

A CRYSTALLINE INCLUSION IN THE CHLOROPLASTS OF THE OUTER HYPODERMAL CELLS OF THE BANANA FRUIT

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[Manuscript received November 6, 1967]

Summary

Crystalline inclusions have been found in chloroplasts of the outer hypodermal cells of the peel of the banana fruit. Their structure has been observed at varying stages of ripeness of the fruit and, in line with earlier suggestions, a possible storage function is ascribed to them.

I. INTRODUCTION

Several references have been made to the occurrence of crystalline inclusions in various tissues and parts of plant cells, e.g. in the chloroplasts of leaves of beet (Englebrecht and Esau 1963), *Abutilon* (Sun 1965), coconut palm (Price, Martinez, and Warmke 1967), *Macadamia* (Price and Thomson 1967), *Vicia faba* (Shumway, Weier, and Stocking 1967), and of the sporangial wall of *Equisetum* (Manton 1966); in cytoplasm (Cronshaw 1964; Thornton and Thimann 1964; Marinos 1965; Arnott 1967; Arnott and Smith 1967; Petzold 1967); in xylem vessels (Arnott and Dauwalder 1967); and in mitochondria (Arnott 1967). Early observations (Englebrecht and Esau 1963; Sun 1965) associated the inclusions with a virus infection, but later observations (Cronshaw, Hoefert, and Esau 1966; Price, Martinez, and Warmke 1966; Price and Thompson 1967) have refuted this possibility. Most of the crystalline inclusions have been shown surrounded by a membrane.

In this work, crystalline inclusions are now reported in the chloroplasts of the hypodermal cells of the banana fruit, their presence being observed while following the ultrastructural changes occurring in the fruit tissues during ripening. They appear to be of general occurrence, having been found in banana fruit taken from widely separated areas and over a period of several years. The ultrastructural changes observed in them indicate that they could serve as storage areas in the tissue, their breakdown and depletion being evident as the cells senesce.

II. MATERIALS AND METHODS

Pieces of tissue, approximately 1 mm³ and including the hypodermis, were cut from the surface of banana fruits in various stages of ripeness (dark green, green-yellow, and yellow) and prepared for electron microscopy. The tissue was placed either in 2% KMnO₄ (Luft 1956) for 1–2 hr, in 1% OsO₄ (Palade 1952) for 2.5–4 hr, or overnight in 2.5% glutaraldehyde in phosphate buffer (pH 6.8) and post-fixed in 1% OsO₄. It was then washed, stained in 2% uranyl acetate for 1 hr, dehydrated in an alcohol series, embedded in Araldite (Glauert and Glauert 1958), sectioned, and examined in a Siemens Elmiskop 1 electron microscope at 80 kV. In some instances the sections were stained on the grid with lead citrate (Reynolds 1963).

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Pieces of banana leaf were also prepared as above. Samples were taken from a yellow unrolled portion of the youngest leaf, from a green area of the same leaf, from the second youngest leaf (fully expanded), from a green area on the oldest leaf, and from a yellow area on the oldest leaf. These samples were examined to see if the crystalline inclusions of the peel were present in the chloroplasts of the leaves and to relate any structural changes observed during ripening to those occurring during senescence of the leaf. If the crystalline inclusions were located in the banana leaf, then a greater amount of material would be available for their extraction and for subsequent attempts to determine their content.

III. RESULTS AND DISCUSSION

The anatomy of the banana peel is very complex (Ram, Ram, and Steward 1962) and consequently the tissue was not very satisfactory for ultrastructural investigation. As in previous investigations of fruit (Bain and Mercer 1963, 1964), the hypodermal tissue was the most rewarding area to examine. The cells of this tissue were elongated parallel to the outer surface of the fruit, were irregular in shape, and had very thick walls which sometimes made embedding and sectioning difficult. The vacuoles contained conspicuous electron-dense deposits, characteristic of the hypodermal cells of fruits. Chloroplasts were conspicuous in the outer hypodermal cells of the unripe peel, showing more development of grana and thus appearing to have more of a synthetic function than fruit plastids examined previously, e.g. the plastids in apple and pear (Bain and Mercer 1963, 1964). Starch was lacking in these hypodermal banana cells. Crystalline inclusions were obvious in their chloroplasts after fixation with osmium tetroxide (Figs. 1 and 2) or glutaraldehyde, but not with potassium permanganate.

The crystalline inclusions were frequent in the cells (up to four per cell), but were not always in each chloroplast in the section. Usually they occurred singly, although in one instance a second smaller body was seen in a chloroplast. The observed shape of the inclusions varied. They were square, rectangular, diamond-shaped, or triangular according to the direction of sectioning and their shape was reflected in the surrounding membrane structure of the plastid. The crystalline nature of the inclusion was made more obvious by staining with lead citrate. It was made up of subunits which appeared either as parallel lines in longitudinal section (Figs. 3 and 4) or as hexagonal structures in transverse section (Fig. 5). Crystallization occurred in many planes, groups of units being seen in transverse or longitudinal

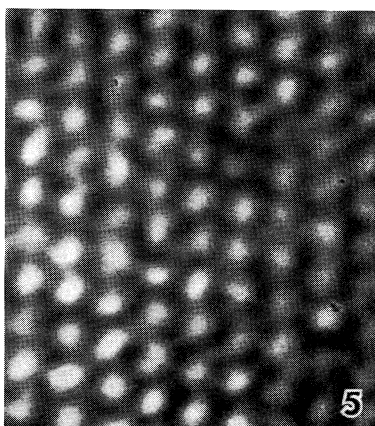
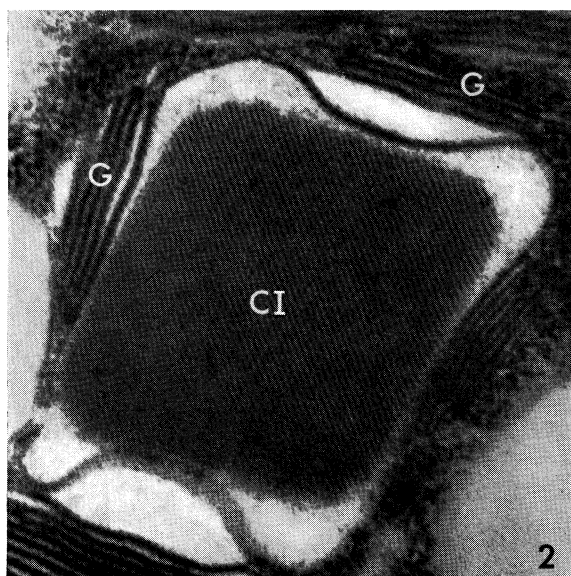
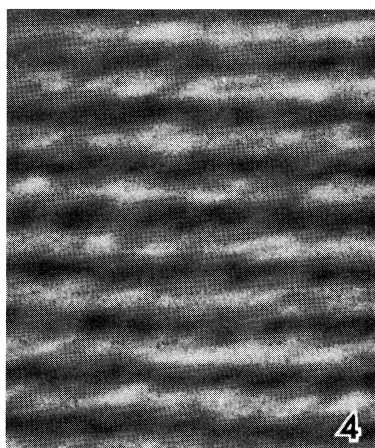
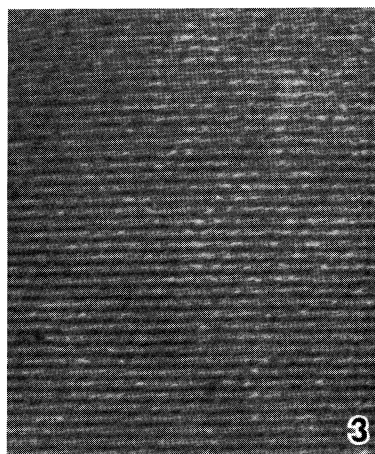
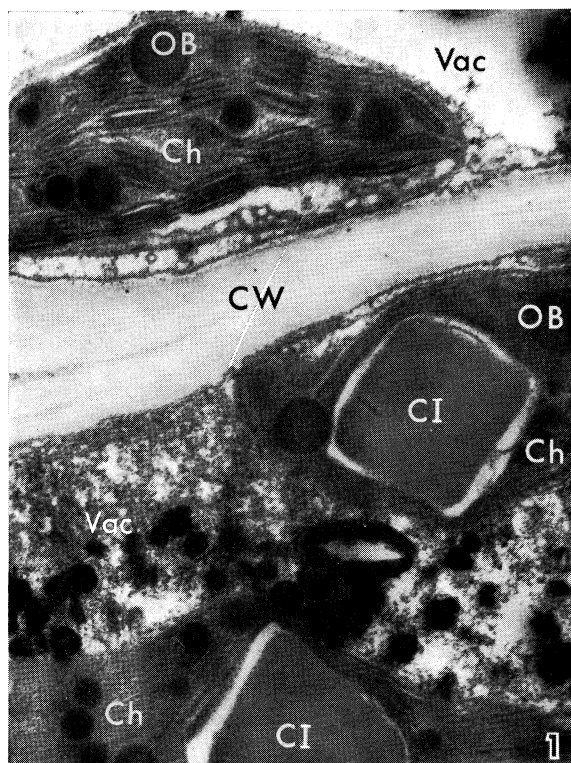
Fig. 1.—Crystalline inclusions (*CI*) are evident in two chloroplasts (*Ch*) in the hypodermal cells of a green fruit. The chloroplasts contain osmiophilic bodies (*OB*) and other electron-dense material is conspicuous in the vacuoles (*Vac*) of the lower cell. The cell wall (*CW*) is thick. 2×8000 .

Fig. 2.—Section of a crystalline inclusion (*CI*) showing the longitudinal pattern of the units. The shape of the body is reflected in the plastid structure, the grana (*G*) surrounding the space in which the crystalline inclusion developed. $1.5 \times 40,000$.

Fig. 3.—Units of the crystalline inclusion seen in longitudinal section, each unit consisting of part of two outer dense layers and an inner lighter area. $3 \times 40,000$.

Fig. 4.—Greater magnification of the structure shown in Figure 3, showing connections between the electron-dense bands in the units of the crystalline inclusion. $14 \times 40,000$.

Fig. 5.—Transverse section showing the hexagonal structure of units of the crystalline inclusion. $12 \times 40,000$.



Electron micrographs shown in Figures 1-9 are of the hypodermal cells of banana fruit. The material was fixed in 1% buffered osmium tetroxide, stained with uranyl acetate, and embedded in Araldite. Sections were stained with lead citrate.

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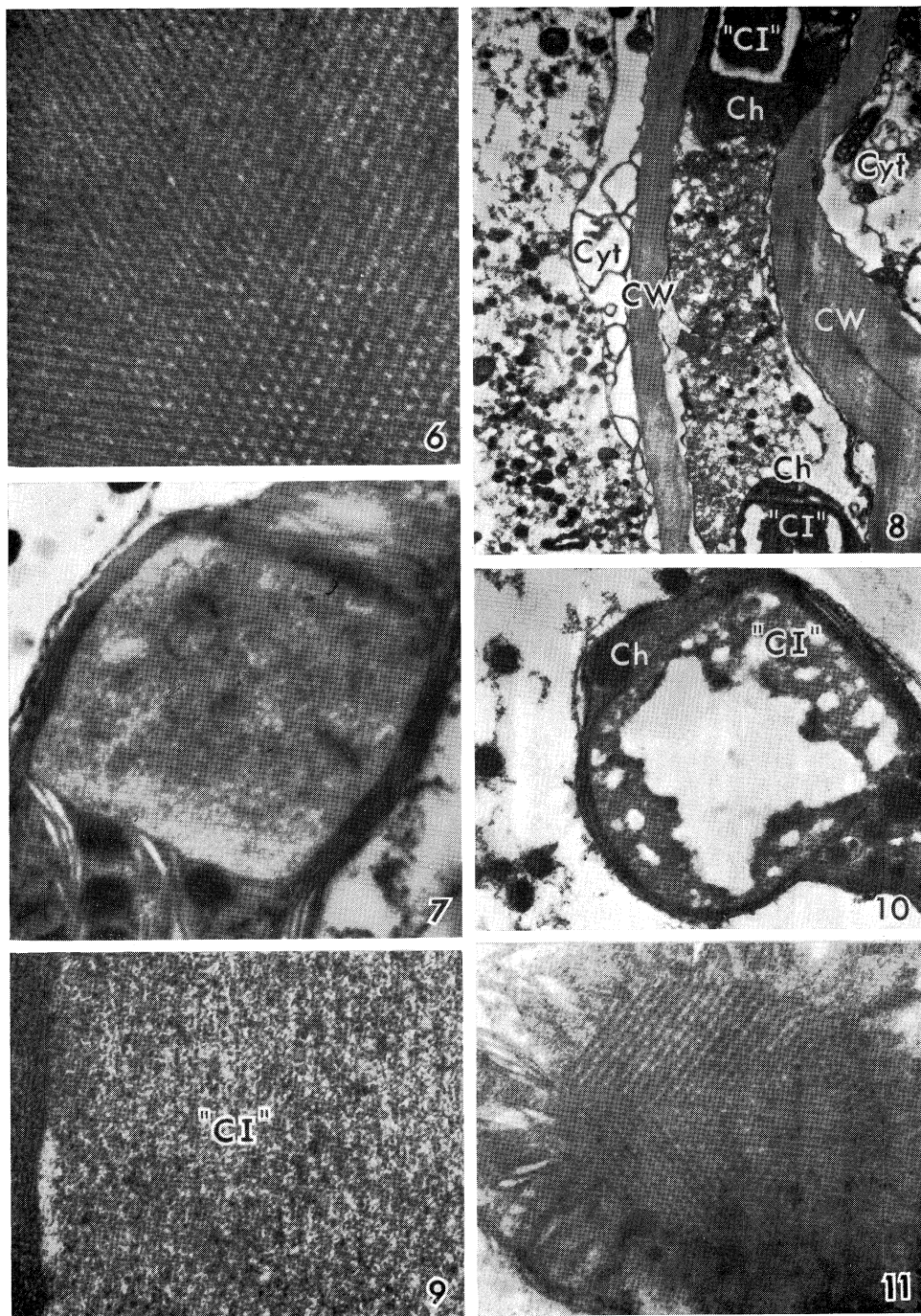


Fig. 6.—Showing varying orientation of groups of units in a section of the crystalline inclusion in a green fruit. $6 \times 30,000$.

[For continuation of legend, see opposite page.]

view in a single section of the inclusion. Units showing the parallel structure were grouped in areas which showed varying orientation (Fig. 6). The spacing of the units after osmium tetroxide fixation was from 104–130 Å, with an average of 120 Å, a value similar to that given by Price, Martinez, and Warmke (1966), but less than that of 160 Å given for the crystalline inclusion in the coconut palm after glutaraldehyde fixation (Price and Thomson 1967). The spacing in the inclusion in the banana after glutaraldehyde fixation was approximately 130 Å. Occasionally an area was observed in the chloroplast similar in shape to that of a crystalline inclusion, but filled with amorphous material (Fig. 7), representing perhaps the stage prior to crystallization.

The association of the inclusion and the chloroplast varied with the fixative used. After osmium tetroxide fixation (Figs. 1 and 2) there was a space surrounding the inclusion, as if it had been pulled away from the plastid structure while maintaining its original shape. A similar effect is observed in electron micrographs of Price, Martinez, and Warmke (1966) after osmium fixation. After glutaraldehyde fixation, the inclusion was closely associated with the plastid structure; Price and Thomson (1967) and Shumway, Weier, and Stocking (1967) also showed such an association. The inclusion was not surrounded by a membrane. Evidence of this is given in Figure 2 where the body is shown separated from the lamellar system of the chloroplast. This observation is in agreement with Price and Thomson (1967), but not with Price, Martinez, and Warmke (1966) as the latter stated that all the crystalline inclusions observed in the chloroplasts of coconut palm leaves were enclosed by a membrane. Although a membrane clearly surrounds the space in which the inclusion occurs in the plastid in Figure 1 of their paper, it does seem possible that the plastid could have come from a mature leaf beginning to senesce and that the surrounding membrane is one of the few membranes left in the plastid structure. The size and number of the osmiophilic bodies and the lack of grana in the plastid of the leaf also indicate this. Ultrastructural changes associated with senescence (increased size and number of osmiophilic bodies, loss of grana, and vacuolation of the stroma) occur in an organ before any morphological change due to aging is evident (Bain 1964; Bain and Mercer 1964).

Marked ultrastructural changes occurred in all parts of the cell during banana ripening including the crystalline inclusions. The disorganization of the cell contents

Fig. 7.—Representing a developmental stage of the crystalline inclusion in a green fruit. The material has not yet crystallized and is closely associated with the components of the plastid. $2.5 \times 15,000$.

Fig. 8.—The hypodermal cells of the ripened banana fruit are senescent. The cytoplasm (*Cyt*) has become vacuolated. Crystalline inclusions (“*CI*”) are recognizable in the disorganized plastids (*Ch*). Cell walls (*CW*) are thickened. 1.4×5000 .

Fig. 9.—Detail of a crystalline inclusion (“*CI*”) in a plastid from a ripened banana fruit. Its crystalline structure is now disorganized. It was only in contact with the plastid in a small area. $4 \times 40,000$.

Fig. 10.—The shape of the former crystalline inclusion (“*CI*”) is indicated in the disorganized plastid (*Ch*) of the ripe fruit, but much of the material has been lost from it. The remaining material is “vacuolated”. $2 \times 10,000$.

Fig. 11.—Crystalline appearance of the prolamellar body in the early stage of plastid development in a banana leaf which was a pale yellow colour and was still unrolling. $2 \times 15,000$.

in the ripened fruit is shown in Figure 8 and is similar to the changes observed during senescence in other fruit (Bain and Mercer 1964). Disorganization began in the cytoplasm and organelles of the hypodermal cells before any marked colour change was evident in the peel of the fruit. Distinct changes had occurred in the crystalline inclusions by the time the peel was yellow. Early disorganization with loss of the regular unit structure was evident in some cases (Fig. 9), but in most cases further degradation was obvious (Fig. 10). The breakdown of crystalline structure, vacuolation, and loss of material during ripening could be related to a storage function.

The composition of the crystalline inclusions in the chloroplasts is not known, but their particle size has been compared to that of high molecular weight proteins found in plants. Cronshaw (1964) suggested that crystalline inclusions were probably storage sites for hydrolytic enzymes. Price and Thomson (1967) suggested that they may be functionally or developmentally related to the grana and intergrana membranes or both, or represent storage sites for excess protein produced in the plastids. Marinos (1965) suggested that the crystal-containing bodies appearing in the cytoplasm of dormant potato tubers were sites of protein storage. Newcomb (1967) has reported storage protein in tubular, not crystalline form, in plastids of bean root tips. Gunning (1965) demonstrated a proteinaceous "stromacentre" or area of aggregated fibrils, 85 Å in diameter, in chloroplasts of *Avena sativa*. Shumway, Weier, and Stocking (1967) have observed crystals with 105–115 Å spacing in chloroplasts of intact *Vicia faba* leaves following treatment with hypertonic solutions. To date, examination of the ultrastructure of banana leaves in varying stages of development has not shown any crystalline structure other than that of the prolamellar body in developing plastids of leaves which were still unrolling (Fig. 11).

If the crystalline inclusions in the banana fruit are indeed protein storage bodies, this form of protein storage capacity appears to be limited to those chloroplasts which are not associated with maintaining starch reserves, no starch being observed in the chloroplasts in the outer hypodermal cells.

IV. ACKNOWLEDGMENTS

The technical assistance of Mrs. S. Stephens and Mrs. J. Hall is acknowledged gratefully.

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