DEVELOPMENT OF THE STRUCTURE OF THE NORMAL, SMOOTH CUTICLE OF THE APPLE "GOLDEN DELICIOUS"

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SUMMARY

The epidermal and cuticular layers of the apple "Golden Delicious" during growth were studied electron microscopically with special reference to the cutin containing layer.

The structure of the cuticle does not change essentially during the increase in thickness of the cuticle. A fibrillar structure of electron dense material occurs in the cuticle, most clearly in the young apple or in older stages when the wax is extracted. The cutinous substance expands inwardly during growth and in the full-grown stage the epidermal cells can be surrounded with cutinous material. Below a damaged cuticle a very strong cutinization has been observed.

1. INTRODUCTION

The epidermis of higher plants is coated with a cuticle, and in many plants – especially in xerophytes – it has an underlying cuticular layer (FREY-WYSSLING & MÜHLETHALER 1965; SITTE 1965). In the cuticle, cutin substances are found in pure form; wax molecules occur there (ROELOFSEN 1952; FREY-WYSSLING & MÜHLETHALER 1965). The cuticular layer (cutinized layer, LINSKENS *c.s.* 1965) contains cutin, waxes, and carbohydrates. When a cuticular layer is formed, a distinction between the cuticle and the cuticular layer is possible in the polarizing microscope; but it is possible in the light microscope only after staining the present polysaccharides or after dissolving partially the cutin with alkali (*e.g.* FRITZ 1935; ROELOFSEN 1952; SITTE & RENNIER 1963; HÜLSBRUCH 1966). But a proper distinction of the cutin substances is not yet possible in light microscopy, nor in electron microscopy (SITTE & RENNIER 1963; FREY-WYSSLING & MÜHLETHALER 1965; LINSKENS & GELISSEN 1966). Because there is no uniformity in the nomenclature of the epidermal outer cell wall layers we have called the cuticle: the cuticle sensu stricto (ROELOFSEN 1952; O'BRIEN 1967; SITTE & RENNIER 1963: Cuticle proper). The cuticle sensu stricto and the cuticular layer together are called the cuticle (LINSKENS & GELISSEN 1966, use the term in this sense).

The cuticles of the examined apple varieties (TETLEY 1930) show about the same cutin reactions (Sudan stains) in the light microscope, and also all other plants examined. Differences in the thickness of the cuticles sensu stricto and the composition of the cuticular layers exist from species to species (SITTE & RENNIER 1963). In some plants the production of cutin substances is so large that a cuticular epithelium is found (DAMM 1902; FRITZ 1935). TETLEY (1930) observed that the cuticle deposited on the mature epidermal cells was found to vary considerably in the different apple varieties ("Golden Delicious" not examined).

Thus far the osmiophilic structures in the epidermal outer cell wall of the following objects have been described by means of the electron microscope:

a. the oat coleoptile *(Avena sativa)*

The cuticle consists of an apparently structureless matrix through which ramifies a reticulum of electron dense fibrillar material. Multivesicular elements are found in the inner region of the outer cell wall. Often they contain material of low electron contrast, but on other occasions they appear to be empty (O'BRIEN 1967).

b. leaves

The cuticles are structureless in *Philodendron scandens* (BOLLIGER 1959), in *Echeveria secunda* and *Clivia nobilis* (FREY-WYSSLING & MÜHLETHALER 1959, 1965). The cuticle has a very fine lamella structure in *Ficus elastica* (SITTE 1962; SITTE & RENNIER 1963). It has dotted bead-like structures arranged to parallel lamellae in *Gasteria verrucosa* (DE VRIES, BREDEMEIJER & HEINEN 1967).

c. fruits

The cuticle has a very fine lamella structure in several apple varieties (HILKENBÄUMER 1958), and this is confirmed by LINSKENS & GELISSEN (1966) in "Golden Delicious".

Perhaps the difficulties involved in getting good sections for electron microscopy have prevented more work to be done on the cuticle. There are few successful preparation procedures for cuticles, due to the low penetration of resins into the cuticle (O'BRIEN 1967). Because of the difference in solidity of the cuticle, tissues underneath, and the resin that is used it is hard to get good sections (MAZLIAK 1963).

The work described in this paper is undertaken as a part of the research into the nature of russeting in fruits of "Golden Delicious". It is a continuation of the publication of LINSKENS & GELISSEN (1966). A comprised study of the cuticle of the smooth apple is the object of the present investigation, because russeting consists in an alteration of epidermal and cuticular layers (LINSKENS & GELISSEN 1966).

2. MATERIAL AND METHODS

Small pieces of the skin from "Golden Delicious" apples were cut out half-way to the calyx and the stem of the apple. They were fixed for two hours in glutaraldehyde in phosphate buffer pH 7.2, and afterwards rinsed with the same buffer for at least 12 hours and postfixed with 2% OsO_4 solution.

Postfixation with 2% $KMnO_4$ is only suitable in the youngest stages because the cuticle loosens very easily from the cells during sectioning (DRAWERT & MIX 1963). Fixation and rinsing was done at 4°C. During the fixation in glutaraldehyde, the objects were evacuated vigorously, dehydrated in ethanol, and finally transferred to Epon 812 via epoxypropane for flat embedments. Transverse sections were cut with a diamond knife on a L.K.B. ultratome 4801 A, mostly stained with lead citrate (REYNOLDS 1963), and examined in a Philips EM 100 C at 60 kV.

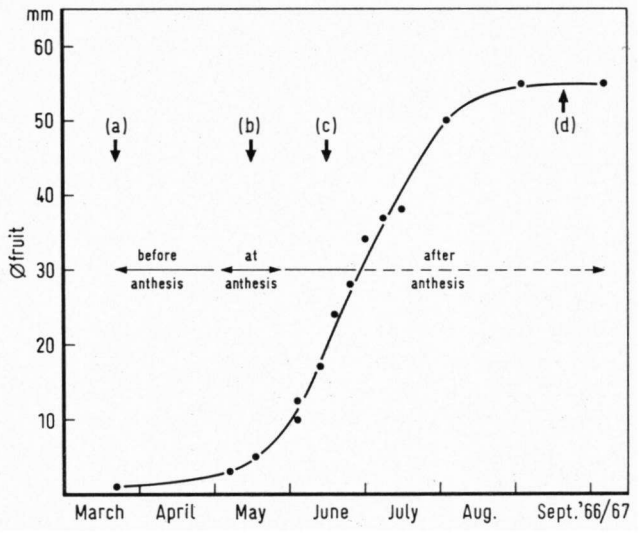

Fig. 1.
Diameter apple *vs.* growth period. The horizontal lines represent developmental stages mentioned in the text. The vertical arrows (a) to (d) refer to the electron micrographs taken at these stages, similar as in *fig.* 2.

3. Observations and results

3.1. Growth of the cuticle

The increase of the diameter of the apple – half way to the calyx and the stem – versus the growth period shows the classical growth curve. At anthesis, likely after pollination (the setting stage), the apple increases considerably in size untill the beginning of September *(fig. 1)*. The shape of our growth curve for "Golden Delicious" is about the same as the growth curve for "Cox's Orange Pippin", which was obtained by the average weight of the apple *vs.* growth period (Hulme *c.s.* 1966). The increase in thickness of the cuticle initiates before anthesis *(fig. 2)* and is about linear *vs.* the diameter of the apple.

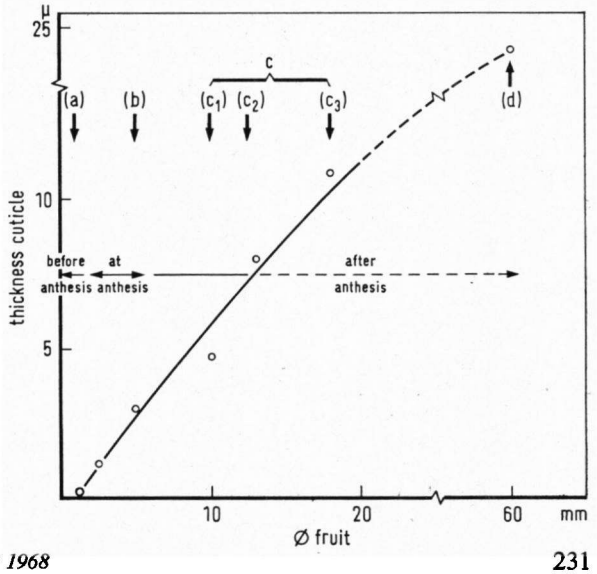

Fig. 2.
The thickness of the cuticle *vs.* the diameter of the apple. Explanation as in *fig.* 1.

We have studied different developmental stages of the outer epidermis of the peel tissue (with special reference to the cutin layer) before, at, and after anthesis of the apple.

3.2. The cuticle of the outer epidermis before anthesis

The epidermis of the floral tube (also called the extracarpellary part, the hypanthium, or the concave receptacle) was taken before anthesis in March (stage (a), *fig. 2*).

It has only a cuticle sensu stricto, which is very osmiophilic and is composed of droplets as seen by electron microscopy *(fig. 3a)*. In the sections postfixed with $KMnO_4$ the same droplet-like structure is seen *(fig. 3b)*. In this period electron dense material below the cuticle sensu stricto is only seen in the outer cell wall of the basal part of a hair *(fig. 6a*, marked with the arrow a) and in the inner cell wall (same figure, arrow b).

3.3. The cuticle of the outer epidermis at anthesis

At anthesis (stage (b), *fig. 2)* there is already a well developed cuticle *(fig. 4a)*. A distinction between the cuticular layer and the cuticle sensu stricto can be seen in the basal part of the hairs where the further cutinization has proceeded. Here the cuticle sensu stricto merges gradually into the cuticular layer (*fig. 6b*, marked with the arrow). The cuticle in this period is more osmiophilic at the inside than at the outside *(fig. 4a)*. The inside layer often has a droplet-like structure, and the outside layer shows a fine-drawn fibrillar structure only detectable after staining with lead *(fig. 4b)*. This structure resembles the fibrillar structure in the cuticle of the oat coleoptile described by O'BRIEN (1967).

3.4. The cuticle after anthesis

a. The increase in thickness

The apple grows considerably in size during the month of June (*fig. 1*, stage (c)). The thickness of the cuticle in this period increases nearly linear with time (*fig. 2*, stage (c)). But throughout the middle lamellae, the cutinous substance expands inwardly, so that it is anchored between the anticlinal walls forming wedges of cutin *(fig. 5)*. In full-grown apples (*fig. 1*, stage (d)), the cuticle has reached its maximum thickness (*fig. 2*, stage (d)). The cutin wedges may reach just below the epidermal cells. Sometimes the epidermal cells are cutinized on all sides. A so called "cuticular epithelium" is formed *(fig. 10)*.

Fig. 3a. Outer epidermal cell wall before anthesis (stage (a)). Fix. glut. ald. + OsO_4. Stained with lead citrate.

Fig. 3b. As *fig. 3a*. Fix. glut. ald. + $KMnO_4$. Stained with lead citrate.

Fig. 4a. Outer epidermal cell wall with epidermal cells at anthesis (stage (b)). Fix. glut. ald. + OsO_4. Stained with lead citrate.

Fig. 4b. As *fig. 4a*. The inside layer of the cuticle is composed of droplets (arrow), the outside shows a fibrillar structure.

Fig. 5. The epidermis with the cuticle after anthesis (stage (c_3)). Fix. glut. ald. + OsO_4. Stained with lead citrate.

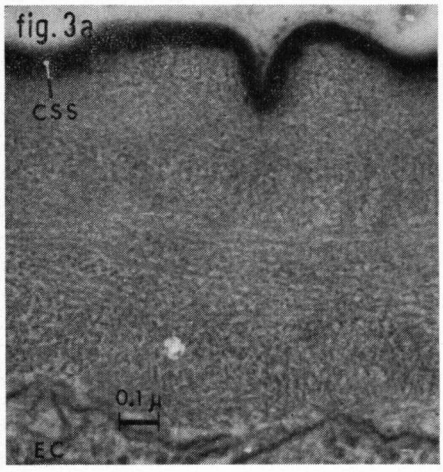
fig. 3a
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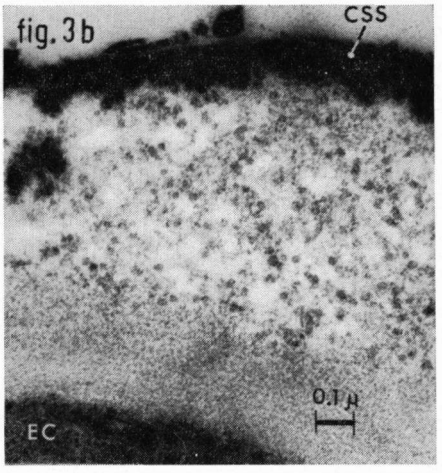
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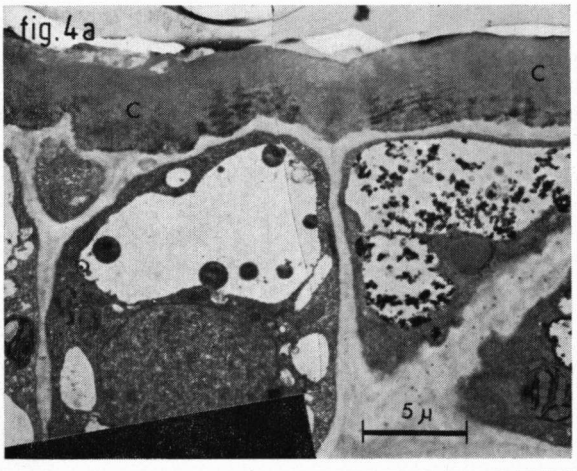
fig. 4a
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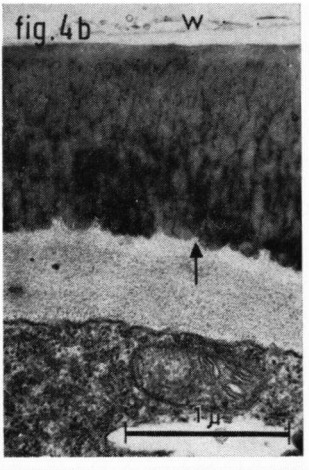
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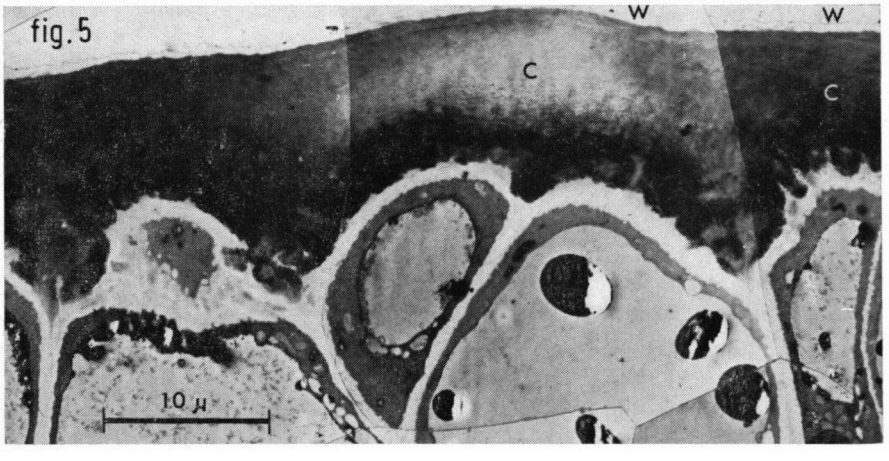
fig. 5
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b. The structure of the cuticle

During growth of the apple the structure of the cuticle does not change, essentially, compared to that of the flowering stage. The cuticle remains more osmiophilic at the inside *(fig. 5)*. The fibrillar structure at the outside is visible. In the outer part of the cuticle this structure becomes indistinct in the process of aging; until it seems to have disappeared entirely in the full-grown apple. But extraction of the waxes with hot 100% methanol (boiled for 72 hours, changed every 24 hours) reveals this structure in the whole cuticle. But it is seen most clearly in the inner part of the cuticle and in the middle of the cutin wedges *(fig. 8*, arrow a). Also many holes appear, previously filled with waxes (*fig. 8*, arrow b).

3.5. The epidermal cells at and after anthesis

While the cuticle increases in thickness, the epidermal cells remain surrounded by a non-osmiophilic cellulose wall. Three types of multivesicular elements may be found in the cell walls, especially in the fullgrown apples:

1. Empty elements. They are sometimes situated within the cuticle, mostly in the anticlinal and periclinal epidermal walls *(fig. 9c)*.
2. A second type of elements, which have little electron density, is found in the radial walls of the epidermal cells *(fig. 9a)*.
3. Elements of considerable electronic contrast were found in the inner periclinal walls of the epidermis *(fig. 9b)*.

The multivesicular elements have been observed also in invaginations of the plasmalemma of the epidermal cells (the first type in *fig. 9d*, arrow b, and the second type in *fig. 9e*, arrow). The elements of the third type are found in an invagination of a hypodermal cell (*fig. 9a*, marked with an arrow).

Osmiophilic lipid droplets (the procutin according to BOLLIGER 1959) can be found sometimes (*fig. 7a*, arrow, and *fig. 7b*, arrow a) in the wall. They also occur in invaginations of the plasmalemma, which is an indication of pinocytotic activity (*fig. 7b*, arrow b, and *fig. 9d*, arrow a). Vesicles of the same osmiophilia and size were also observed within the epidermal cells, situated in multivesicular bodies (*fig. 7c*, arrow b).

A very strong cutinization is seen below a damaged cuticle *(fig. 11a)*. Not

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Fig. 6. Cutinization of the basal part of a hair. a. Before anthesis (stage (a)). Fix. glut. ald. + $KMnO_4$. Stained with lead citrate.
b. At anthesis (stage (b)). Fix. glut. ald. + OsO_4.

Fig. 7. Osmiophilic lipid droplets in the cellulose wall (arrow a)
a. Stage (b). Fix. glut. ald. + OsO_4.
b. Stage (c_2). Fix. glut. ald. + OsO_4. Stained with lead citrate. Pinocytotic activity at arrow b.
c. As b. Multivesicular body with lipid droplets marked with the arrow b.

Fig. 8. Cuticle of a full-grown apple after extraction of the waxes. Notes the many holes that appear now, and the hexagonal structure marked with the arrows. Fix. glut. ald. + OsO_4. Stained with lead citrate.

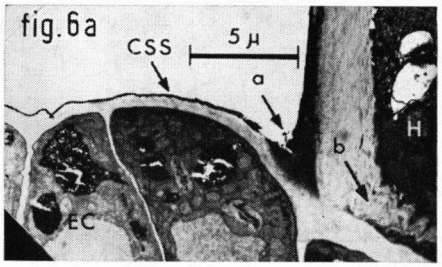
fig. 6a
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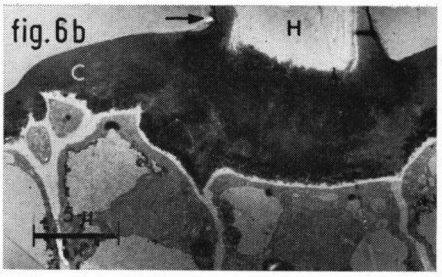
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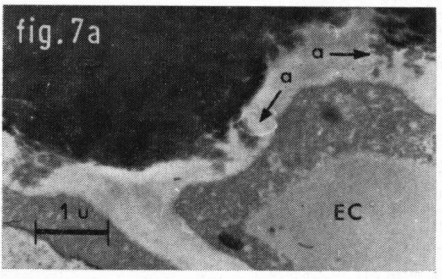
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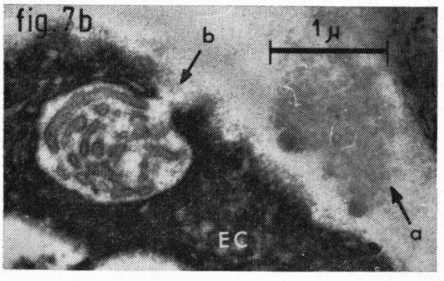
fig. 7b
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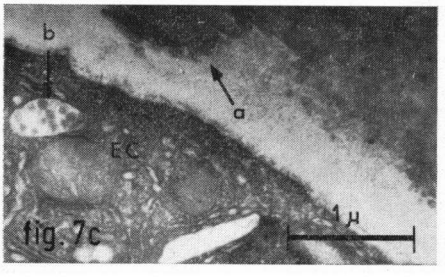
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fig. 7c
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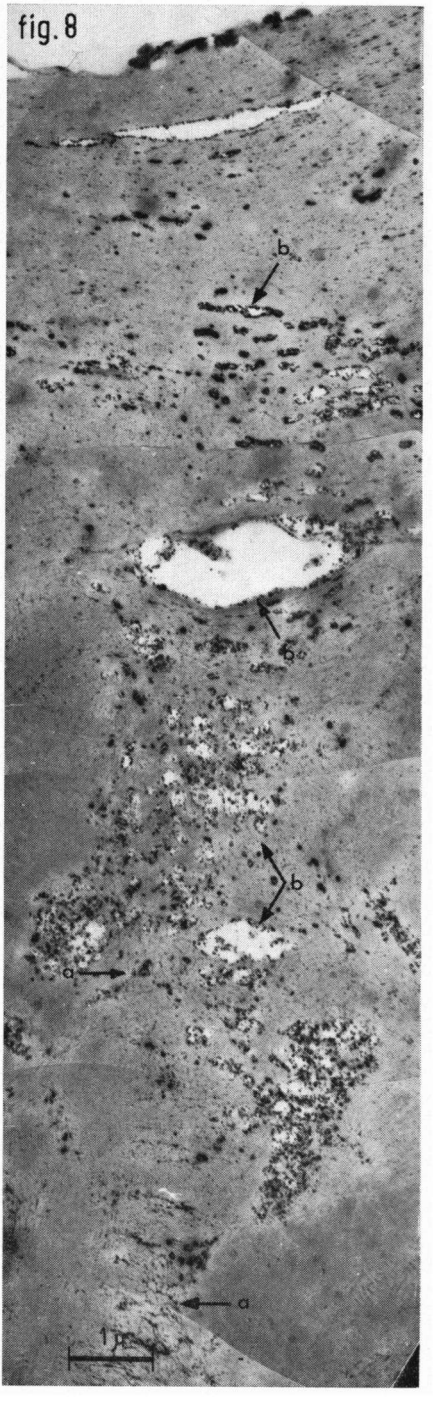
fig. 8
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b
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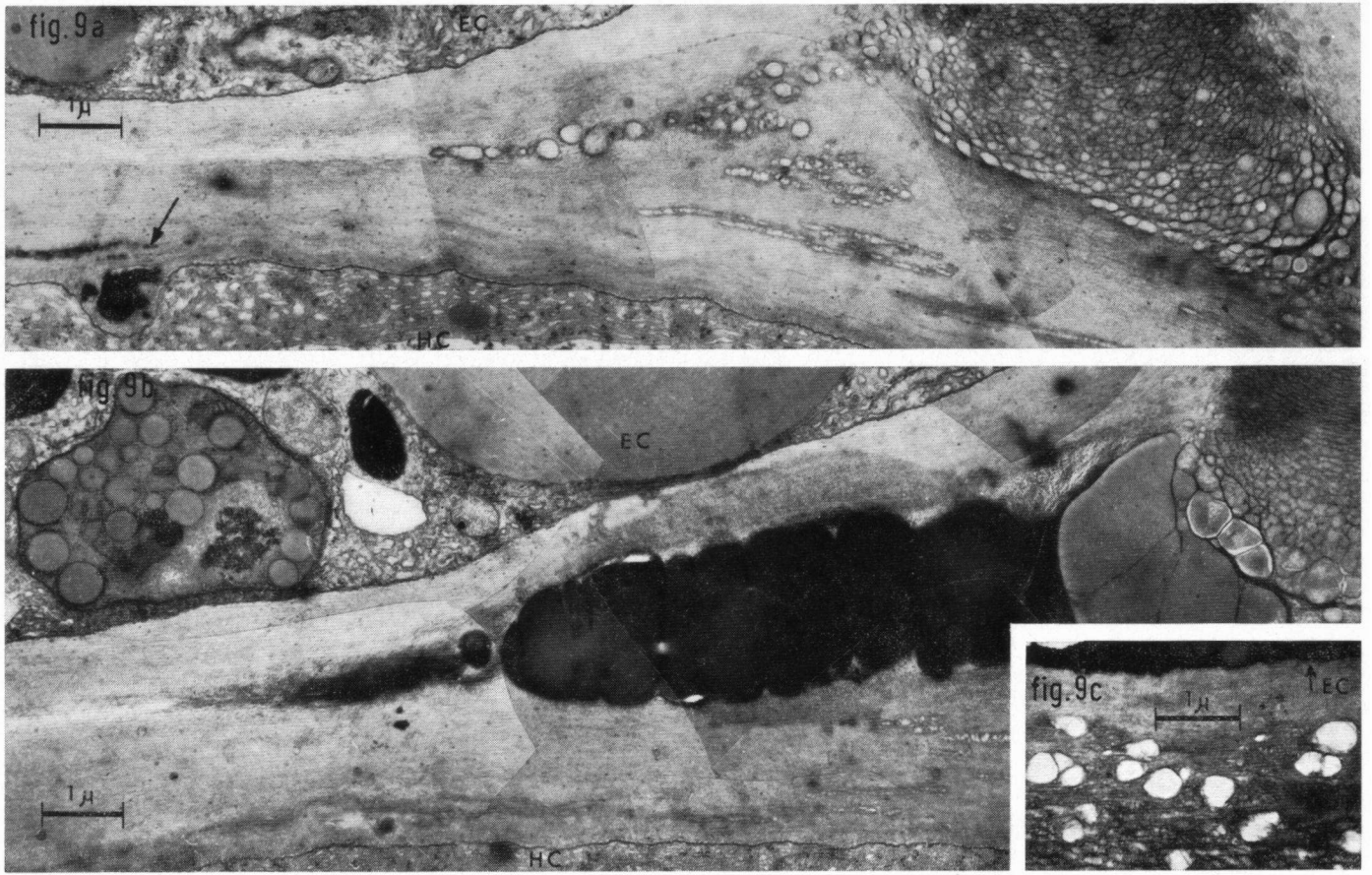

Fig. 9. Multivesicular elements in the epidermal cell walls of full-grown apples. Fix. glut. ald. + OsO_4. Stained with lead citrate.

a. elements with little electron density are seen at the end of a cutin wedge and in the inner periclinal cell wall. An invagination of the plasmalemma of the hypodermal cell is seen, filled with elements of high electron density: arrow.

b. Elements of low and high electron density in the inner epidermal cell wall.

c. Elements in the inner epidermal cell wall that look empty.

only osmiophilic lipid droplets emerge from the cell but also the above-mentioned multivesicular elements, the parts of which look empty *(fig. 11b)*.

WRISCHER (1965) has found that in a cell during nekrobiosis the cytoplasm becomes first electron dense, and has afterwards little electronic contrast, the nucleus has many electron dense granules, the mitochondria are much larger and have many forms, the plastids show lipophanerosis, there is an increase in the amount of endoplasmic reticulum, and many vacuoles appear. In our object epidermal cells below a damaged cuticle *(fig. 11a)*, and cells that are surrounded with cutinous material – particularly in the full-grown stage *(fig. 10)* – show signs of degeneration. Sometimes we found an increase of endoplasmic reticulum, many small vacuoles, and electron dense cytoplasm *(fig. 11a)*. The ground substance of the cytoplasm becomes homogeneously osmiophilic, and cell organelles can hardly be found *(fig. 10)*. Such cells still remain surrounded, however, with a non-osmiophilic cellulose wall.

4. DISCUSSION

The inner part of the cuticle consists of osmiophilic lipid droplets which are made up from unsaturated fatty acids. More outwards in the cuticle the fibrillar structure occurs after staining with lead citrate, and in later stages also in the place of the above mentioned lipid droplets. The electron dense fibrils form a honeycomb or almost hexagonal structure, the diameter of which corresponds to the lipid droplets that are situated in the innermost part. The information so far available from the morphological results on the formation of the cuticular structure is schematically summarized in *fig. 12*. The structure of the cuticle starts with a continuous approach of the droplets, which generally merge and appear to be flattened at the edges. The boundaries become clearly visible after lead staining, when the osmiophilia is decreased. The unsaturated fatty acids are thus possibly oxidized.

It is most likely that the carboxyl groups of the fatty acids, which are the main components of cutin, are the free sites for attachment of the lead stain. It is also possible, that pectin- and hemicellulose-molecules or phenolic compounds occur between the lipid droplets, and that these molecules give rise to this structure.

The fibrillar structure seldom appears in the older stages of the apple cuticle. The structure, however, becomes visible after extraction of the wax. Apart from the holes, the non-osmiophilic wax molecules evidently appear also between the lipid droplets. O'BRIEN (1967) has found in coleoptile cells the same type of fibrillar structure as we have done in the cuticle of the apple fruit.

The cellulose wall, that always surrounds the epidermal cell, does not show any pronounced lamellation, or other distinct structure.

The lipid droplets, which also occur in the basal part of the cuticle, are expelled from the cell by pinocytotic acticity. In later stages, we see how the cells begin to degenerate, before they increase the production of cutinous material and the multivesicular elements. The relation between the multivesicular elements

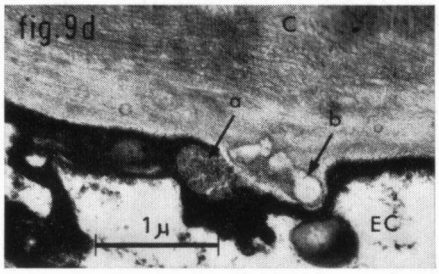
fig.9d
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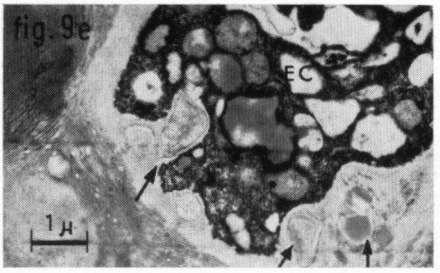
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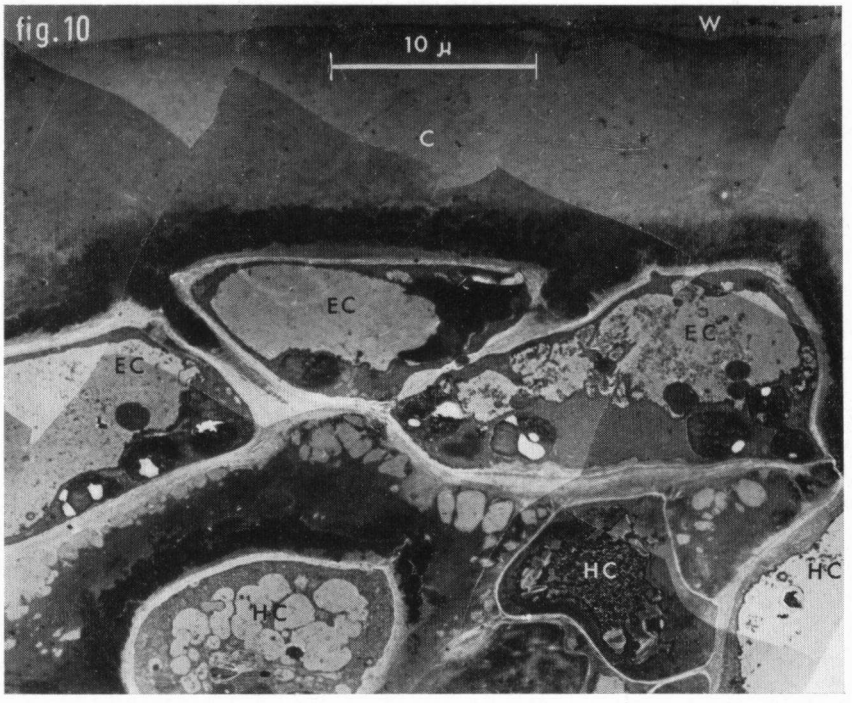
fig.10
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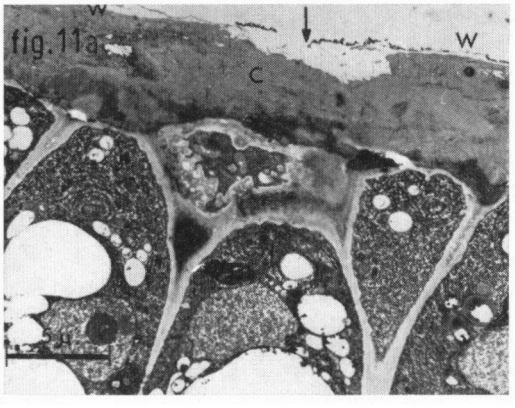
fig.11a
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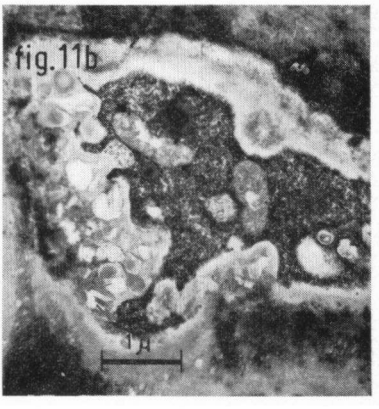
fig.11b
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Fig. 9d. Invagination of the plasmalemma of the epidermal cell, filled with lipid droplets (arrow a) and with elements that look empty (arrow b).
e. Degenerating epidermal cell with elements of low electron density (arrows)

Fig. 10. Cuticular epithelium in full-grown apple (stage (d)). The "second cuticle" that is formed beneath the epidermal cells contains many elements that look empty. Some cells are homogeneously osmiophilic and no organelles can be found in these cells. Fix. glut. ald. + OsO_4.

Fig. 11a. Epidermal cells below a damaged cuticle (arrow), stage (c_1). The cells show signs typical for degeneration. Fix. glut. ald. + OsO_4. Stained with lead citrate.

Fig. 11b. Magnification of *fig. 11a*. The degenerating epidermal cell forms multivesicular elements.

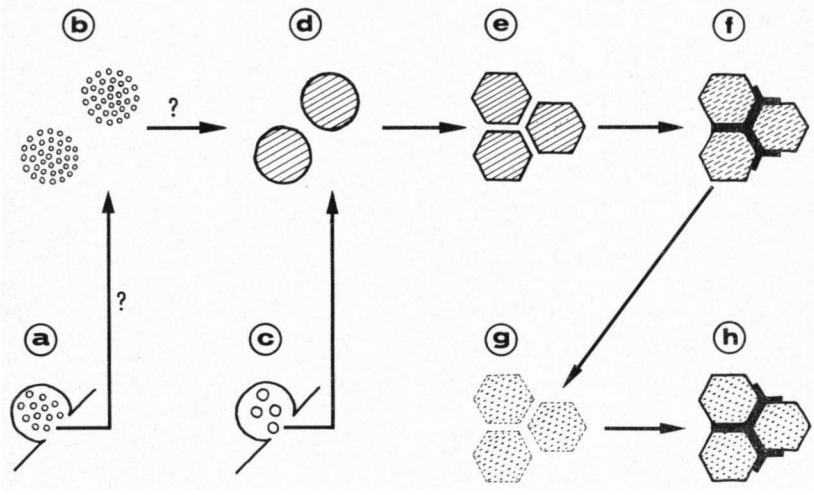

Fig. 12. Formation of the cuticular structure schematically summarized.
a. Pinocytosis: small osmiophilic droplets in an invagination of the plasmalemma. Possibly in very young stages.
b. Small osmiophilic droplets arranging into larger droplets in the outer cell wall.
c. Pinocytosis: these larger osmiophilic droplets in an invagination of the plasmalemma.
d. Magnification of the droplets which are found in the outer cell wall.
e. Osmiophilic droplets flattening at the edges.
f. Osmiophilia of the droplets decreased: the boundaries become visible after lead-staining.
g. In older stages the fibrillar structure disappears.
h. After wax extraction the fibrillar structure becomes visible in older stages.

and the cutin substances is not clear as yet. However, the localization of both in the pictures suggests some connection.

During growth the cutin substances are increasing between the epidermal cells, leading to the formation of the cutin wedges. This phenomenon, however, depends on the size of the apple and the condition of the cuticle. Therefore, it is possible that the differences in cutin deposits in the different apple varieties, as observed by TETLEY (1930), are due to these causes. She concluded that there is a correlation between cutin deposit and russeting: russeting occurs often in that variety, that has a great cutin deposit.

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KEY TO LABELING

C = CUTICLE
CSS = CUTICLE SENSU STRICTO
EC = EPIDERMAL CELL
HC = HYPODERMAL CELL
H = HAIR
W = WAX LAYER

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