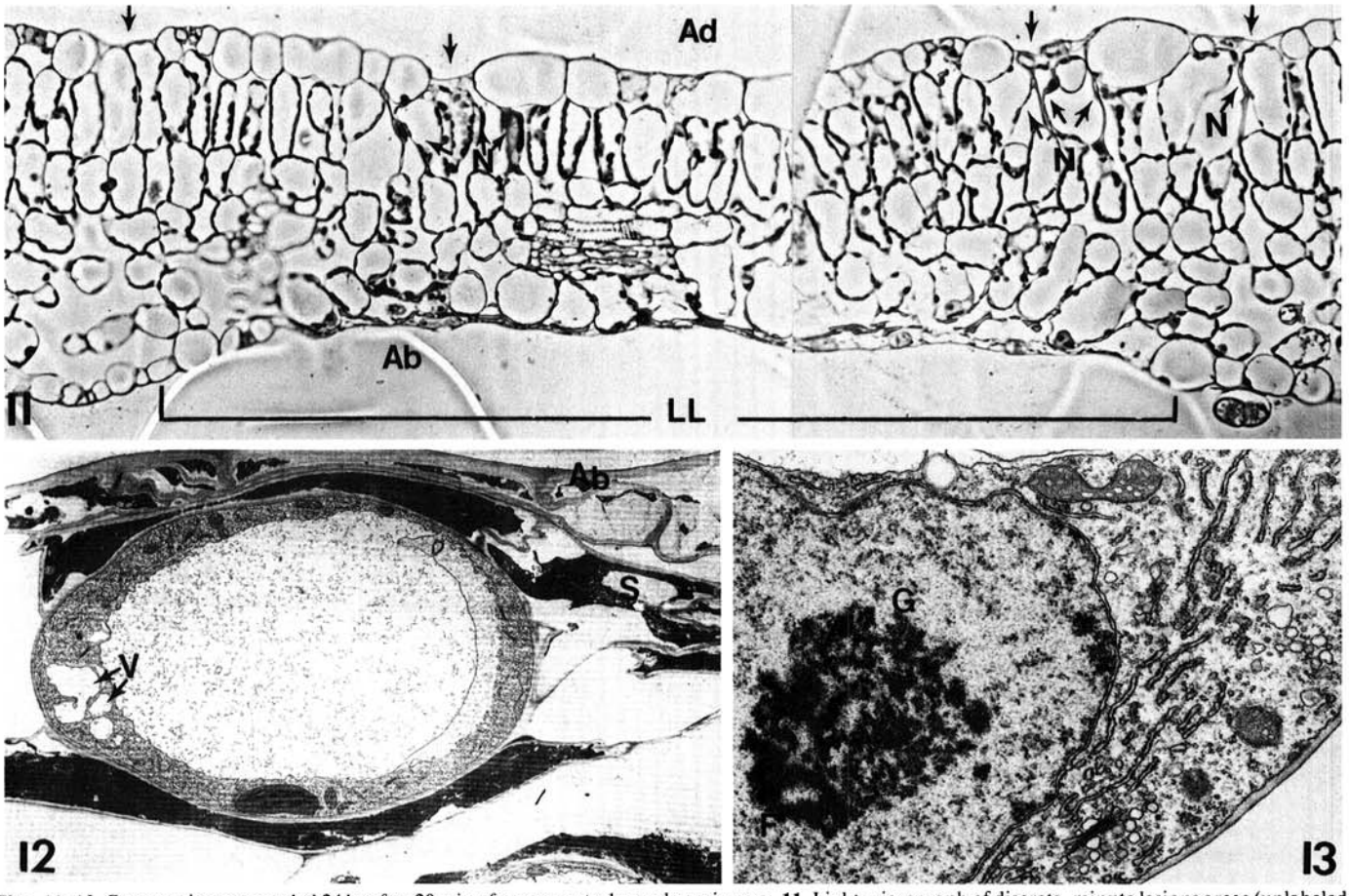
Electron microscopy. Although light microscopy showed less severe disruption of the abaxial epidermis (Fig. 4), electron microscopy revealed extensive damage including total loss of cell integrity and degeneration of plasmalemma, tonoplast, and membraneous organelles (Fig. 6). In less-affected areas of the epidermis, vacuoles of various shapes and sizes were formed within the cytoplasm, and intact organelles appeared to be distributed as they would have been in control cells (Figs. 2 and 7). Regardless of the extent of injury, amorphous fibrillar material was dispersed throughout the central vacuole and anticlinal cell wall interdigitation was evident (Figs. 6 and 7).

Most mesophyll cells appeared unaffected except for the formation of vacuoles within the cytoplasm (Fig. 8) and separation of the granular and fibrillar regions of the nucleoli (Fig. 9). Some palisade cells located immediately beneath a severely injured stoma exhibited characteristic necrosis including: plasmolysis, distortion, or disappearance of the membrane system of cell organelles, and filling of the vacuolar region with disrupted cytoplasmic components (Fig. 10).

Effects 24 hr after 1 ppm bromine. *Light microscopy.* The adaxial depressions due to interdigitation of the anticlinal walls were not observed 24 hr after exposure. However, discrete lesion areas were present on both adaxial and abaxial surfaces (Fig. 11). On the adaxial surface, small sunken areas resulted from the collapse of one or two epidermal cells, often guard cells, which covered heavily stained, necrotic palisade cells (Fig. 11). On the abaxial surface, larger lesions developed with the collapse of 20–30 epidermal cells. These depressions caused a considerable decrease in leaf blade thickness. Spongy mesophyll cells underlying these larger lesions were often completely collapsed (Fig. 11).

Electron microscopy. The adaxial and abaxial epidermal cells in lesion areas exhibited extreme anticlinal wall collapse. The cytoplasm was electron dense, and cell organelles were not distinguishable (Fig. 12). Epidermal cells, not involved in lesions but previously exhibiting anticlinal wall interdigitation appeared to resume original conformation, and the fibrillar material observed in the central vacuole was significantly reduced.

Cells of both palisade and spongy mesophyll associated with lesions were also collapsed and appeared as electron-dense strands that were appressed to adjacent cells (Fig. 12). Other mesophyll cells that may correspond to those appearing intact under the light microscope showed changes in their cell organelles; chloroplasts were not elongated or linearly arranged within the cytoplasm. Ground cytoplasm and nuclear matrix appeared less dense than those of unaffected cells (Fig. 13), and nucleolar components appeared somewhat fragmented with granular and fibrillar regions



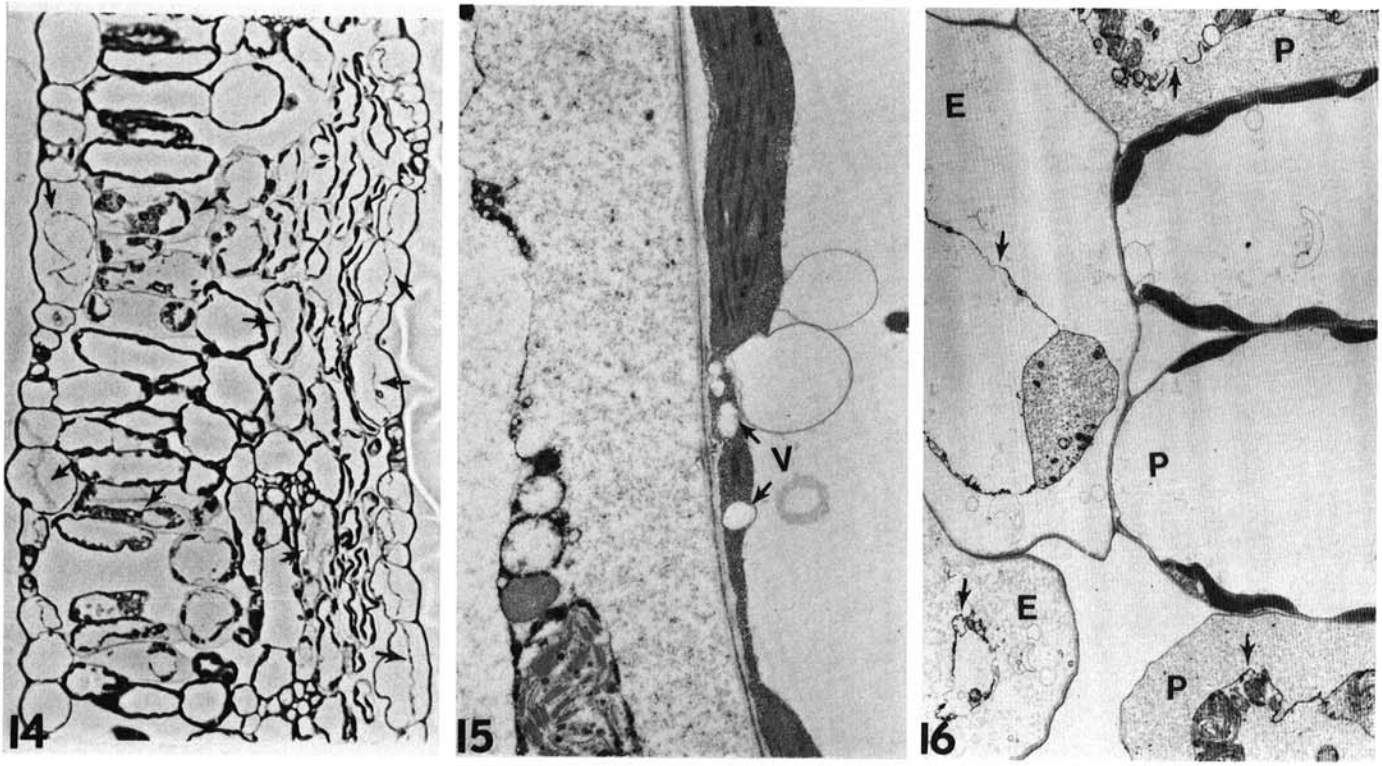
Figs. 11–13. Cowpea tissues sampled 24 hr after 20 min of exposure to 1 ppm bromine gas. **11,** Light micrograph of discrete, minute lesions areas (unlabeled arrows) of the adaxial (Ad) epidermis and a large lesion (LL) of abaxial (Ab) epidermis are shown. Note necrotic cells (N) associated with stomatal regions (×264). **12,** Electron micrograph showing collapsed abaxial epidermal (Ab) and spongy mesophyll (S) cells adjacent to a spongy mesophyll cell with several vacuoles (V) (×2,600). **13,** Palisade mesophyll cell with leached cytoplasm, nucleolus with fragmented and separated fibrillar (F) and granular (G) portions, mitochondria and golgi body with vesicles (×12,540).

well separated.

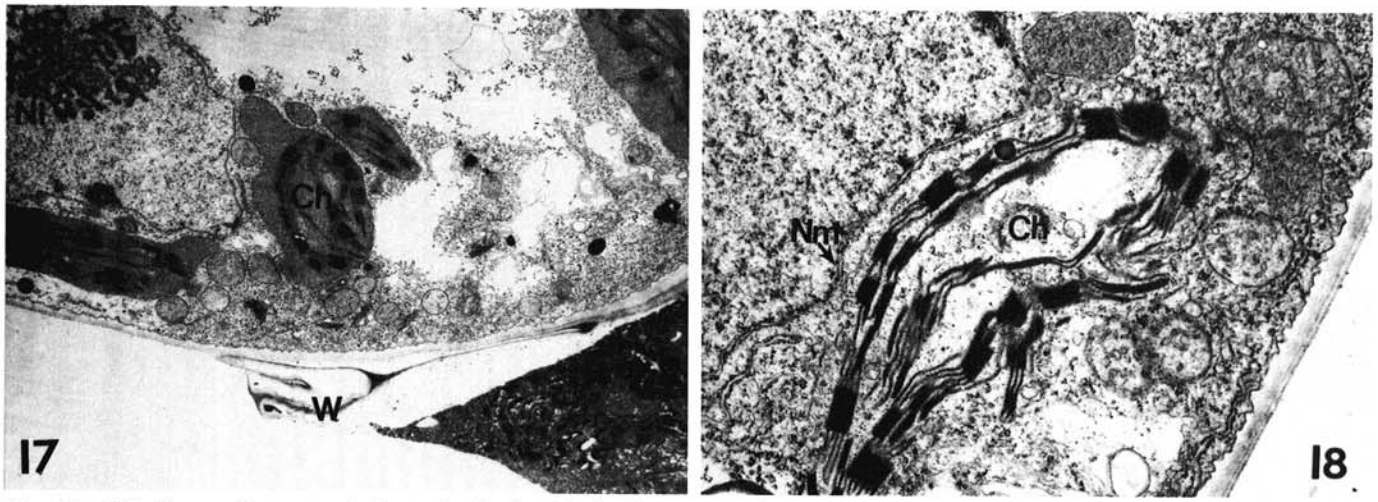
The extent of injury and the reaction of the mesophyll cells were related directly to proximity of stomata and/or lesion areas. It appeared that more spongy mesophyll cells than palisade cells were affected; a multilayered, wavy contour was by far the most consistent and striking response of spongy mesophyll (Fig. 12). Cell wall collapse and interdigitation were extreme and resulted in sunken abaxial lesions. Occasionally an intact cell was evident between multilayered necrotic cells. Such cells contained vesicles and vacuoles of various sizes and shapes and an elaborate system of ER distributed throughout the cytoplasm (Fig. 12).

Immediate effects of 3 ppm bromine. *Light microscopy.* The most striking feature of epidermal cells was retraction of the protoplast, as a result of plasmolysis, into strands that generally ran parallel to the outer periclinal wall (Fig. 14) of the abaxial epidermis. Anticlinal wall interdigitation of epidermal cells was not evident.

Most mesophyll cells showed some degree of protoplast retraction independent of lesion or stomata; however, changes in spongy mesophyll cells were more striking than in palisade cells (Fig. 14). Occasionally palisade cells were comparable to control cells in general shape (Figs. 1 and 14).



Figs. 14–16. Cowpea tissues sampled immediately after 20 min exposure to 3 ppm bromine gas. **14,** Light micrograph showing protoplast retraction in both epidermal and mesophyll cells (arrows). **15,** Electron micrograph showing variable response in adjacent mesophyll cells contrasts organelle degeneration in the cell on the left with cytoplasmic vacuolation (V) in the one on the right (×7,500). **16,** Electron micrograph showing retracted protoplasts (arrows) in adaxial epidermal (E) and palisade mesophyll cells (P) (×1,820).



Figs. 17 and 18. Cowpea tissues sampled immediately after 20 min of exposure to 3 ppm bromine gas. **17,** Adjacent mesophyll cells display differential responses: the upper cell contains distorted and displaced chloroplasts (Ch), fragmented nucleolus (Nl) and ruptured tonoplast while the lower exists as a compact, electron dense cytoplasmic mass within collapsed cell wall (W) (×7,000). **18,** Mesophyll cell with an intact nuclear membrane (Nm) and a disrupted chloroplast without limiting membrane (×15,000).

Electron microscopy. Four types of injury were distinguishable among different mesophyll cells. The first type, which appeared to be a mild response, was characterized by occurrence of vacuoles within the cytoplasm (Fig. 15); however, cytological detail was comparable to that of control cells (Figs. 3 and 10).

In the second type of injury, the tonoplast ruptured, and cell constituents were spread throughout the cell. Vacuoles within the cytoplasm extended into the central vacuole, and many chloroplasts were not confined to the cell periphery (Fig. 17) as was the case in most unaffected cells. Occasionally, chloroplasts lost their limiting membranes and general organization, although the grana stacks were still discernible (Fig. 18). In these cells, the granular and fibrillar components of nucleoli were fragmented and dispersed (Fig. 17).

The third type of injury involved a marked alteration of cellular organization. The entire cytoplasm was pulled away from the cell wall and assumed a central position (Figs. 15 and 16). Cytoplasmic contents were lost, leaving only a remnant of darkly stained material in the center of the cell (Fig. 16). All organelles were disorganized, and the remains were appressed to the ruptured, electron-dense plasmalemma. The only recognizable structures were the grana membranes of the chloroplasts (Figs. 15 and 16). Electron-dense granular material often filled both the central vacuole and the area between the cell wall and the plasmalemma (Fig. 15); however, cell walls were not collapsed (Figs. 15 and 16).

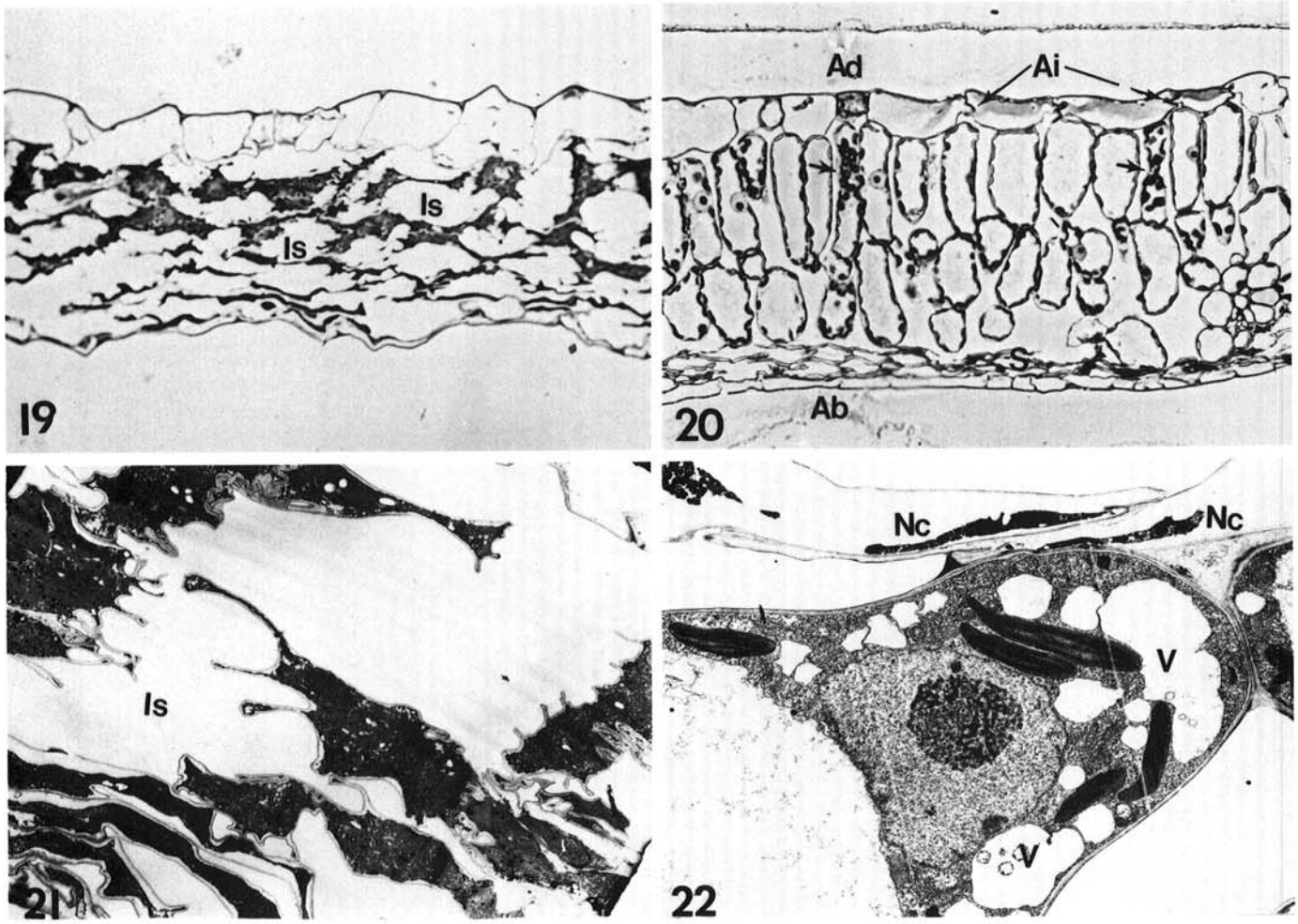
The fourth and most severe type of injury was characterized by a totally collapsed, electron-dense cytoplasmic mass appressed to adjacent cells (Fig. 17). Different types of injury were observed throughout the tissues, and cells exhibiting different injury responses were often adjacent to each other (Figs. 15–17).

Effects 24 hr after 3 ppm bromine. *Light microscopy.* Necrotic areas that were not observed immediately after fumigation developed 24 hr after exposure to 3 ppm bromine. Light microscopy showed collapse and compression regardless of cell type. Collapse was so uniform and extreme that individual cells appeared as darkly stained strands surrounded by enlarged intercellular spaces (Fig. 19). Leaf blade thickness was significantly reduced as the result of tissue compression.

Non-necrotic areas were comparable to tissues observed immediately after exposure to 3 ppm bromine, although the extent of collapse of spongy mesophyll was more severe (Fig. 20). All spongy mesophyll cells collapsed parallel to the leaf axis and were appressed to each other.

Electron microscopy. A striking feature of the cells in the lesions was fingerlike appearances of collapsed cell walls in the large intercellular spaces (Fig. 21). Cell constituents were so compressed that they appeared as an electron-dense mass (Fig. 21). In mesophyll cells, darkly stained globules of various sizes and shapes were randomly distributed throughout the cytoplasmic mass (Fig. 21).

Injury response varied in nonlesioned areas, but was comparable to that observed immediately after exposure to bromine. Necrotic spongy mesophyll cells were often observed adjacent to palisade cells, which were not as severely affected. The latter cells showed



Figs. 19–22. Cowpea tissues sampled 24 hr after 20 min exposure to 3 ppm bromine gas. **19,** Light micrograph of a necrotic area of leaf in which all cells have collapsed leaving large intercellular spaces (Is) (×264). **20,** Light micrograph showing anticlinal cell wall interdigitation (Ai) in adaxial epidermis (Ad), periclinal wall collapse of some palisade mesophyll cells (unlabeled arrows), compression of the spongy mesophyll cells (S) and collapse of the abaxial epidermis (Ab) (×264). **21,** Electron micrograph of a collapsed lesion area showing necrotic palisade mesophyll cells and large intercellular spaces (Is) (×2,000). **22,** Mesophyll cell with abundant cytoplasmic vacuolation (V) and displaced chloroplast next to a completely necrotic cell (Nc) (×3,300).

increased stain intensity and large numbers of vacuoles within the cytoplasm (Fig. 22). Nucleolar changes, similar to those previously noted, were observed (Figs. 13 and 22).

Immediate effects of 5 ppm bromine. *Light microscopy.* At this bromine concentration, the entire leaf wilted, but no lesions were formed. Except for retraction of the protoplasts seen in some epidermal and mesophyll cells, most cells appeared to be comparable to those of untreated leaves (Figs. 1 and 23). No collapsed cells were observed.

Electron microscopy. All cells of the leaf were irreversibly shocked (Fig. 24). Cell constituents showed the response to 5 ppm bromine most vividly. At low magnification, the peripheral cytoplasm appeared as a homogeneous electron-dense mass with all cytological details distorted (Fig. 24). At higher magnification, the only identifiable organelles were the chloroplast and nuclei (Fig. 25). The chloroplasts were appreciably distended and contained remnants of dilated thylakoids (Figs. 24 and 25). The nucleoplasm failed to display its usual granular nature and contained scattered, small patches of electron dense material. The nucleolus was extremely electron dense and compact, and granular and fibrillar regions could not be distinguished.

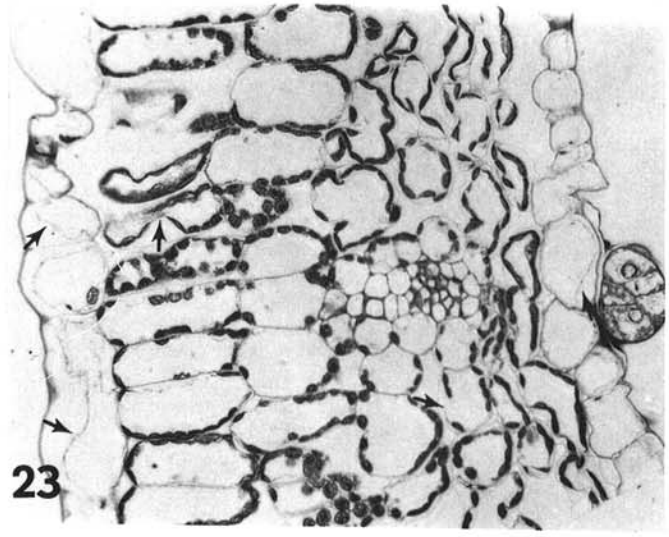
nucleoplasm failed to display its usual granular nature and contained scattered, small patches of electron dense material. The nucleolus was extremely electron dense and compact, and granular and fibrillar regions could not be distinguished.

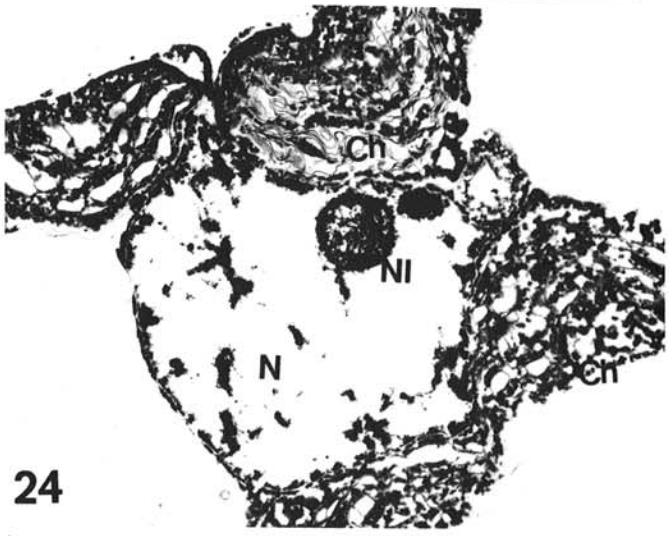
DISCUSSION

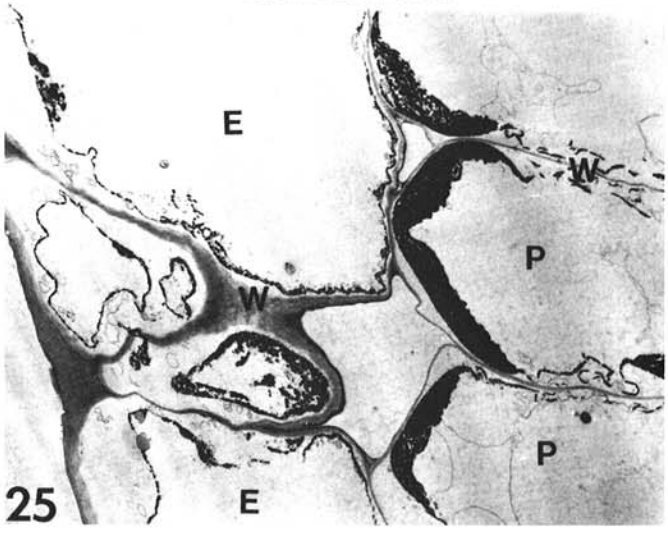
The most noticeable cytological changes caused by bromine fumigation were observed in the epidermis (Figs. 4, 6, and 7) immediately after exposure to 1 ppm bromine gas. The fact that the mesophyll cells were not as seriously or uniformly affected may indicate that bromine gas penetrated the cuticle of the epidermal cells. Initial action of bromine on cowpea leaves appeared to be a surface phenomenon similar to that reported for exposure to hydrogen chloride gas (4) or peroxyacetonitrite (13). Occasional collapse and/or necrosis of the mesophyll cells bordering substomatal chambers (Figs. 4 and 10) apparently resulted from bromine uptake through the stomata. Furthermore, the adaxial and abaxial surfaces showed differential response to bromine exposure. When samples were collected immediately after exposure, most of the adaxial epidermal cells contained darkly staining material in the central vacuole and exhibited anticlinal wall interdigitation, whereas only a few abaxial epidermal cells showed such changes (Figs. 4 and 7). When samples were collected 24 hr after exposure, many adaxial epidermal cells had recovered, and sporadic, minute lesions consisting of one or two necrotic epidermal cells and/or the underlying palisade cells comprised the only observable effects. By contrast, the abaxial epidermis retained the large lesions, which resulted from the collapse of 20–30 epidermal cells and a large number of nearby spongy mesophyll cells. Spongy mesophyll cells appeared to be far more susceptible to bromine gas than palisade mesophyll cells. This difference may be explained by the fact spongy mesophyll has larger intercellular spaces than the palisade mesophyll (5). The bromine gas that enters the leaf may more readily saturate such large intercellular space and gradually affect spongy mesophyll and abaxial epidermal cells causing the large lesions (Fig. 11).

At the light microscopic level, the abaxial epidermal cells appeared least injured (Fig. 4), but ultrastructurally they exhibited more severe alteration than the adaxial cells (Figs. 6 and 7). Cytological interpretation of pollution damages on plant cells by light microscopy alone may not always represent the true extent of injury. Similar interpretation would be given for plants exposed to 5 ppm of bromine. At this concentration the critical threshold might have been reached, and the entire cell population was instantly and irreversibly shocked beyond recovery. Whereas light microscopy of leaf cells treated with 5 ppm bromine revealed little or no damage when compared to cells either untreated or treated with 1 or 3 ppm, electron microscopy showed that all these cells were severely damaged.

Changes induced in the epidermis by bromine included retraction







Figs. 23–25, Cowpea tissue sampled immediately after 20 min of exposure to 5 ppm bromine gas. **23,** Light micrograph showing protoplast retraction (unlabeled arrows) in some epidermal and mesophyll cells (×488). **24,** Electron micrograph showing completely distorted nucleus (N), nucleolus (NI), and chloroplast (Ch) (×2,600). **25,** A low magnification of adaxial epidermal (E) and palisade (P) cells showing convoluted, necrotic cytoplasm. Cell walls (W) are not collapsed (×7,800).

of the protoplast, formation of circular lesions, plasmolysis, and interdigitation of anticlinal walls of adjacent cells. Some cells were capable of recovery, and osmotic movement of water could account for the reversible anticlinal wall interdigitation and recovery. Such histological changes have been observed by other investigators (3,6,15) in studies involving other pollutants. Perhaps individual cells differ in susceptibility to an air pollutant, or directional movement of a pollutant within leaves exposes different cells to different pollutant concentrations. Differential effects on adjacent cells have been observed in virtually all ultrastructural studies of plants exposed to air pollutants (14,20,21,23,24).

Vacuolation in the cytoplasm observed in the present study has also been reported to be a consistent response to other air pollutants. Wei and Miller (25) and Endress et al (3) found that the presence of small vacuoles was the first noticeable change in cells of leguminous plants exposed to hydrogen fluoride or hydrogen chloride, respectively. Vacuoles and/or vesicles were reported to increase in number as injury progressed. Ultrastructural evidence (2) suggests that formation of vauoles, as lytic compartments of the cell (1,11,12), parallels cell senescence, and micrographs of extensive vacuolation in pears during ripening, support this hypothesis (10).

The accumulation of electron-dense material in the central vacuole of epidermal cells (Figs. 4 and 7), the loss of the chloroplast membranes (Fig. 18), and the leached appearance of ground cytoplasm (Fig. 13) suggest that the effects of bromine observed in cowpea leaf cells resulted from changes in cellular permeability. Either the "leaky" membrane (7,21,24) or subsequent breakdown of the vacuolar membrane with release of both hydrolytic enzymes and bromine ions into the cytoplasm may precede senescence and the loss of the cell's internal structure.

Another consistent ultrastructural change induced by bromine was the alteration of the nucleolus. This effect has not been reported for other air pollutants but appeared similar to changes that occur in animal cells irradiated or treated with actinomycin D (17). Although the significance of the nucleolar change is not known at the present time, it could represent a consequence of disturbance of RNA systhesis.

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