

ELECTRON MICROSCOPY OF CURED AND FERMENTED CIGAR TOBACCO

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The ultrastructure of cured and fermented cigar filler tobacco was examined for the first time by electron microscopy. No consistent differences between the cured and fermented leaf were found. Both contained numerous small, electron-dense areas believed to be lipid in character. In addition, lightly stained linear structures were observed which appeared to be chloroplastic in origin. These lines are believed to have resulted from the loss of the lipid portion of the lamellae during the general deterioration of the cell in curing.

INTRODUCTION

Electron microscopy has been widely used to investigate the ultrastructure of many species of green leaves including tobacco. There have been, however, no previously published reports on electron microscopy of leaf tissue after prolonged senescence or death. The curing of tobacco could be considered a forced senescence, leading ultimately to the death of the cells. The presence of enzymatic activity in the fully cured leaf (1) prompted us to examine the ultrastructure of this material for comparison with the green tissue. In addition, the ultrastructure of the fermented leaf was studied to see if changes in structure could be related to the fermentation process.

Green Pennsylvania filler tobacco was prepared for electron microscopy immediately after harvest. The cured leaf samples had been stored under ambient conditions for four years after being air cured on the stalk immediately after harvest. Samples of fermented leaves were prepared for microscopy shortly after the fermentation process was complete. Fermentation of cigar filler tobacco is an active process which requires addition of moisture to the leaves and bulking them under high temperature and humidity conditions. Under these conditions the bulk is allowed to develop internal heat to a certain temperature and then the entire bulk is cooled. This cycle is continued until the fermentation is considered to be complete.

In preparation of the samples, small pieces, approximately 1 by 5 mm, were cut from each leaf parallel to the midrib. Pieces were fixed in 2 per cent glutaraldehyde, in pH 7.0, 0.05 M phosphate buffer, for 24 hours and then placed in unbuffered 1 per cent osmium tetroxide for one hour. The samples were then washed in buffer and dehydrated with increasing concentrations of ethanol in the usual manner (2). Each sample was oriented in the final embedding medium so that each microtome section represented a cross section of the leaf.

Cured and fermented tobacco present a special problem with respect to embedding for sectioning. Standard embedding media such as Epon and Araldite do not penetrate these tissues. Low viscosity embedding media are necessary and two procedures using

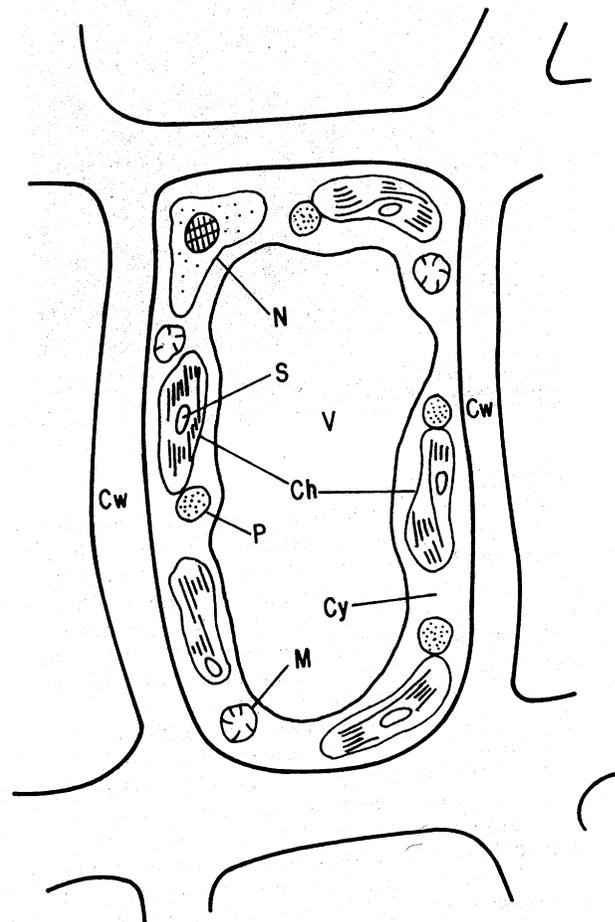


Figure 1. Drawing of a typical leaf cell. N—nucleus, Ch—chloroplast, S—starch granule, P—peroxisome, Cy—cytoplasm, M—mitochondria, CW—cell wall, V—vacuole.

this type of resin were employed.

In all, three methods of embedding were used. The green tissue was embedded in Epon according to the procedure of Luft (3). The following two methods were used for the cured and fermented samples:

1. In the first procedure infiltration was obtained using an 8:2 ratio of butyl to methyl methacrylate (2). Samples were embedded by placing them first in a mixture of equal volumes of resin and absolute ethanol for one hour and then pure resin for 72 hours. Fully infiltrated samples were oriented in BEEM capsules and then methacrylate mixture plus 8 to 12 granules of benzoyl peroxide added. Polymerization was catalyzed by an ultraviolet lamp (360 mu max.)

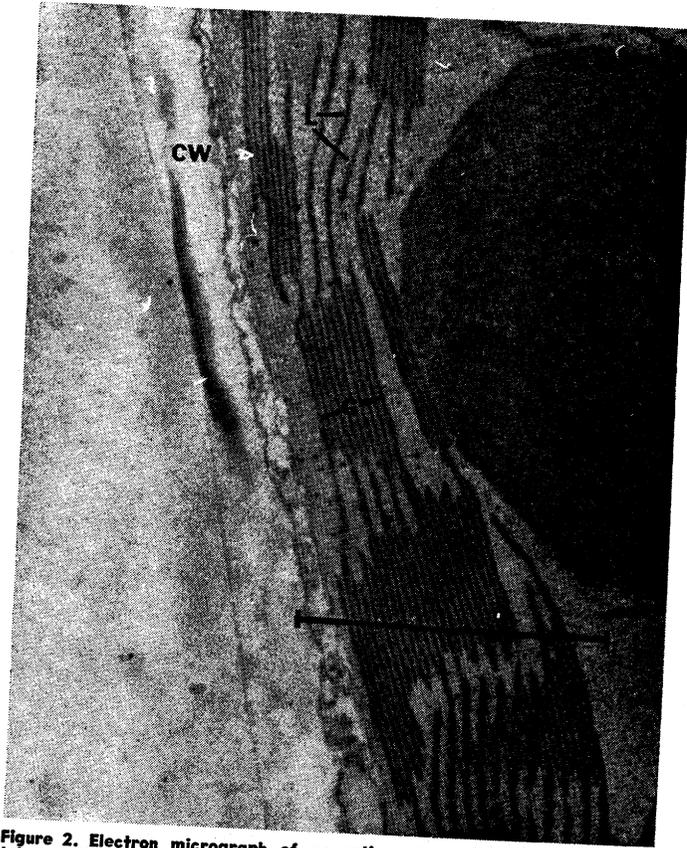


Figure 2. Electron micrograph of a portion of a chloroplast from green tobacco leaf. Sample embedded in Epon. L—lamella, G—granum, CW—cell wall. All tissues were initially fixed in glutaraldehyde and osmium and post-stained with uranyl acetate and lead. Marker indicates 1 micron.



Figure 3. Electron micrograph of cured tobacco. Sample embedded in butyl-methyl methacrylate. Marker indicates 1 micron.

at room temperature for two days.

2. The second procedure used was based on that developed by Spurr (4). This method utilizes a low viscosity embedding medium based on vinylcyclohexane dioxide. This procedure became the method of choice for these tissues because it was more rapid. There was no noticeable difference between these two methods with regard to ease of final sectioning or in the quality of the final electron micrographs.

Thin sections were obtained using an LKB Ultratome III. Electron micrographs were taken with an RCA 3-G microscope operating at 100 KV and using a 50 micron aperture. Section contrast was enhanced with uranyl acetate and lead citrate staining.

Green tobacco leaves are composed of several types of cells each of which contains a number of distinct and recognizable organelles such as chloroplasts, mitochondria, nuclei etc. A typical green leaf cell is illustrated in the drawing in Figure 1. Figure 2 is an electron micrograph of a portion of such a cell. Each mature cell is approximately 70 per cent vacuole. The cells are arranged in a honeycomb with the cell walls forming open or box-like structures around each cell.

Curing causes drastic changes in the appearance of the cells and in the leaf. The leaf shrinks to about 20 to 30 per cent of its original width. At the same time the cell arrangement collapses with the individual cell walls remaining largely unbroken but crushed and twisted (Figure 3). The process of fermentation, although responsible for major changes in the chemical composition of the leaf and in the quality of the smoke, does not have any observable effect on the appearance of the tissue under the electron microscope (Figure 4). Examination of several hundred electron micrographs of both cured and fermented leaf did not reveal any noticeable consistent differences

between the two.

Cells of both the cured and fermented leaves generally contained numerous small, electron-dense, oval masses ranging in size from 0.1 to 1.0 microns. Osmium, one of the reagents used to fix the samples, is a lipophilic reagent and stains most heavily in portions of tissue containing lipids. This suggests that the above electron-dense areas are composed, at least in part, of lipids. To experimentally test this idea, samples of cured and fermented leaves were extracted with absolute ethanol and ether after the glutaraldehyde fixation but prior to the addition of osmium. As a result of this extraction, a significant decrease in the number of electron-dense areas was apparent after subsequent fixation with osmium (Figure 5). Therefore, electron-dense areas could be globules of lipid formed by coalescence of lipid materials released by cytoplasmic deterioration during curing.

In both the fermented and cured leaves one additional feature was frequently observed (Figure 6). A series of light lines were present in some cells, the orientation of which suggested that they were of chloroplastic origin. Support for this explanation is found in experiments reported by Pease (2). In his work, tissue rich in mitochondria was fixed in glutaraldehyde and then dehydrated with ethanol solutions. Post-fixation with osmium yielded mitochondria with a ghost-like appearance similar to what we have found. If the same tissue was fixed in osmium prior to dehydration, normal mitochondria were observed. Pease explained these results by indicating that glutaraldehyde fixation alone was not adequate to prevent solvent extraction of lipids from the membranes during dehydration; osmium fixation was also required. Since, in our studies, osmium fixation was performed prior to dehydration in the standard prep-

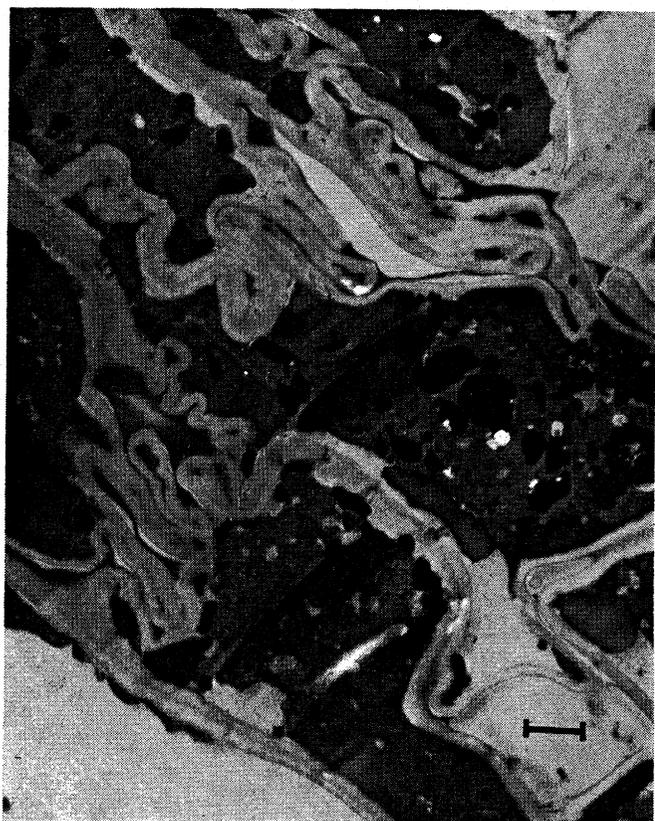


Figure 4. Electron micrograph of fermented tobacco. Embedded in Spurr medium. Marker indicates 1 micron.



Figure 5. Electron micrograph of cured tobacco extracted with absolute ethanol and ether before osmium fixation. Embedded in Spurr medium. Marker indicates 1 micron.

aration of the samples, it is unlikely that the chloroplast ghosts are fixation artifacts. Rather, these results suggest that lipids gradually migrate out of the lamellae as part of the general cytoplasmic breakdown that takes place during curing and fermentation. Part of the lamellae (presumably the protein portion) remains in place, hence the appearance of the ghost lines.

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Figure 6. Electron micrograph of cured tobacco containing structure which appears to be of chloroplastic origin. Embedded in butyl-methyl methacrylate. Marker indicates 0.1 micron.