THE SUBMICROSCOPIC CYTOLOGY OF SUPERFICIAL SCALD, A PHYSIOLOGICAL DISEASE OF APPLES

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Summary

Superficial scald in Granny Smith apples has been examined by electron microscopy, and a detailed picture of the changes in cell structure as macroscopic scald symptoms develop has been obtained.

Two types of disorganization have been recognized in scalded tissue. One is similar to the disorganization accompanying normal senescence; the other, the scald condition, is superimposed on the first. Scalding is distinct from normal aging.

Scalding is first detected in the outer hypodermal cells when the lesion is light brown, and the inner hypodermal cells become affected as the disorder progresses. The first submicroscopic symptom of scalding detected is the formation of an additional electron-dense material in close association with a normal constituent of the vacuoles of hypodermal cells. The additional material increases and accumulates on the tonoplast as scalding becomes more severe, and the protoplasts become disorganized and undergo overall "tanning", becoming increasingly electron-dense. Following this the cells collapse radially, forming the dark brown sunken lesion associated with severe scalding.

It is tentatively suggested that these submicroscopic changes are consistent with the breakdown of the control mechanism of the entire polyphenol system in the cytoplasm and vacuole, and that the electron-dense materials may be derived from polyphenols. The electron-dense materials are submicroscopic symptoms of scald, not the cause.

The localization of the disorder in the hypodermis is correlated with the electron-dense material observed in the vacuoles of normal hypodermal cells.

I. INTRODUCTION

Superficial scald, as shown by light-microscope studies (Bain 1956), is confined to the hypodermis and is characterized by a browning of the cell contents. The outer hypodermal cells only are affected in slight scald, and the entire hypodermis of five or six layers when the disorder is severe. In very severe scald the cells collapse in a radial direction causing the area to sink. Scald, though confined to cells with chloroplasts, is not correlated with microscopic changes in these bodies. In the present study the cell structure of non-scalded and scalded tissue has been examined with an electron microscope.

II. MATERIALS AND METHODS

Granny Smith apples, stored at 0°C for approximately 4 months and developing patches of scald on removal to 25°C, were used in the investigation. Stages in the development of the disorder were obtained by taking successive samples from

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single fruits over a period of several days as scald developed. The structure of the scalded cells was compared with that of cells taken from a non-scalded area of the same fruit.

Pieces of tissue, approximately 1 mm^3, were cut to include the cuticle, hypodermis, and outer flesh of the fruit, and fixed in a 1% buffered solution of osmium tetroxide (Palade 1952) for 4 hr at approximately 5°C. These were then dehydrated in an ethanol series, and embedded in "Araldite" according to the procedure given by Mercer and Birbeck (1961). Each piece of tissue was embedded with the cuticular surface parallel to the long axis of the gelatine capsule. Sections were cut with a diamond knife in a Porter–Blum microtome, and examined in a Siemens Elmiskop I electron microscope.

After the general picture of the changes in ultrastructure associated with the development of scald had been determined, attempts were made to induce similar changes in normal tissue by rupturing the cell membranes by various treatments. Pieces of tissue were removed as above; some were frozen for half an hour and then thawed, while others were placed in chloroform vapour for half an hour. The browned tissue was then fixed and embedded as above.

III. Results

Problems of fixation were presented throughout the investigation by the nature of the material used. The difficulty of fixing severely disorganized or dead cells was a serious one; fixation artefacts were not considered such a problem in the early stages of the disorder, as the structure of mitochondria and plastids in slightly scalded cells and in non-scalded cells compared favourably. The nature of the material often made embedding difficult as the thick walls of the hypodermal cells and the thick cuticle characteristic of Granny Smith apples impeded penetration of the "Araldite". As the result, only a small proportion of the sections cut were suitable for electron microscopy.

Since the fruit used in this investigation was mature when stored and senescent when sampled, it was necessary to distinguish changes in ultrastructure due to normal ripening from those occasioned by the development of scald symptoms. To do this, non-scalded but senescent tissue was used as a control.

(*a*) *Structure of Non-scalded Tissue*

(i) *Epidermis.*—The epidermal cells appear elongated tangentially with granular contents and covered by a thick cuticle when examined under the light microscope. With the electron microscope, their ultrastructure indicated that they were more functional than supposed from light microscopy. The dense cytoplasm contained structures resembling mitochondria, and also other dense bodies of similar size which were probably disorganized mitochondria. Plastids were not observed. Aggregates of a dense material (material *A*) were scattered through the vacuoles.

(ii) *Hypodermis.*—The hypodermis is made up of five or six rows of thick-walled tangentially elongated cells with numerous chloroplasts, and with very small inter-

cellular spaces. The cells increase in size away from the epidermis merging into the underlying, large, thinner-walled cortical cells.

In electron micrographs the cytoplasm occupied a high proportion of the area of some of the outer hypodermal cells but became a very thin layer surrounding the vacuole in the larger inner cells. An electron-dense material was found in the vacuoles. It was in large aggregates in the vacuoles of the outer cells and frequently against the tonoplast (Plate 1, Fig. 1), but it was finely dispersed in the inner cells (Plate 1, Fig. 2) and not apparent in the cortical cells. This substance (material *A*) is presumed to be a polyphenol, such substances being known to be present in the outer layers of apple fruit (Williams 1960). Chloroplasts were a distinctive feature of the hypodermal cells, especially in the outer cells. These were easily identified by their lamellate structure, and by the numerous dense osmiophilic bodies and starch grains. Some disorganization of the cytoplasm was evident but this was considered an effect of tissue aging rather than one of fixation and embedding; the chloroplasts at this stage were showing some slight swelling, while the mitochondria appeared normal (Plate 1, Fig. 3).

(*b*) *Structure of Scalded Tissue*

The changes in ultrastructure which are described have been put in sequence from examination of numerous electron micrographs taken from tissue showing increasing severity of scald, i.e. from light-brown, brown, and dark-brown lesions. The structure of the cells in the completely collapsed lesions of the final stages of the disorder has been included but the possibility of artefact is admitted.

Cells taken from very light-brown lesions showed some cytoplasmic disorganization in the outer hypodermal cells but the basic structure of the cells was identifiable. These changes are considered as normal for senescent tissue (Bain and Mercer, unpublished data). Vacuoles and vesicles were often developed in the cytoplasm and in the stroma of the chloroplasts, but the mitochondria appeared unaltered. An additional electron-dense material (*B*) had developed in close association with the original aggregated dense material in the vacuole (Plate 2, Fig. 1). This material, which appeared "pitted" compared with the original material (*A*) was considered responsible for the macroscopic cell browning observed in the early stages of the development of scald.

This new electron-dense material increased in quantity in the vacuole, and also became concentrated on the tonoplast as the disorder increased in severity (Plate 2, Fig. 2). At the same time vesicles in the cytoplasm increased in size and number, and the chloroplast membranes became disorganized, dispersing fragments of lamellae, vesicles, starch grains, and osmiophilic bodies into the cytoplasm (Plate 2, Fig. 3). The mitochondria became denser, though cristae structure was still recognizable in many instances. The lesion was now browner and the inner hypodermal cells affected.

The material *B* on the tonoplast increased in amount as the disorder became more severe, and an additional layer of dense material (solid, not pitted) appeared between the cell wall and the plasmalemma (material *C*, Plate 3, Fig. 1). Striking changes took place in the cytoplasm at the same time (Plate 3, Fig. 2). The vesicles

shrank and the cytoplasm showed an overall increase in density and became compacted. The cytoplasmic membranes and the chloroplast lamellae disappeared, leaving only osmiophilic bodies to indicate the former existence of the chloroplasts. The mitochondria became vague in appearance, the cristae disappeared, the matrix became denser, and eventually dense homogeneous bodies were all that remained. In the final stages of the disorder, the cytoplasm and the vacuoles became increasingly electron-dense, losing their identity as the cells collapsed radially (Plate 4, Fig. 1). The tissue became sunken and dark brown. For brevity this progression of events from a recognizable protoplast to an electron-dense body (material *D*) will be referred to in the discussion as the "tanning" of the protoplast.

(*c*) *Structure of Damaged Tissue*

As the colour changes associated with scalding resemble the browning of tissue following injury, electron micrographs were made of cells after treatment with chloroform vapour and after freezing and thawing. Both treatments caused extensive destruction of the protoplasts, but the position of the former vacuoles, cytoplasm, and some organelles could be identified in the hypodermis, except in the outermost cells, after freezing and thawing. The appearance of these cells was comparable to that of severely scalded cells. Two electron-dense materials were present in the vacuoles of cells treated with chloroform. These were in close association in the vacuole, and resembled materials *A* and *B* of scalded cells; material "*B*" was also concentrated at the former tonoplast (Plate 4, Fig. 2). Similar materials were found in the vacuoles of frozen cells; and a substance similar to substance *C* of the scalded cell was also found on the outside of the protoplast (Plate 4, Fig. 3).

Aggregates of a dense material resembling material *D* were observed scattered throughout the disorganized protoplasts (Plate 4, Fig. 2) but it was difficult to determine the site of formation of this material. The general impression given by the electron micrographs is that it was formed in the cytoplasm, but, because the protoplasts were destroyed by the treatments, the exact location cannot be established definitely.

IV. Discussion

From the electron micrographs two distinct types of disorganization can be identified in scalded tissue. One, most obvious in the early stages of the disorder, is characterized by the formation of vesicles in the chloroplasts and throughout the cytoplasm. These changes closely resemble those occurring in normal tissues undergoing normal senescence (Bain and Mercer, unpublished data). As the apples from which the scalded tissue was obtained would have been undergoing senescence it is considered that the vesicle formation seen in scalded cells corresponds to the normal breakdown of cell structure due to aging and not to the scald condition (Plate 1, Fig. 3). The other type of disorganization which becomes pronounced as the macroscopic browning symptoms develop is characterized by the formation of new electron-dense substances, and by an overall tanning and collapse of the protoplasts. This second type is considered to be due to the scald condition, and is superimposed on the disorganization due to normal senescence.

A feature of the submicroscopic symptoms of scalding is the presence of electron-dense substances in the protoplasts, and at least four have been recognized. These are: *A*, the aggregated material in the vacuole which has the appearance of the dense material seen in the vacuoles of normal cells; *B*, the pitted material of the vacuoles; *C*, the material of the dense layer outside the plasmalemma, and *D*, the material which is dispersed throughout the cytoplasm during the tanning of the protoplasts. If the order of appearance of these materials represents a developmental sequence, then two distinct phases of the disorder can be recognized. In the first or vacuolar phase a normal constituent of the vacuole (material *A*) is partially replaced by an additional substance (material *B*) which gradually aggregates on the tonoplast (Plate 2, Figs. 1 and 2). At the same time the colour of the tissue changes from yellow green to light brown. The initial browning of the tissue appears associated with the formation of the material *B* in the vacuoles, and not with changes in the cytoplasm. In the second or cytoplasmic phase the protoplasts shrink, the vesicles disappear, and the protoplasts become converted into more or less uniformly dense compressed bodies (material *D*), and a layer of electron-dense material (*C*) accumulates outside the plasmalemma; the tissue becomes dark brown.

The macroscopic browning of scald lesions can be likened to the macroscopic browning of tissue after injury by freezing and thawing or with chloroform vapour. Electron micrographs of apple tissue treated in this way (Plate 4, Figs. 2 and 3) show electron-dense materials similar in appearance to materials *A*, *B*, and *C* (Plate 2, Fig. 1; Plate 3, Fig. 1), and possibly to material *D* of scalded cells, but as pointed out previously (Section III(*c*)) this point is difficult to establish because the two treatments (chloroform vapour and freezing–thawing) almost completely destroyed the protoplasts, making the identification of the site of formation of the dense aggregates difficult. These observations suggest that the substances responsible for macroscopic browning and the additional electron-dense substances seen in both scald and the treatments could be identical.

In normal plant tissues macroscopic browning is known to be due to the oxidation of phenolic and tannin compounds following damage of the cells. A list of the polyphenols found in apple tissue is given by Williams (1960) and includes chlorogenic acid, epicatechin, and catechin. Siegelman (1955) claims that (−)epicatechin is the principal substrate for the browning reaction in the skin of Grimes Golden and Golden Delicious apples. Polyphenol compounds are likely to reduce or be precipitated by the osmium tetroxide fixative. Electron-dense material *A*, therefore, could be derived from a normal polyphenol of the vacuoles (such as (−)epicatechin). Similarly the oxidation products, which are responsible for the brown colour in the normal browning reaction, are likely to be reactive towards osmium tetroxide, and also give rise to electron-dense materials. In addition, local differences in the cell milieu may affect the degree of oxidation and polymerization of the oxidation products, and so affect the texture of the electron-dense materials. Thus the normal polyphenols and their oxidation products from uncontrolled polyphenolase activity following cell damage (normal browning) are likely to appear as electron-dense regions in electron micrographs. Hence it is suggested that material *A* corresponds to the normal polyphenols of vacuole and materials *B*, *C*, and *D* to oxidation products.

The first submicroscopic sign of scalding is the appearance of material *B* and the gradual depletion of material *A* in the vacuoles of tissue which has become light brown; as far as is known, the structure of the cytoplasm and cell membranes remains normal at this time. Material *B*, therefore, could be the pigment responsible for the brown colour of the tissue at this stage in the development of the disorder. The similarity between the symptoms of damaged and scalded cells suggests that material *B* could be an oxidation product of the normal polyphenol in the vacuole. As the disorder progresses, material *B* accumulates on the tonoplast, the tissue becomes browner, and the cells lose turgor and gradually collapse. These changes are to be expected if material *B* damages the organization of the tonoplast, increasing permeability. A loss of permeability would allow vacuolar sap, containing polyphenols, to leak into the cytoplasm. If the polyphenolases are in the cytoplasm, enzymatic oxidation of the polyphenols would occur. Possibly local differences in the cell milieu may determine the degree of polymerization of the oxidation products. For example, to the outside of the protoplast, possibly through contact with the external atmosphere, oxidation and polymerization might be complete and give a compact layer of material such as material *C*, which is seen as a layer adjacent to the cell walls in the electron micrographs, while in the bulk cytoplasm the oxidation products may be more evenly distributed and contribute to the increasing electron density of the protoplasts in the later stages of the disorder. The material responsible for the increasing electron density of the cytoplasm, as the disorder progresses, has been described as material *D*.

A further consequence of the mixing of vacuolar sap with the cytoplasm is that the oxidation products of the polyphenols may form complexes with the cytoplasmic proteins similar to those formed in the tanning of proteins by certain types of tannins (Byrde, Fielding, and Williams 1960). Compounds with 15 carbon atoms, such as catechin and epicatechin, are believed to be precursors of the above type of tannins. It is possible that their oxidation products will possess some tannin properties including the ability to precipitate proteins. Such protein–oxidation product complexes would be dispersed throughout the cytoplasm, and so contribute to the overall electron density of the protoplasts. Material *D* could be heterogeneous in composition.

It is suggested, therefore, that uncontrolled polyphenolase activity could account for the formation of electron-dense materials in both scalded and damaged cells. Cell browning and the formation of the electron-dense materials are seen as symptoms of scald, not a cause. If this interpretation is essentially correct an early physiological change due to the scald state must involve processes which normally maintain the polyphenols in the oxidized state and, if the polyphenolases are localized in the cytoplasm, the initiation of the vacuolar phase (material *A* disappearing as material *B* accumulates) would require a non-enzymic oxidation of the phenol.

The postulated increase of cell permeability would account for the shrinkage of the vacuole and vesicles, which is a characteristic feature of the later stages of the disorder, since it would lead gradually to a loss of turgor, allowing the greater turgor of the underlying cells to compress the hypodermis between cortex and cuticle. This compression or squashing of the cells combined with the tanning action of

the uncontrolled polyphenolase system would explain the apparent homogeneous structure and density of the protoplasts in the final stages of scalding.

The present observations do not necessarily conflict with the view that scald is caused by a volatile substance which becomes toxic if the concentration within the protoplasts rises beyond a critical level. For, it could be argued that the toxic volatile compound is responsible for the breakdown of the physiological control of the polyphenolase system. The observations demonstrate that scald is distinct from normal aging and its localization to the hypodermis may be determined by the presence of a substance in the vacuoles of the outer cells which is not found in the cortical cells. Material *A* is localized to the hypodermis.

V. References

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Explanation of Plates 1–4

All plates are electron micrographs of Granny Smith apple tissue which was fixed in buffered osmium tetroxide (following transfer of the apples to room temperature after several months in cold storage), embedded in "Araldite", and sectioned

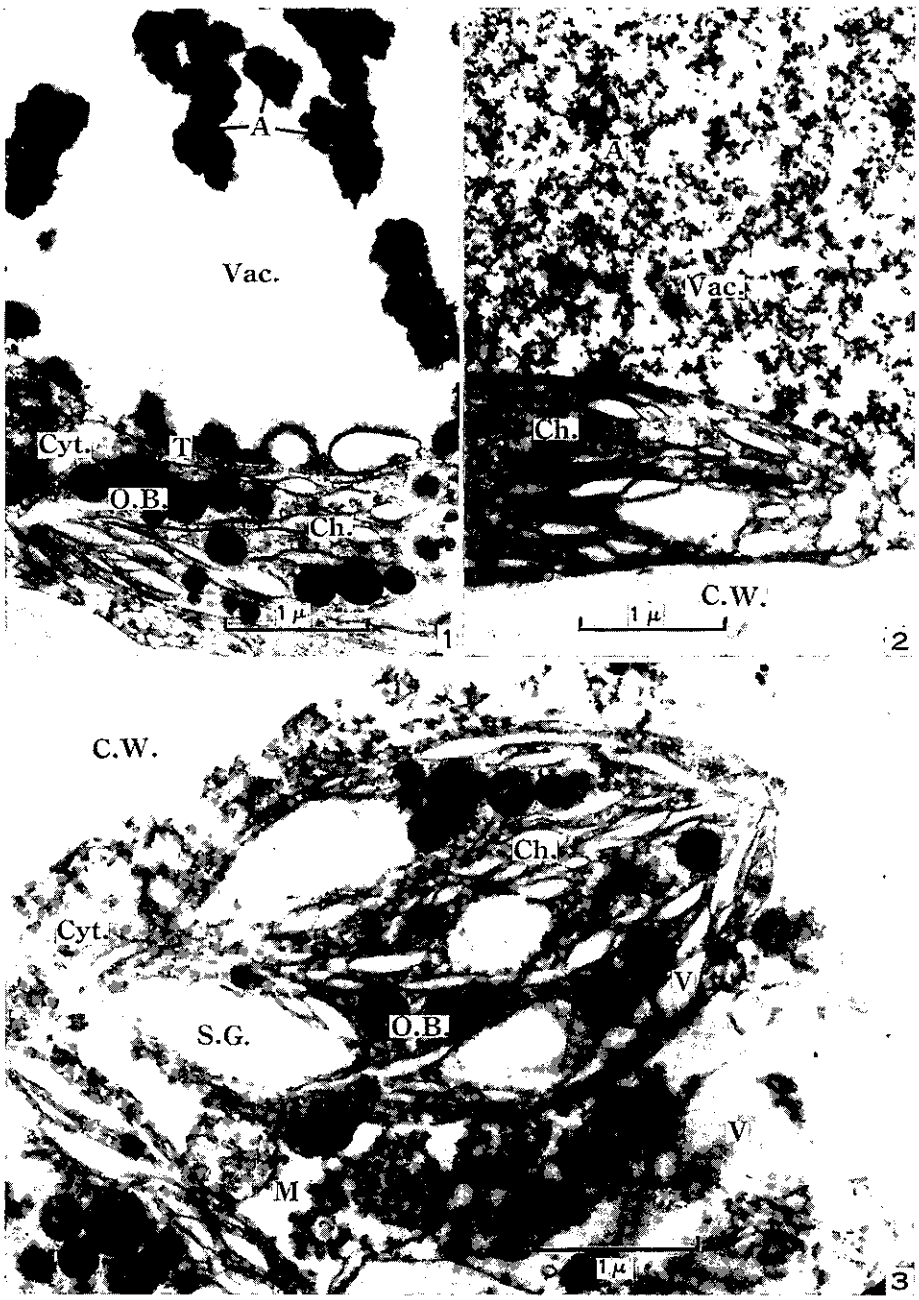
Plate 1

Fig. 1.—Outer hypodermal cell of non-scalded tissue showing the form of the electron-dense material (*A*) commonly found aggregated throughout the vacuole (*Vac.*) and in frequent association with the tonoplast (*T*). Part of the cytoplasm (*Cyt.*) and a chloroplast (*Ch.*) with osmiophilic bodies (*O.B.*) are included. ×20,000.

Fig. 2.—Inner hypodermal cell of non-scalded tissue, showing the electron-dense material (*A*) finely dispersed throughout the vacuole (*Vac.*). Part of a chloroplast (*Ch.*) and the adjacent cell wall (*C.W.*) are also shown. ×20,000.

Fig. 3.—Structure of the protoplast in an outer, non-scalded, hypodermal cell, and representing the normal structure of a senescent pre-scald cell. The cytoplasm (*Cyt.*) and chloroplast (*Ch.*) appear slightly swollen through the formation of small vesicles (*V*) but the mitochondrion (*M*) is normal. Dense spherical osmiophilic bodies (*O.B.*) and possible starch grains (*S.G.*) occur in the swollen chloroplast. Vesicle formation is considered to result from the disorganization of the protoplast associated with normal senescence and not fixation. The cytoplasm is close against the cell wall (*C.W.*). ×23,000.

SUBMICROSCOPIC CYTOLOGY OF APPLE SCALD



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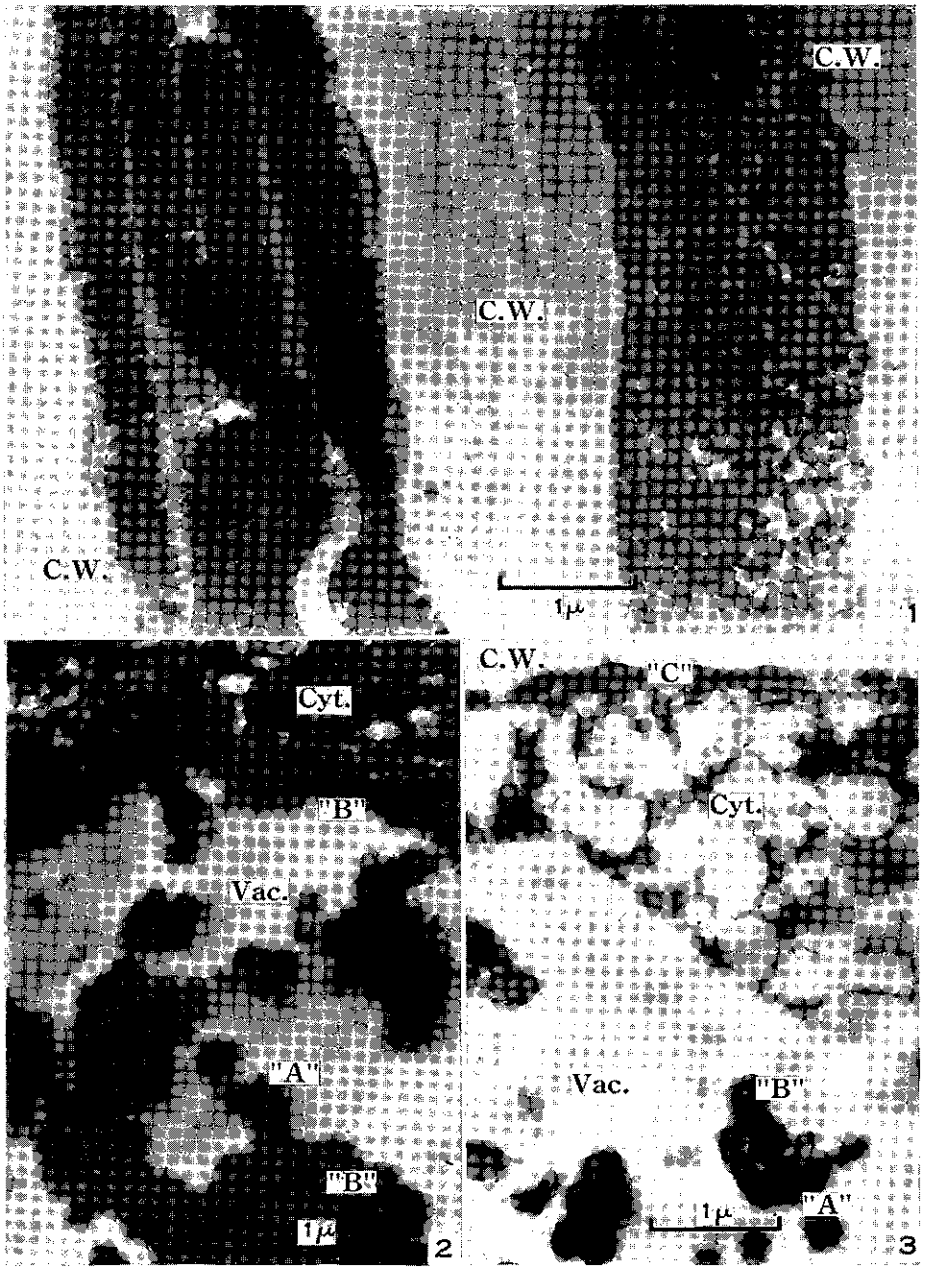

PLATE 2

Fig. 1.—Slightly scalded outer hypodermal cell showing the disorganization of the cytoplasm due to normal aging and the first signs of scalding in the vacuole (*Vac.*). An additional electron-dense material with a pitted structure (*B*) is present in association with material *A* which is typical of the normal vacuole. Other structures in the cell are the cell wall (*C.W.*), the tonoplast (*T*), endoplasmic reticulum (*E.R.*), mitochondrion (*M*), chloroplast (*Ch.*), and osmiophilic body (*O.B.*). ×20,000.

Fig. 2.—Inner hypodermal cell showing the electron-dense material *B* formed in the vacuole (*Vac.*) and concentrated on the tonoplast (*T*). The scald lesion had changed from light brown to brown when this inner hypodermal cell was fixed. The cytoplasm (*Cyt.*) bounded on the outside by the cell wall (*C.W.*) is noticeably vesiculated but the structure of the mitochondrion (*M*) appears little altered. ×24,000.

Fig. 3.—Disorganization of the cytoplasm, following accumulation of the pitted material (*B*) on the tonoplast (*T*), has proceeded further than in Plate 2, Figure 2, with increasing severity of disorder. Vesicles (*V*) have increased in size and the chloroplasts have become disorganized, dispersing fragments of lamellae (*L*), vesicles, starch grains (*S.G.*), and osmiophilic bodies (*O.B.*) into the cytoplasm (*Cyt.*). The cell wall (*C.W.*) and the vacuole (*Vac.*) are also indicated. ×14,000.

PLATE 3

Fig. 1.—Cell structure in an inner hypodermal cell of severely scalded tissue showing material *B* concentrated at the position of the tonoplast with a third electron-dense material (*C*) present outside the plasmalemma and adjacent to the cell wall (*C.W.*). The cytoplasm (*Cyt.*) is becoming denser. Cristae can still be distinguished in the mitochondrion (*M*). ×27,500.

Fig. 2.—Showing the "tanning" of the protoplast in severely scalded tissue. The cytoplasm (*Cyt.*) has become compacted and the previous position of chloroplasts is shown by the distribution of osmiophilic bodies (*O.B.*) and lamellae (*L*). The form of a mitochondrion (*M*) is still recognizable. Material *B* is shown concentrated at the outer limit of the vacuole (*Vac.*) and an indication of material *C* is found adjacent to the cell wall (*C.W.*). ×27,500.

PLATE 4

Fig. 1.—Cells in the final stages of the disorder. The protoplasts, separated by the cell wall (*C.W.*), have become very dense and the identity of vacuole and cytoplasm is lost; the cells are collapsed radially. ×20,000.

Fig. 2.—Outer hypodermal cell taken from tissue placed in chloroform vapour for half an hour. Cell structure has been greatly affected by this treatment but it is possible to discern substances in the former vacuole (*Vac.*) resembling materials *A* and *B* of the scalded tissue. Substance "*B*" is associated with substance "*A*" and is also concentrated on the limit of the cytoplasm (*Cyt.*) which has been "tanned" by the treatment. ×14,000.

Fig. 3.—Portion of an inner hypodermal cell, frozen and thawed prior to fixation, is shown. Electron-dense materials ("*A*", "*B*") resembling material *A* of normal cells and material *B* in scalded cells respectively are identifiable in the vacuole (*Vac.*). The cytoplasm (*Cyt.*) has become vesiculated and an electron-dense material ("*C*") which resembles material *C* in scalded cells is found between the plasmalemma and the cell wall (*C.W.*). ×18,000.