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Mechanisms for the infection of potato tubers by the
soft rot organism Erwinia carotovora var. atroseptica
(van Hall) Holland, and associated defence mechanisms.

A thesis submitted for the degree of
Doctor of philosophy
in the
faculty of Science
by
R.T.V.Fox BSc..

Department of Botany
University of Southampton

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ABSTRACT

FACULTY OF SCIENCE

BOTANY

Doctor of Philosophy

MECHANISMS FOR THE INFECTION OF POTATO TUBERS BY THE
SOFT ROT ORGANISM ERWINIA CAROTOVORA VAR.

ATROSEPTICA (VAN HALL) HOLLAND, AND ASSOCIATED DEFENCE
MECHANISMS.

By Roland Thomas Victor Fox.

The increasing losses of potato tubers in storage due to soft rot spoilage have emphasised the need for detailed studies of the entry and spread of the pathogen and the action of the defence mechanisms of the potato at an ultrastructural level.

The studies have shown that the tuber may be infected through fresh wounds or lenticels under conditions of high relative humidity, providing sufficient inoculum is present and, in the case of lenticels, the conditions before inoculation have been suitable.

The effects of the environment on the physical barriers and consequently bacterial spread are complex. Observation of the formation of suberin and melanin at the infection interface has shown that the former may

hinder bacterial spread: the role of the latter is more obscure.

Light microscopy has shown that the pattern of initial colonisation is highly temperature dependant. Colonisation through lenticels is quicker at first than through fresh wounds probably due to the differences in anatomy, although quantitatively large amounts of inoculum are involved in the latter under most conditions.

Once inside the tuber the bacterium spreads through the storage parenchyma as zoned colonies aligned in one direction during the early stages, but after the host cells have been macerated the cells of the pathogen become uniformly distributed between the potato cells.

Vascular spread is less common, and the bacteria are enclosed in the xylem and phloem by the suberised, closely packed cells of the vascular parenchyma, although some pectolysis and subsequent melanin formation occurs outside the bundles for distances of up to 500 μ m.

Tissue degradation involves the destruction of the cytoplasm, as well as the cell wall. Characteristically membrane rupture is associated with the enlargement of microbodies. Although cells containing calcium oxalate monohydrate are colonised intracellularly, the crystals do not appear to be utilised.

The significance of these findings in relation to the

disease as a whole are discussed, and possible practical implications for the grower and user of potatoes are considered.

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Introduction.

Blackleg and soft rot of potato tubers, caused by Erwinia carotovora var. atroseptica (van Hall) Holland, are important diseases of growing and stored potatoes respectively wherever this crop is grown, and have generated many problems of both practical and academic interest.

The history of the taxonomy and nomenclature of this organism is rather confused, but 'soft rot' is now recognised as being caused almost exclusively by this variety of the widespread plant pathogen. However it appears that the identification tests used at present are equivocal and there is no adequate pathogenicity test yet devised (Graham, 1964; Moustafa, 1966; Logan, 1965).

Very little work has been concerned with the ecology of E.c. var. atroseptica and antagonistic organisms such as Pseudomonas spp. (Waksman & Woodruff, 1940). It seems unlikely that E.c. var. atroseptica overwinters in the soil (Logan, 1968), and the tuber appears to be the main means by which the disease survives from one season to the next. Infected mother tubers may breakdown to release inoculum which causes lenticel infection after wet weather (Pérombelon, 1969). The mechanisms by which lenticel and fresh wound infection occur has been studied in this investigation, with particular reference to the environmental conditions necessary.

It has been suggested that once the bacteria enter the potato tuber their spread may be checked either by a suberin barrier (Rudd Jones & Dowson, 1950), or an oxidised polyphenol barrier (Lovrekovich, Lovrekovich & Stahmann, 1967). The spatial relationships of these barriers were investigated at a cellular level using an electron microscope during this study, in order to obtain evidence of their possible mode of action.

The practical problems involved in storing potatoes in bulk are often made more acute due to incomplete knowledge of the history of the crop, its state of infection if this is not visible to the naked eye, and the exact environmental conditions at different positions in the store. This often means that the exact conditions before and after inoculation are not known with any degree of accuracy. Because of the small scale of this investigation, the exact conditions before and after inoculation and subsequent infection are known, as they were controlled as was infection itself.

The importance of producing disease free stocks is emphasised by the fact that infection appears to be spread by infected tubers, and that the control or monitoring of the storage environment appears most costly and difficult.

The role of the various organisms involved in the

secondary colonisation of E.c. var. atroseptica rots is not clear. Whether these or Erwinia are latent, and whether other organisms play a significant part in soft rotting, is not known although Hollis (1951) has isolated Bacillus megatherium from storage parenchyma and vascular tissue of healthy tubers. Bacillus spp. and their behaviour in the potato tissue after soft rotting are discussed in this investigation. The mode of spread of E.c. var. atroseptica through the tissue has not hitherto ~~here~~ been definitely known; although Smith (1905) suggested that intercellular spread was likely he was faced with the difficulties of section thickness and resolution inseparable from light microscopy. Vascular spread has been an enigma which has been recorded but not studied. Both forms of spread are examined and discussed in the present investigation.

The enzymology of the infection has generated much research (Wood, 1967). The effect of bacterial action on the cytoplasm has received much less attention than the enzymes of cell wall degradation, which is unfortunate since a great deal of economic loss is due to the decomposition of the cytoplasm causing tainting of uninfected tubers. The process of cytoplasmic damage is described at the ultrastructural level in this investigation.

The nature of E.c. var. atroseptica as a pathogenic

organism, the channels for infection that exist in the potato, and the conditions under which they can be utilised have been investigated by experimental methods together with concurrent electron microscope studies. In the same way the initial colonisation of the potato tuber and the spread of infection leading to tissue degradation and incitement of the host defence mechanisms were investigated by these complementary methods.

The ultrastructure of potato soft rot has not received adequate study, perhaps owing to the difficulties involved in preserving even uninfected potato tissue. The preservation difficulties are due to the large size of the storage parenchyma cells, the presence of starch which is not compatible with glutaraldehyde fixation, and the large vacuoles which make isotonic fixation essential. The problems involved in fixing soft rotted tissue are enhanced by the special requirements of the bacteria (Kellenberger, Ryter & Séchaud, 1958), as well as the slimey, watery nature of the soft rotted tissue which makes dehydration difficult. The lack of study of this aspect of soft rotted tissue was unfortunate as electron microscopy can provide evidence concerning the nature of several features of the disease syndrome such as cytoplasmic damage, vascular spread, the nature of tissue breakdown, host defence mechanisms, and secondary colonisation not obtainable by other methods.

Details of these processes at the ultrastructural level together with the experimental context in which they occur form a large part of this investigation.

Part 1. The nature of the pathogen.

Introduction.

Erwinia carotovora var. atroseptica has been intensively studied from many viewpoints which differ from that taken in this investigation, including taxonomic, ecological, biochemical, and physiological aspects, all of which have a bearing on the mechanisms for infection and associated defence barriers.

1. Taxonomy.

The rather confused taxonomic history of Erwinia carotovora has been discussed elsewhere at length (Leach, 1930b; Malcomson, 1959; Rudd Jones, 1950; Graham, 1964; Hellmers & Dowson, 1953; Logan 1965). For the present investigation the name Erwinia carotovora var. atroseptica (van Hall) Holland has been suggested for the blackleg organism, following Graham (1964).

Inoculation of potato slices, the sucrose test (Moustafa 1966), medium and serological tests have shown that among Erwinia carotovora isolates from soft rotted potatoes var. atroseptica is most common. Pérombelon (1967) using 253 isolates found that 83% were var. atroseptica, 14% var. carotovora and 3% var. aroideae.

2. Ecology.

Neither Graham (1958) nor Logan (1968) accept that inoculum of Erwinia carotovora over winters in British soils, but Leach (1930a) has suggested that it does so in Minnesota and can survive desiccation.

The inoculum which initiates blackleg outbreaks in the field may be due to the breakdown of an infected mother seed tuber (Perombelon 1969). However, from the evidence of those responsible for the introduction of clean seed stocks free from blackleg, (Wight, 1969) it appears that ^{escape from} ~~resistance~~ to infection is considerably reduced when planting takes place in areas with a history of the disease, but in 'virgin' soil almost no infection occurs. It is of course impossible to prove that over wintering in the soil does not play a part in the sources of inoculum initiating blackleg and soft rot. It is certain that the storage of seed and ware potatoes under inappropriate conditions must play a major part in the incidence and spread of the disease (Paton 1969).

At harvest time inoculum already present in the field may be transmitted to healthy tubers by farm machinery, and Lelliott (1969) has suggested that the sterilisation of farm machinery may reduce this method of spread ; such procedures are carried out in the U.S.A. against Corynebacterium sepedonicum. The part played by insects,

nematodes, slugs, and fungi in producing wounds and as vectors for Erwinia has been discussed by many workers (RuddJones,1948; Leach,1930b;Butler & Jones,1949).

Growth cracks due to the metabolic reaction of the tuber to high levels of nitrogen fertilizers (Harper & Boyd 1963) and mechanical damage by rough handling after harvesting present the bacterium with a habitat to colonise.

3. The etiology of the disease.

This aspect has been covered elsewhere. (Smith,1905; Stapp,1961;Dowson,1957;Butler & Jones 1949.)

4.Biochemistry.

Because of its pectolytic enzymes Erwinia has attracted workers in this field; the purification of these enzymes and the effect on plant tissues and substrates have been investigated from biochemical and ultrastructural aspects. (Wood,1955; Wood,1967;Murant & Wood 1957;Starr & Moran,1962; Calonge,Byrde & Fielding,1968).

The interreaction between the pectolytic enzymes and the polyphenols of the host potato has been studied by Lovrekovich,Lovrekovich & Stahmann,1967,using purified enzyme preparations,as well as the pathogen; they suggest that a 'black infection barrier' results in both cases.

The exact pectolytic properties of the enzymes of the pathogen are yet to be histochemically tested on potato tissue at an ultrastructural level.

5. Morphology.

Little attention has been paid to the morphology of Erwinia ; the flagellation has been described under the electron microscope by Speights, Hughes, Horne & Halliwell (1967) who platinum/palladium shadowed an air dried isolate from a tomato stem rot attributed to E. carotovora var. carotovora, which measured 1.0 - 1.8 μm x 0.7 - 0.8 μm , with 8 - 10 flagella. This number of flagella does not fall within the light microscope results for this species quoted elsewhere (Dowson, 1957; Breed et al., 1957).

Materials & Methods.

In order to examine the external features and flagella of Erwinia carotovora var. atroseptica , an 18 h culture from nutrient broth incubated at 25°C in a shaker, was air dried on carbon films on copper 200 grids, ~~and~~ and these preparations were shadowed with platinum/palladium.

The internal structure of the bacteria was examined using the orthodox Kellenberger method for bacteria, (Kellenberger, Ryter and Séchaud, 1958); agar - araldite embedded 18 h, shake culture (nutrient broth maintained at 25°C) bacteria were used.

Erwinia cells present in soft rotted tissue were prepared for examination under the electron microscope by a specially modified method described below. This was found by first attempting to use glutaraldehyde fixation

but this was rejected in favour of the modified schedule on two points; it distorted the bacterial cells completely and caused violent shrinkage in the starch grains of the host cells of the potato tuber.

Fixation and embedding schedule modified for soft rotted potato tissue.

Blocks of tissue with one dimension not more than 1 mm, were cut from the infected tuber.

- 1) Fix overnight in 1 ml of 1% osmic acid in Kellenberger buffer plus 5% sucrose.
- 2) Wash twice in Kellenberger buffer.
- 3) Dehydrate in ethanol, ending in 1 h in absolute alcohol with 1 g silica gel added.
- 4) Embed in the orthodox way (Kay et al. 1965) but allow 3 days in 50:50 1:2 epoxypropane/araldite mixture.
- 5) Polymerise at 50°C in a disposable 100 ml beaker with large surface area for 3 - 5 days. Subdivide before ultratoming, or cut for light microscope on sledge microtome.

By using this schedule for fixation and embedding, the particular disadvantages of soft rotted potato tissue can be overcome.

Results.

i. Shadowed Preparations.

In the strain used in this investigation only four flagella were seen and these were peritrichous.

The same result was obtained by light microscopy using Bailey's method.

Plate 1.1 shows a Pt/Pd shadowed carbon replica of an 18 h culture, some cells are more rodlike (plate 1.2) and dividing cells are also present (plate 1.3). Negative staining with phosphotungstic acid confirmed the general picture. Fimbriae were seen on some cells (plate 1.4), these were not seen on the air dried preparations, which were shadowed. All the shadowed bacteria show a characteristic rough surface.

ii. Sections.

Kellenberger fixed, double embedded bacteria of the same strain and cultured in the same way, also showed a characteristic 'wrinkled' nature to the cell wall, (plate 1.5) which suggests that as well as being diagnostic this character is probably not an artifact. Similar corrugations but there even more pronounced were seen in Bordetella preparations by Richter & Kress (1967), but otherwise such a cell wall seems uncommon.

The shape and nature of the Erwinia cell is typically gram negative in the way the rods are slightly pointed, indicating that cell division is by 'pinching off' rather than by cross wall formation. The structure of the cell wall also corresponds to the gram negative type, with a thickness of only about 7.5 nm compared with 35 nm for

some bacteria described by Glauert (1962) in her review article.

Neither glycogen nor intracytoplasmic membranes were seen; the cytoplasm is evenly granular with a plasma membrane of about 7.5 nm, and the chromosome is compact and well filled. These diagnostic characters prove very useful when secondary colonisation is considered (part 7), and would possibly be of taxonomic value if sufficient facts were known about the other members of the genus. However, as Stapp (1961) points out, individual variation in cell size is possible and may be controlled to a large extent by environmental conditions. Plate 1.6 shows cells grown at 5°C; it can be seen that this variation in size does not affect the diagnostic characters, such as the cell wall.

An advantage of ultrastructural morphology compared to serology for taxonomic purposes is that the former is recorded on photographic emulsion whereas the latter depends on individual interpretation. Plate 1.7 shows cells of Erwinia present in an intercellular space between cells which have been macerated; the background material becomes organised into filaments in older cultures, the cell contents are seen not to differ from those in the pure culture, fixed by more conventional methods.

The study of morphological differences between the secondary colonising bacteria present after soft rotting and Erwinia itself could give the ecology of soft rot of potato tubers a more precise basis than it enjoys at present. The fact that many different organisms combine to cause wild soft rots is emphasised by Lelliott (1969) who states that between 30 - 40% of the isolates taken from naturally occurring soft rots are not of the genus Erwinia.

This latter point also emphasises the ¹²view taken in this investigation that sterile artificial inoculations should be used throughout.



Plate 1.1 (x 15,000, electron micrograph)
Pt/Pd shadowed replica of an 18 h culture of Erwinia
carotovora var. atroseptica, showing the peritrichous
flagella.



Plate 1.2 (x 30,000, electron micrograph)
Pt/Pd shadowed replica from the same culture as 1.1,
but more rodlike, indicating the range of cell shape
present.

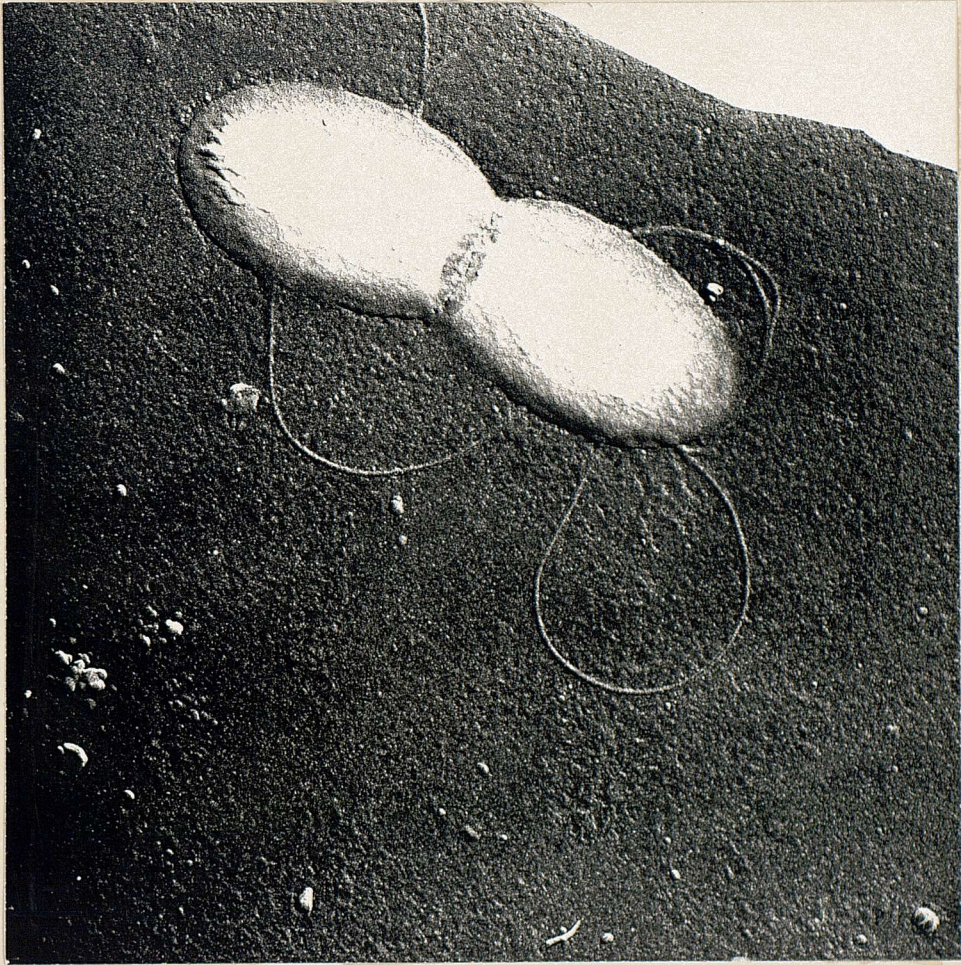


Plate 1.3

(x 30,000, electron micrograph)

Erwinia cell showing typical division by 'pinching in'.

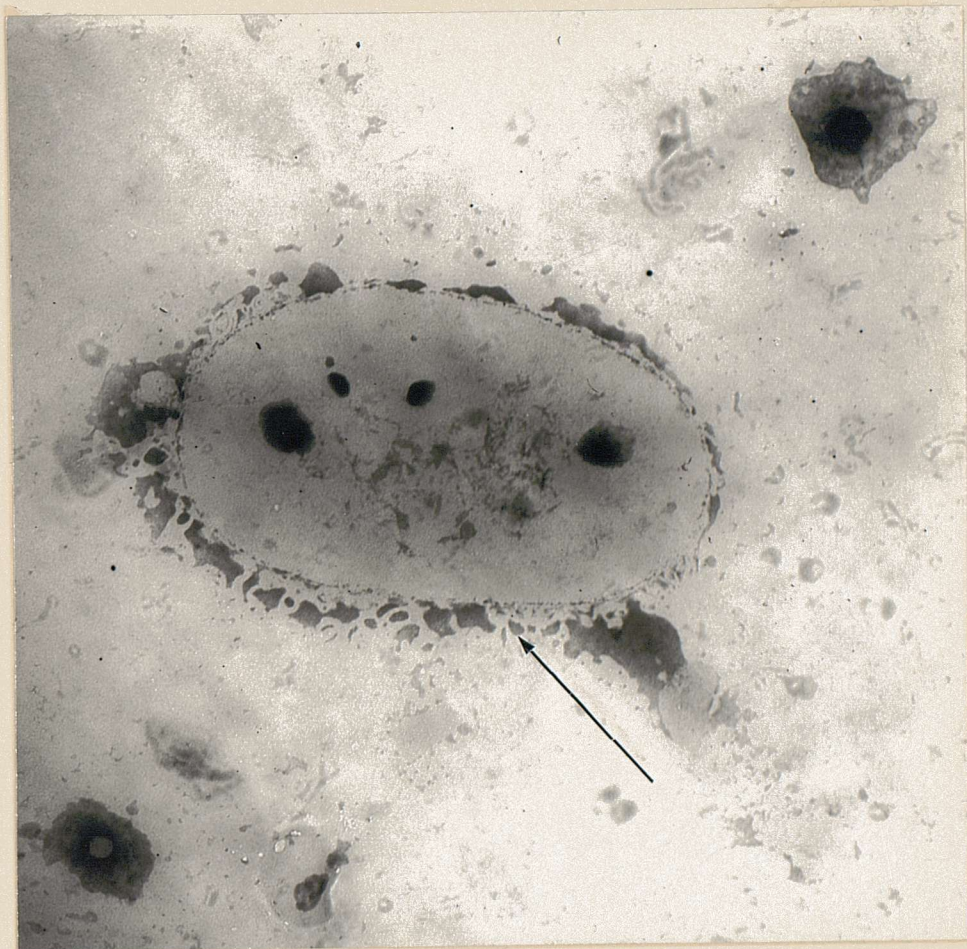


Plate 1.4 (x 30,000, electron micrograph)
Negatively stained Erwinia cell from 18 h culture,
showing fimbriae.

(phosphotungstic acid.)



Plate 1.5

(x 60,000, electron micrograph)

Section through a cell from an 18 h culture of Erwinia
(25°C), showing the 'wrinkled' nature of the cell wall.

(osmic acid, uranyl acetate.)

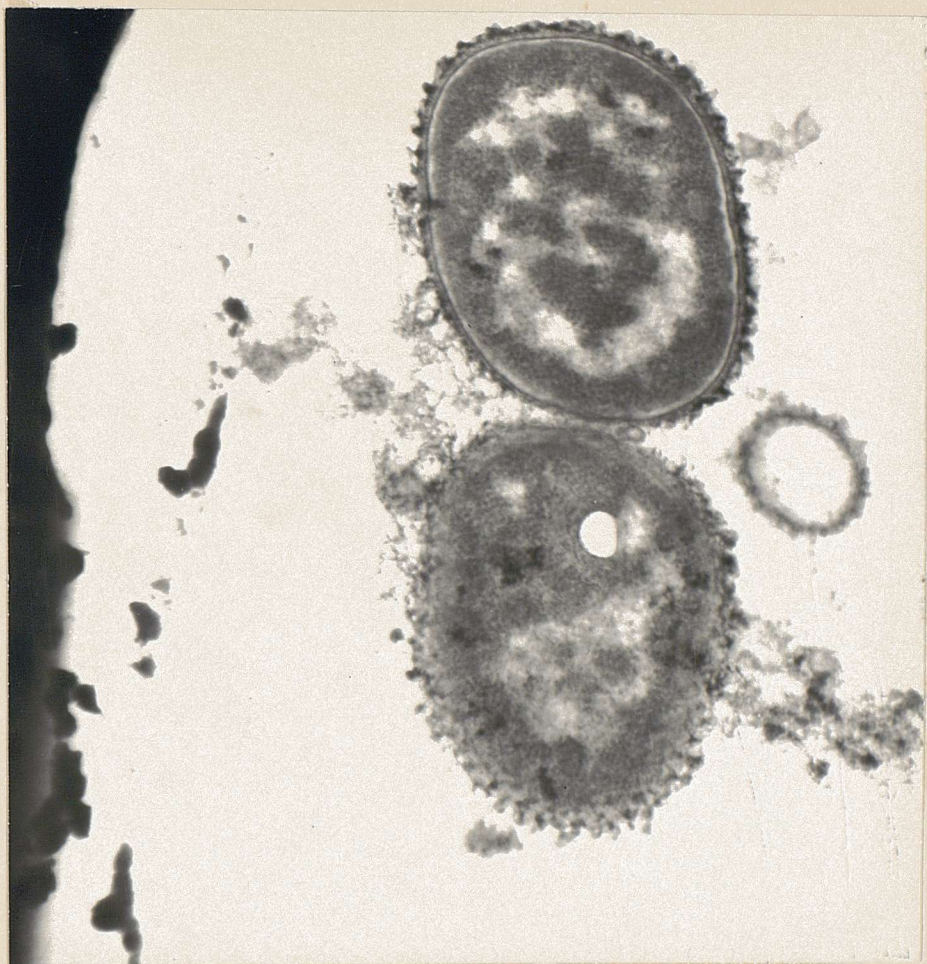


Plate 1.6

(x 60,000, electron micrograph)

Erwinia cell from infection kept at 5°C, note the difference in shape to cells kept at 25°C.

(osmic acid, lead citrate.)

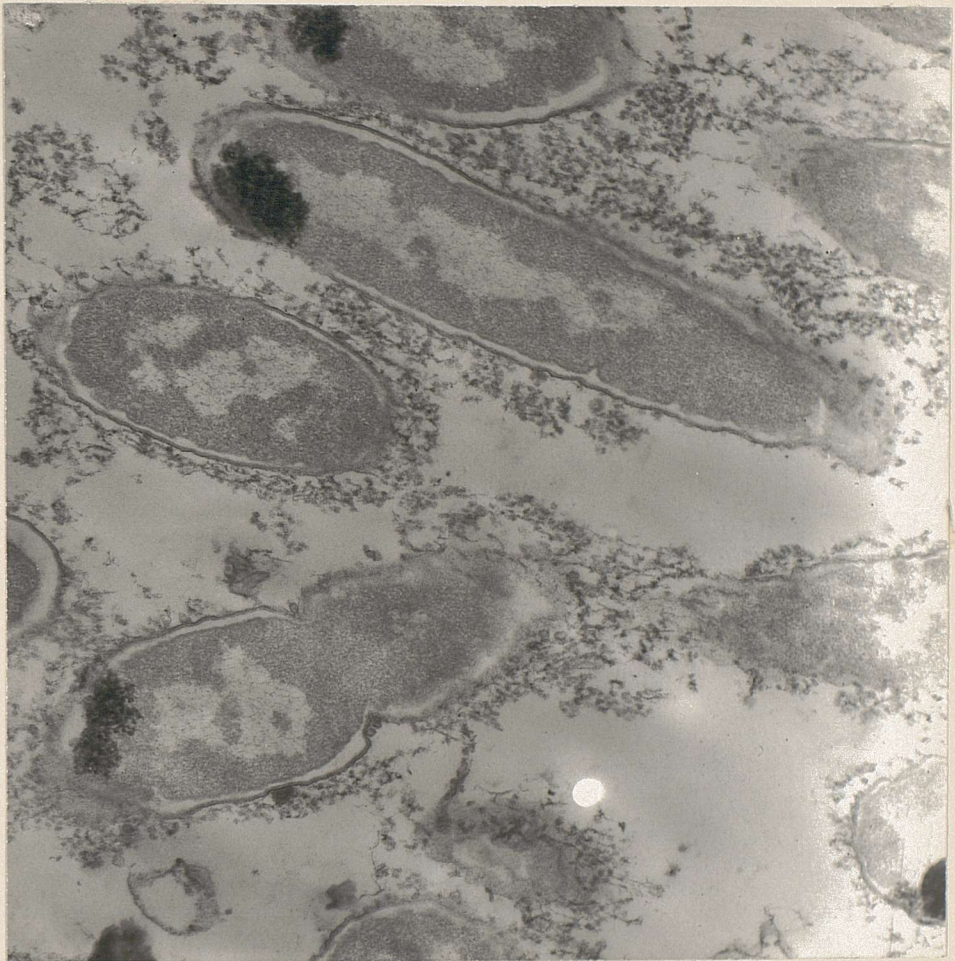


Plate 1.7 (x 30,000, electron micrograph)
Section of Erwinia cells from a colony in soft rotted
tissue kept at 25°C.

(osmic acid, lead citrate)

PART 2: The channels of infection.

Part 2. The channels of infection.

Introduction.

The structure of the potato tuber.

Since Artschwager (1924), described the basic structure of the potato in detail, several other workers have described specialised areas in greater depth (Hayward, 1938; Plaisted, 1957; Nagy & Boyd, 1965; Reeve, 1968), often in connection with pathological studies. As the anatomy of the tuber is basic to considerations of the channels of infection, a review of the former follows.

The enlargement of the potato tuber from a stolon tip is by cell division of the vascular parenchyma which results in the eventual distortion of the vascular ring of bundles and gives rise to some storage parenchyma between the xylem and phloem elements. The vascular ring is therefore rather loose in construction, phloem often being found separated from the xylem by parenchyma.

The cuticularised epidermis of the stolon is replaced in the tuber by a suberised thin walled periderm. The surface of the stolon is covered with stomata which become differentiated into lenticels in the tuber. Burton (1969) suggests that the number of lenticels remains constant throughout the life of the tuber, but in this investigation

it was found that lenticels could appear near already established lenticels under conditions of very high humidity if the latter were blocked with secondary periderm.

The storage parenchyma outside the vascular ring is less extensive than that inside and consists of cells often more than 100 μ m diameter with starch grains packed in a cluster to one side of the nucleus which is positioned in the centre of an extensive ~~tonoplast~~^{central vacuole}; the intercellular spaces were found to be not extensive nor suberised. Burton (1950) has suggested that this area has an oxygen concentration about only 3% less than ambient at 10°C. Sclerified cells may be found in this zone in older tubers.

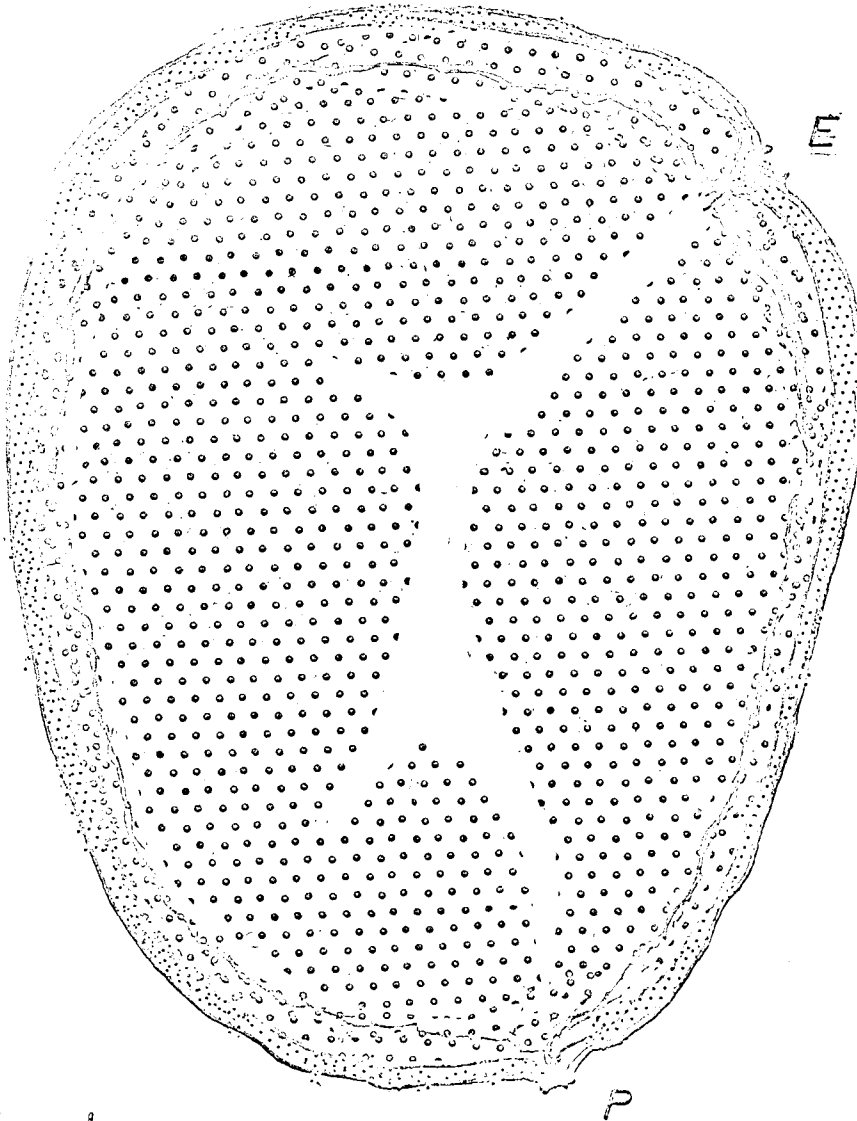
The apical and lateral buds or 'eyes' are covered with cuticle and are well supplied with vascular bundles; rays from the pith also extend to this region. The pith, an area with rather more cytoplasm and less starch than the storage parenchyma, remains intact in the tuber centre. The point of attachment of the tuber to the stolon at the 'heel end' is marked by a patch of secondary periderm.

Varietal differences have been found in the thickness of the periderm, and in the nature of the eye region, and although the former may be correlated with resistance to Oospora, the latter is not (Nagy & Boyd, 1965).







Fig.21 Anatomy of potato tuber.[x 2]

'rose' end

[L.S. King Edward]



'heel' end

Key.  storage parenchyma  cortex  vascular tissues.
 pith  periderm, lenticels/phellogen,  bud.
P, point of attachment to stolon, E, eye.

Channels of infection.

From the survey above it can be seen that the possible sites for bacterial entry include channels with quite different properties. Also the diversity of the the tissue type needs to be taken into account in any consideration of spread within the tuber.

The possible channels of entry open to free swimming bacteria which lack mechanical means of penetration fall into four major groups.

1. Periderm. a. Primary periderm. b. Secondary, wound periderm.
2. Buds or 'eyes'.
3. Wounds where the periderm has been removed mechanically, and not yet replaced.
4. Lenticels of all types.

The potentialities of these as channels of infection were considered separately, but as the techniques employed were similar in each case these are described together.

Materials and methods.

Hand razor sections were used in some investigations but were discarded where possible in favour of Ester wax(1947) sections (Steedman,196⁰), cut on a Cambridge rocking microtome. Other methods tried include freezing microtome sections and sections of paraffin blocks made on a Cambridge

rocking microtome, but these were unsuitable due, inter alia, to their inability to hold the starch grains in their natural position ; they are forced through the tissue making histological observations to any high magnification difficult. The freezing microtome sections were also rendered unstable due to the large vacuole around the nucleus which made satisfactory homogeneous freezing difficult. However as Ester wax is a considerably harder medium than paraffin wax, sections taken from it have a well preserved cytoplasm and cell wall; sections cut at 12 μ m approach in quality 2 μ m araldite sections (taken from blocks yielding satisfactory 50 nm sections used for electron microscopy) viewed under phase contrast microscopy. Araldite 15 μ m sections were also cut but because of the lack of histochemical stains for this medium they were not extensively used.

For the study of gross anatomy , sections were stained with Heidenhain's haematoxylin and counter stained with Bismark brown (Venning,1954).

Microchemical tests were made as follows.

I. For light microscopy. A. Following Ester wax embedding.

- a. For pectin - Ruthenium red (Johansen,1940),
- Albersheim's method (Albersheim et al.,1960).
- b. For suberin - Osmic acid, Sudan III (Venning,1954),
- Ammonical gentian violet (Artschwager,1927).

- c. For melanin + Ferrous iron technique (Lillie,1957),
- Methanamine silver method (Gomori,1952).

B. Following hand sectioning.

- d. For polyphenolic compounds (especially chlorogenic
and caffeic acids) - Hoepfner-Vorsatz test(Reeve,1951;
1968b).
- e. For intact tonoplast - Sections 'fixed' in 4% KOH were
vital stained in Neutral Red, which gives an indication
whether the individual cell is 'dead' or 'alive'.
(Strugger,1935).

II. For the electron microscope. Prior to araldite embedding.

- a. For pectin - Albersheim's method (Q.v.).
- b. For melanin - Ferrous iron technique of Lillie on fresh
tissue which was then embedded in araldite.(Q.v.)
- c. For suberin - Osmic acid (Q.v.) 1% solution in Kellen-
berger's buffer. Similar technique used by
Bonnett (1968) to study endodermis.

Results of study of the potential channels of infection.

1. Periderm.

The periderm was shown not to be a major channel of
infection by a relatively simple test, i.e. soaking a
whole tuber in 2% aqueous Gentian Violet with a trace
of the wetting agent Teepol for 72 h. The stain only
penetrated the outer 1 - 2 cells of the periderm except

at the lenticels where the parenchyma was stained violet. Bacteria, being much larger than Gentian Violet molecules, would be expected to be unable to penetrate the periderm, in the absence of chemical attack.

Non-entry may be explained by the nature of the periderm which consists of ranks of interlocking cells with thin cell walls impregnated with ~~with~~ suberin and devoid of intercellular spaces. Under the electron microscope the intercellular connections and the cell wall suberisation can be seen in thin sections (plate 2.2) and the interlocking nature of cells in surface view in shadowed replicas (plate 2.3). The cells of the phellogen and phellem also are closely packed and exhibit a similar method of cell junction without intercellular spaces; some suberin may be present in this region (plate 2.1). Secondary periderm formed as a result of cambial activity, following the dedifferentiation of storage parenchyma appears to possess the same properties as the primary periderm, after the wound surface has healed completely. So the 'heel end', where the tuber was attached to the stolon, if properly healed should not present any differences from other secondary wound periderm, particularly as the evidence for the dedifferentiation of vascular parenchyma has been reported in potato tubers by Artschwager (1927), and was seen during this investigation, and reported elsewhere in other plants.

2. The region of the 'eyes'.

In the experiment described above, immersion in 2% aqueous Gentian Violet, did not result in any staining of the tissue underlying the intact cuticle of the epidermis in this region, the nature of which is shown in plate 2.4; plate 2.5 for comparison is a section through mature storage parenchyma. It can be seen that the tissue comprising the bud is smaller celled and closely packed compared to the specialised starch storage cells. It also reacted very strongly to the Hoepfner - Vorsatz test of Reeve (1951) which suggests high polyphenol content, in comparison the storage parenchyma did not give a recordable reaction.

3. Wound surfaces.

Introduction.

The causes of wounding are manifold, and it appears that many of the wounds are caused by organisms which also act as vectors for the disease (Stapp, 1961). However the channel of infection is formed, the criterion of major importance is the degree to which the healing has progressed before conditions are such as to start infection. A knowledge of the process by which the susceptible fresh wound dedifferentiates resulting in the formation of resistant periderm, of the time factor

involved, and of the conditions required for periderm formation, are necessary before the importance of the ~~fresh~~ wound as a channel of infection can be assessed.

The apparent contradictions in the literature concerning the processes involved in wound healing, especially suberisation, (Artschwager, 1927; Priestley & Woffenden, 1922; Bloch, 1941; Rudd Jones, 1948; Lapwood, 1957), made it desirable to re-investigate the problem.

A series of experiments was carried out to investigate the effect of suberisation and secondary periderm formation at the light and electron microscope levels.

An ultrastructural survey of wound healing.

From the basic light microscopy of a healed wound (plate 2.6), the characteristic division into layers can be seen. First the cut cells, below them^{are} the suberised cells, then the dedifferentiated cells which have formed the secondary periderm, and the **line** of cells devoid of starch next to the storage parenchyma as described by Olufsen (1903). These layers were examined under the electron microscope using araldite sections of tissue fixed in 1% osmic acid in Kellenberger buffer with 5% sucrose by the modified schedule used for soft rotted tissue (Q.v.), and poststained with Reynolds lead citrate (Reynolds 1963).

The zone of cut cells (plate 2.7), consists of cells

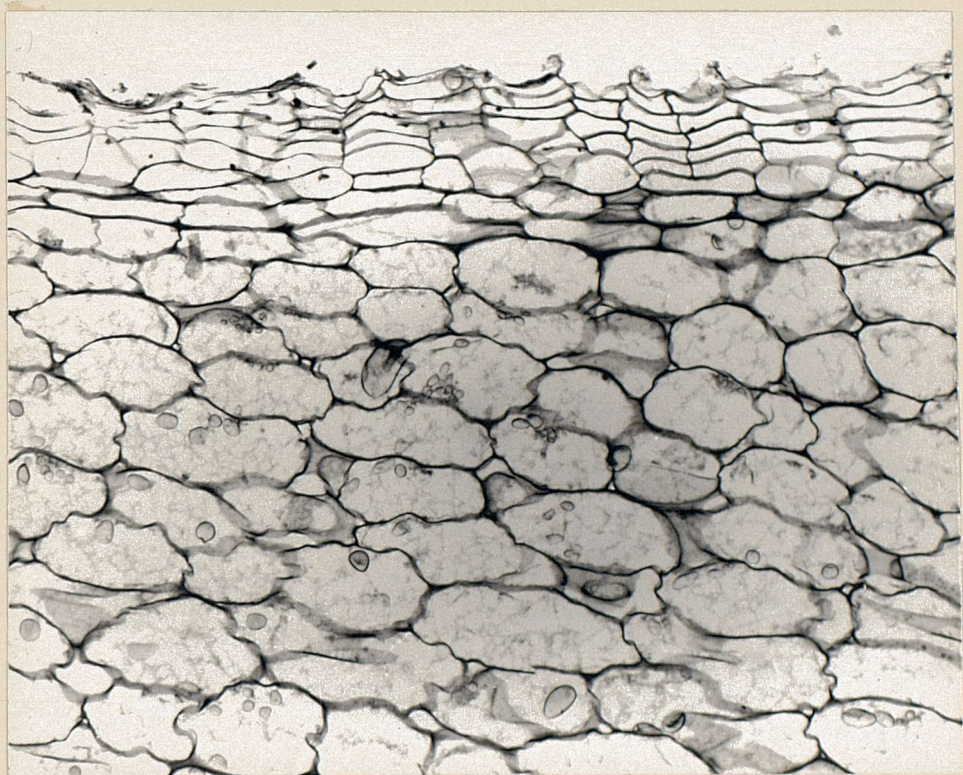


Plate 2.1

(x 120, light micrograph)

Section through tuber tissue showing ranks of primary periderm cells, which lack intercellular spaces compared with underlying cortex and storage parenchyma.

(carbol fuchsin, fast green.)



Plate 2.2

(x 10,000, electron micrograph)

Section through primary periderm, showing suberin
in the intercellular spaces and between the cells.

(osmic acid, lead citrate)



Plate 2.3

(x 6,000, electron micrograph)

Pt/Pd shadowed replica of the surface of the periderm
showing the close junction between the cells.



Plate 2.4

(x 252, light micrograph)

The bud region, the 'eye', showing the relatively small size of the cells of this region, compared with the storage parenchyma.

(Heidenhain's haematoxylin.)

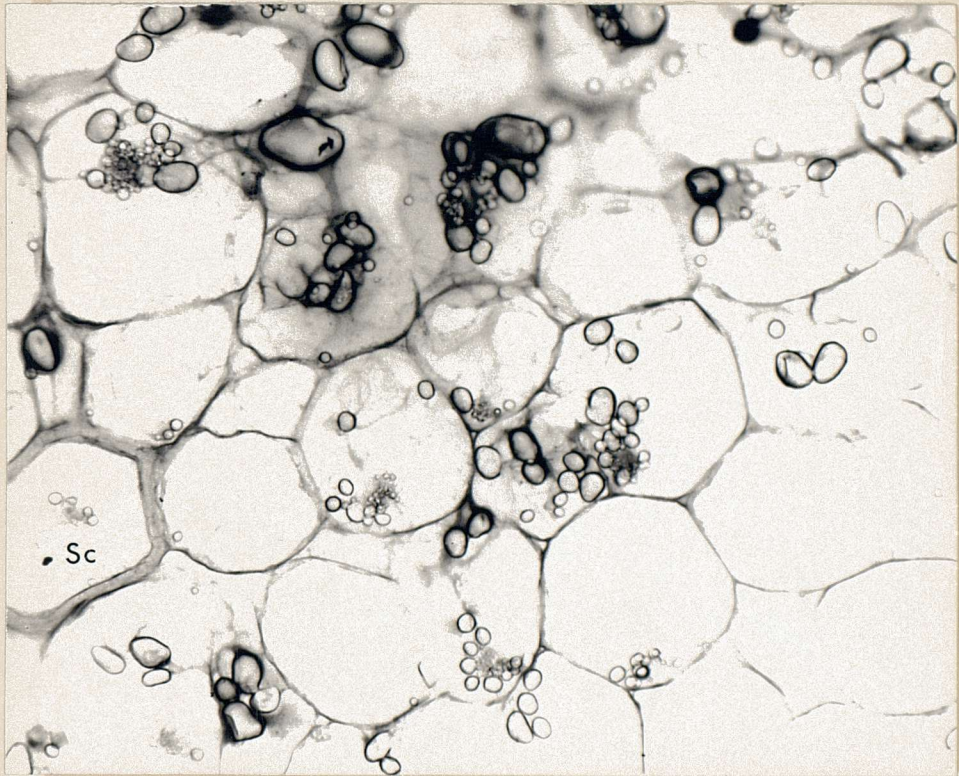


Plate 2.5

(x 252, light micrograph)

Mature storage parenchyma, showing comparatively large size of the cells, clusters of starch grains round the nucleus, the presence of intercellular spaces and the occasional sclerid.^(sc)

(Heidenhain's haematoxylin)

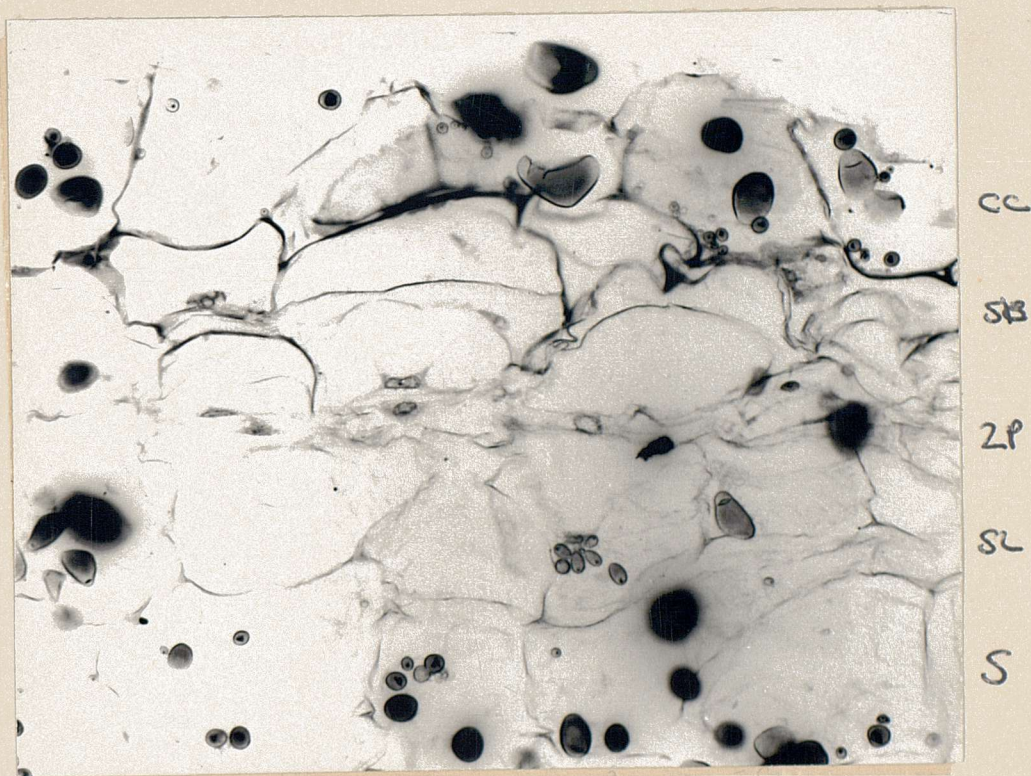


Plate 2.6

(x 252, light micrograph)

Healed wound showing zonation into cut cells,^{CC}
 suberised cells,^{SB} secondary periderm,^{2P} line of cells
 largely devoid of starch,^{SL} and storage parenchyma.^S

(Heidenhain's haematoxylin)

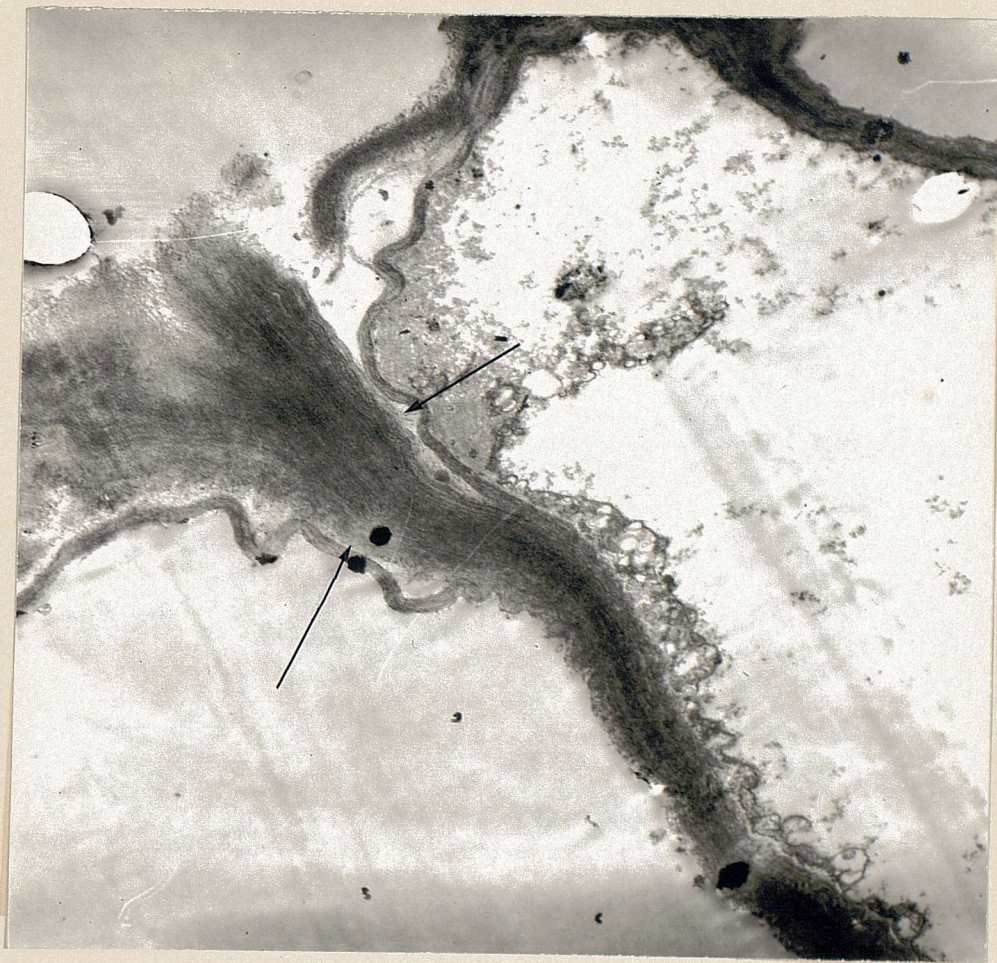


Plate 2.7

(x 10,000)

Cut cell from surface of healed wound showing
disintegration of the cytoplasm and splitting of
the cell wall, ^(disintegrated)
(osmic acid, lead citrate.)



Plate 2.8

(x 30,000)

Plugged intercellular space in suberised layer.

(osmic acid, lead citrate.)

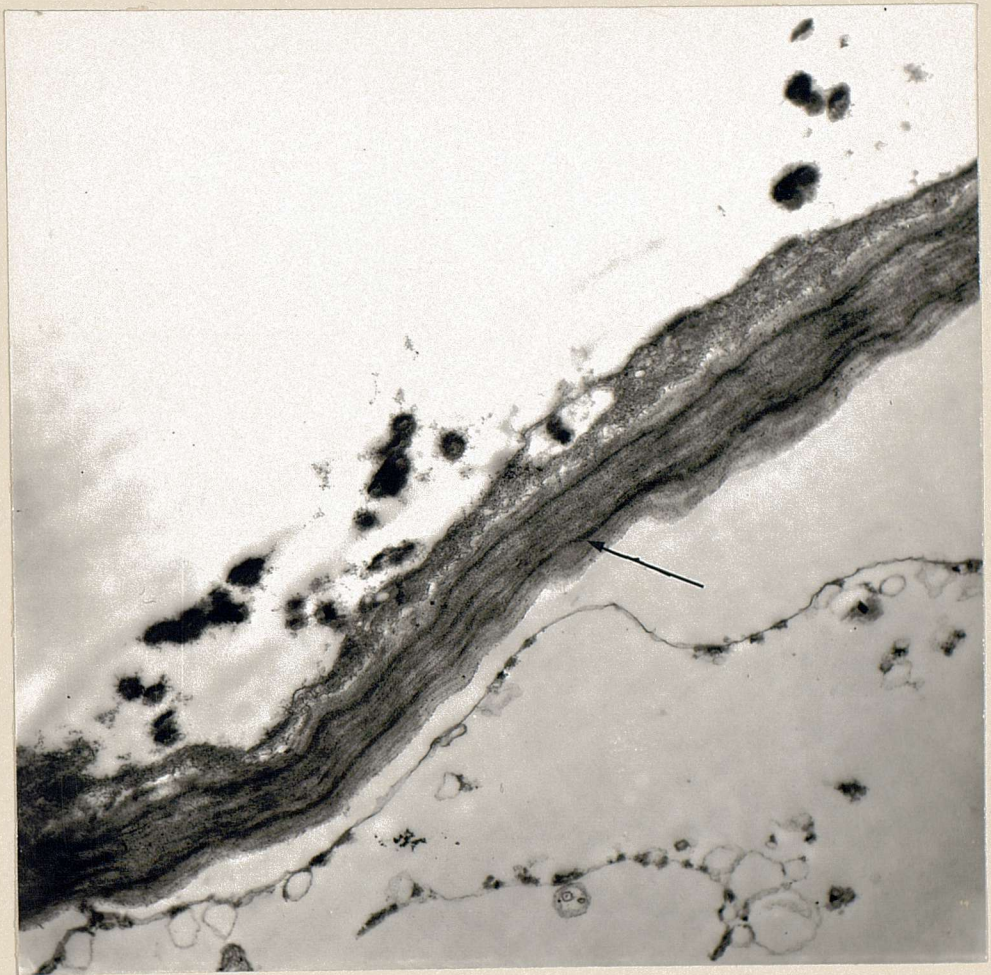


Plate 2.9

(x 12,500, electron micrograph)

Bands of suberin in the cell wall of the suberised
layer *(approved)*.

(osmic acid, lead citrate)

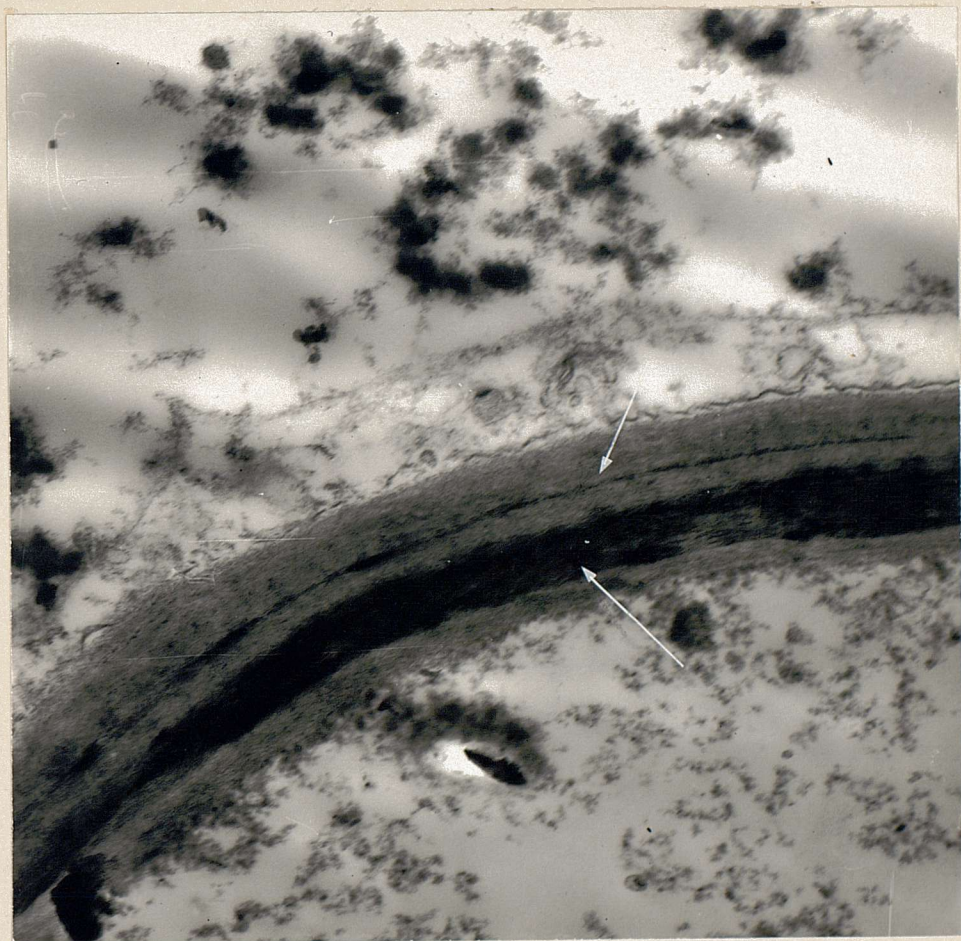


Plate 2.10

(x 30,000)

The bands of suberin may be unequally developed
in the cell wall of the suberised wound surface cells.

(osmic acid, lead citrate.)

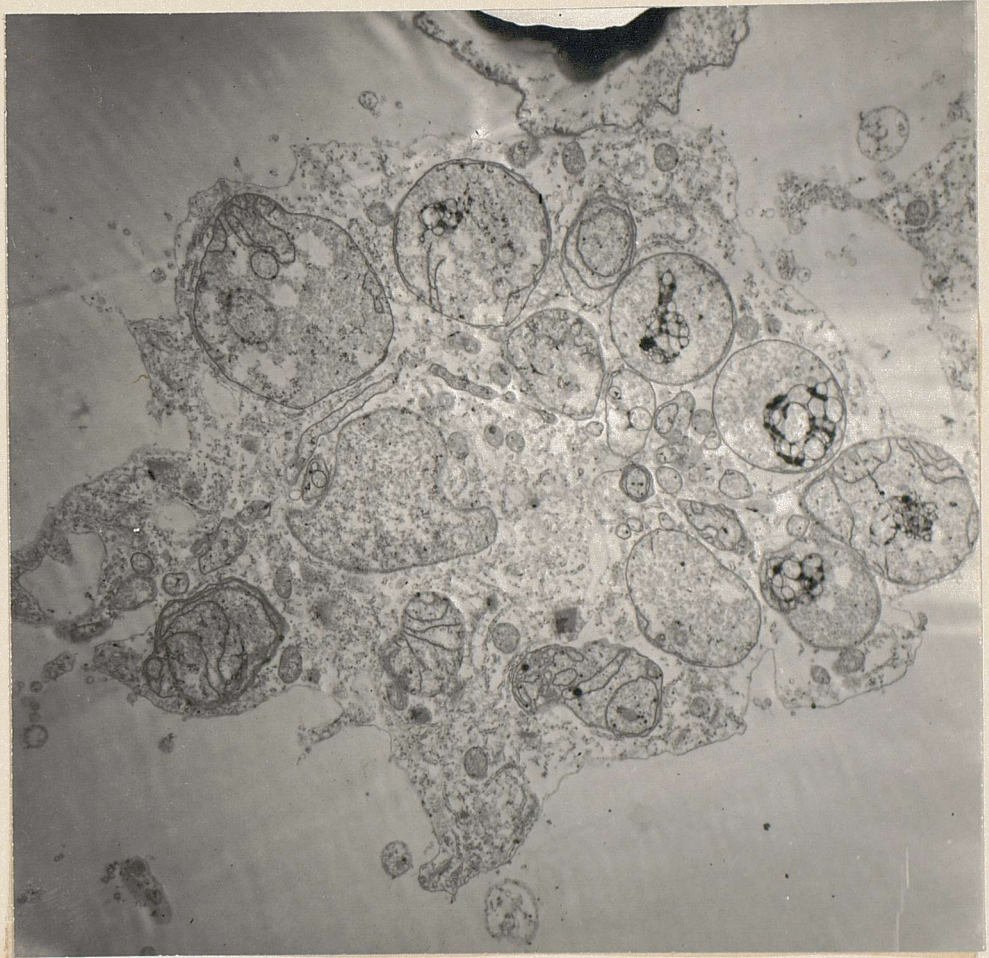


Plate 2.11

(x 6,000)

The cytoplasm of suberising cells contains numerous plastids arranged in clusters.

(osmic acid, lead citrate.)

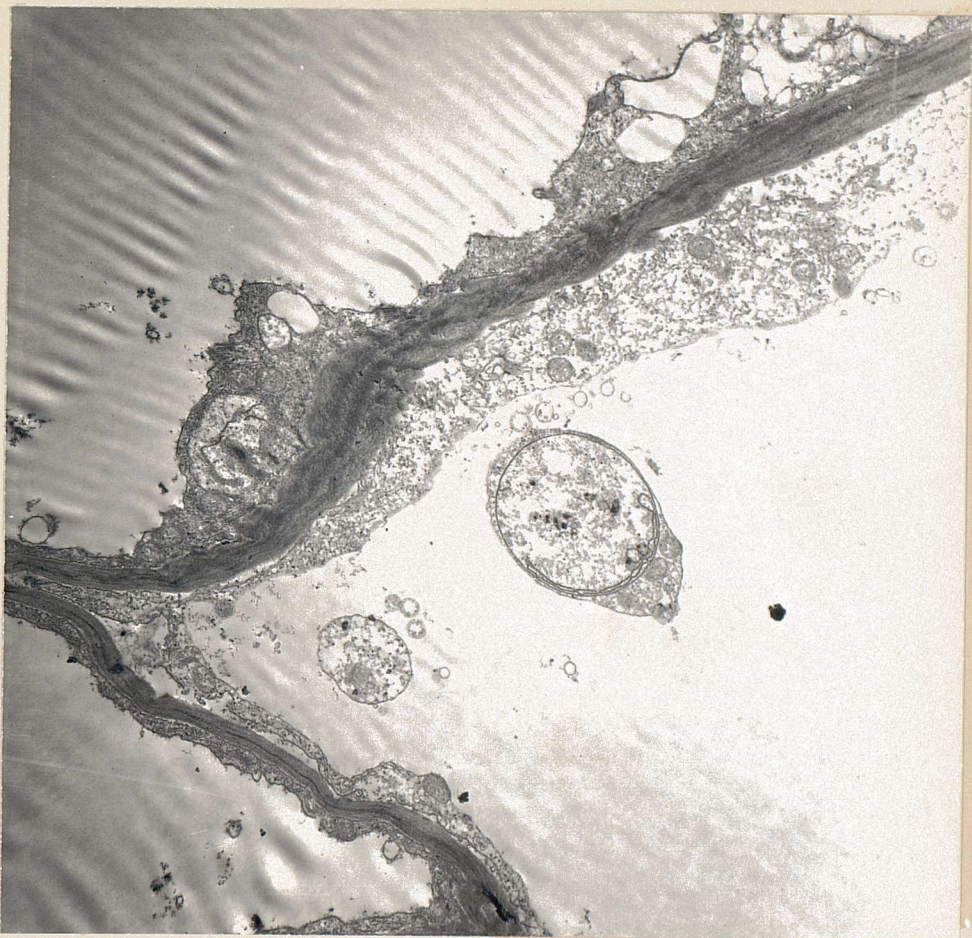


Plate 2.12

(x 6,000)

Secondary periderm from healed wound; the thin cell walls are not yet completely suberised, and there are still traces of cytoplasm.

(osmic acid, lead citrate.)

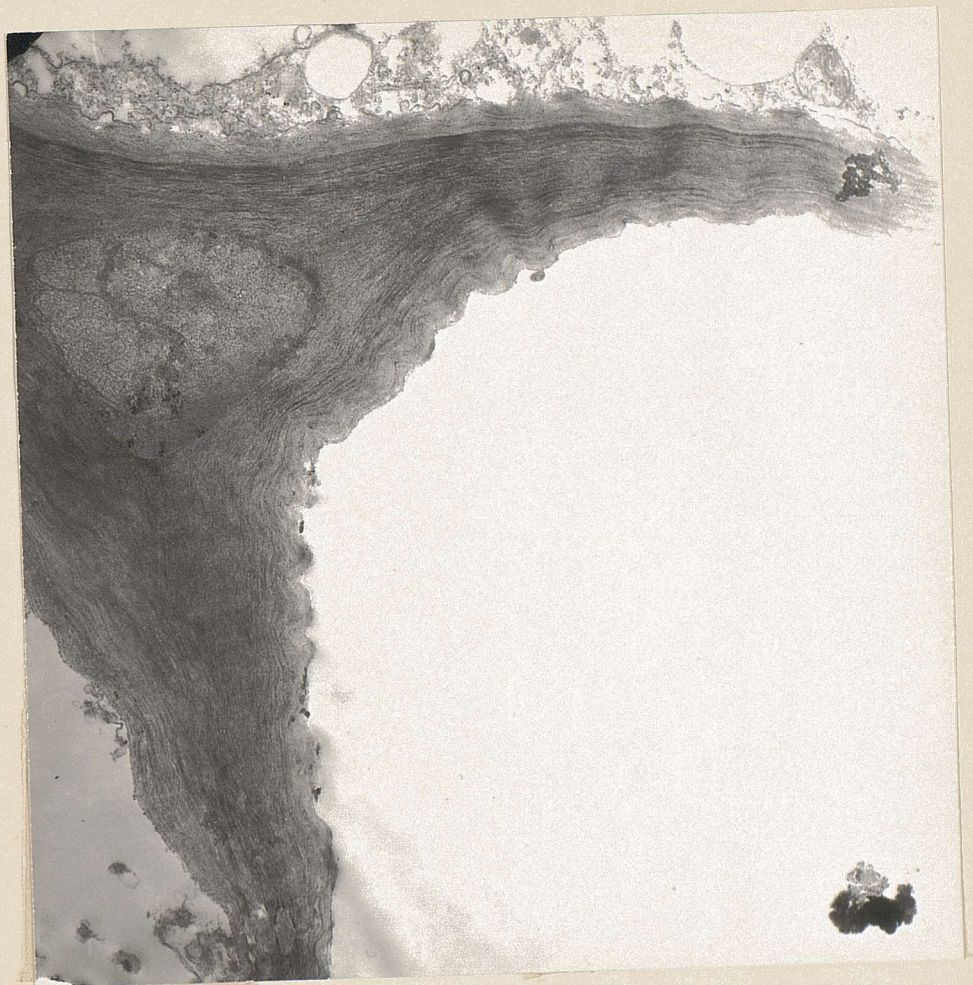


Plate 2.13

(x 15,000)

Intercellular region of the secondary periderm;
no intercellular space, and as yet early stages
of suberisation.

(osmic acid, lead citrate.)

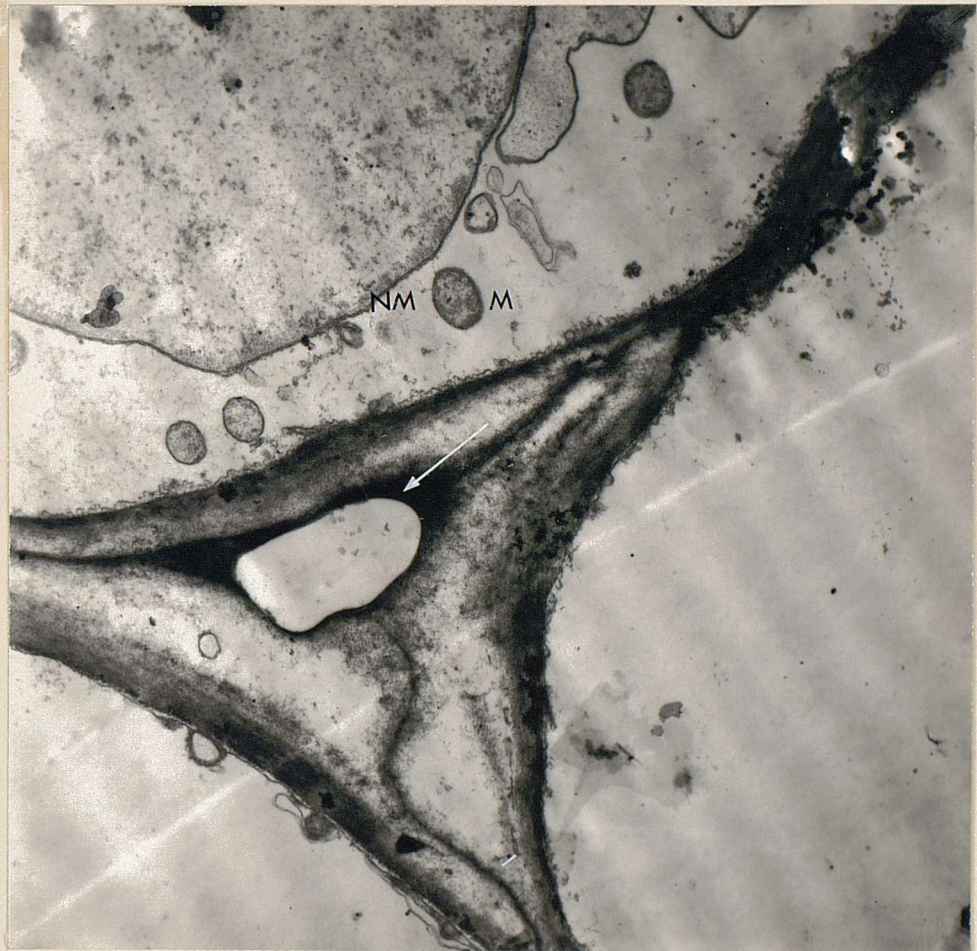


Plate 2.14

(x 10,000, electron micrograph)
(stained)

Isolated incomplete suberin plug from intercellular
region after pectolysis of the surrounding cell wall.

N.b. in this section the mitochondria and the nuclear
membrane. ^M
NM

(osmic acid, lead citrate.)

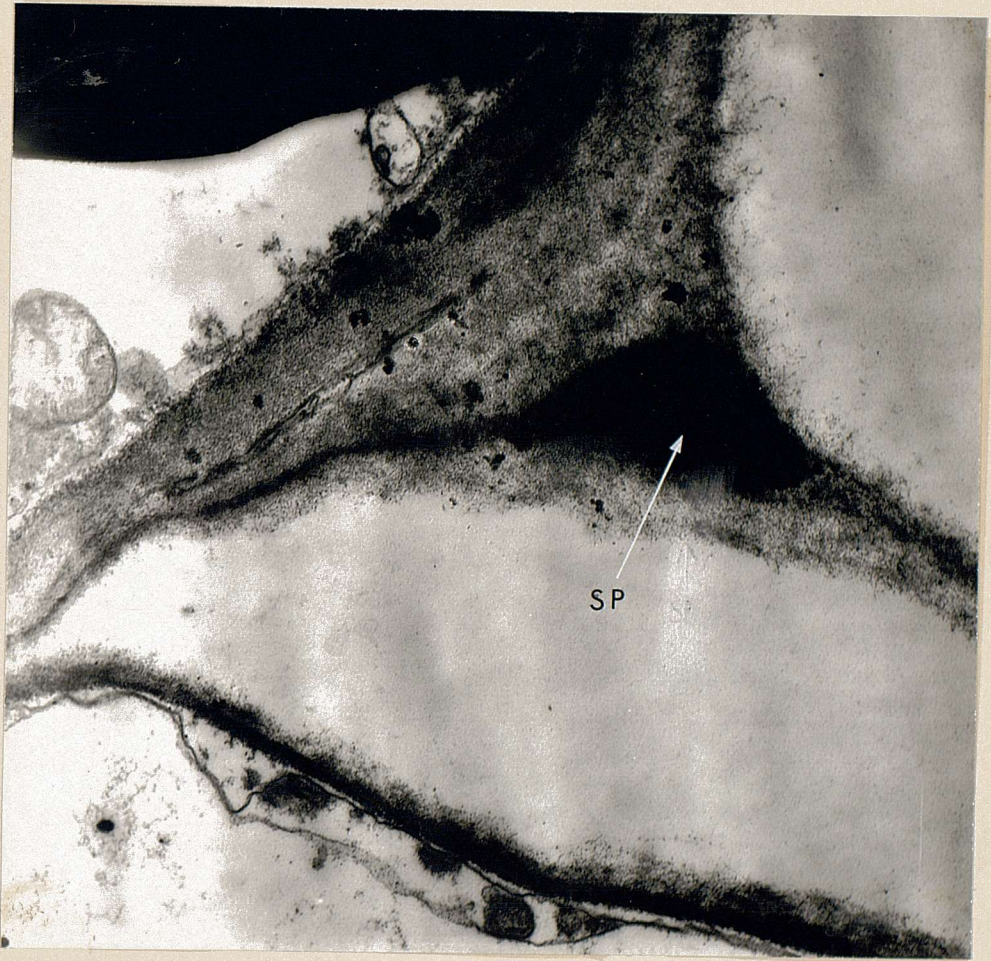


Plate 2.15

(x 20,000, electron micrograph)

(SP)

Disintegrated cell wall surrounding intact suberin plug
originally from an intercellular space.

(osmic acid, lead citrate.)

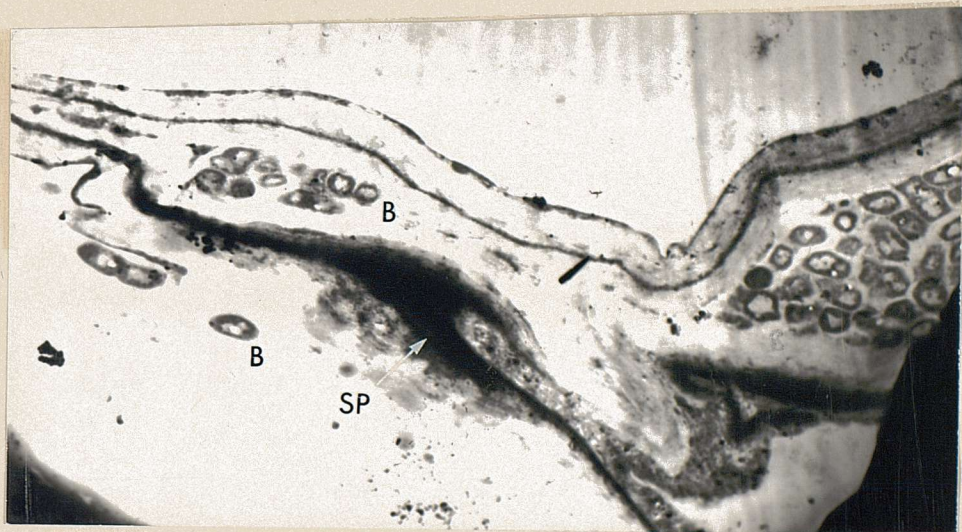


Plate 2.16

(x 6,000, electron micrograph)

Intercellular region; isolated plug of suberin, intact,
but surrounded by bacteria present in space originally
occupied by cell wall now destroyed.

(osmic acid, lead citrate.)

with disorganised cytoplasm and the wall between the cells split into one thick and two thin layers.

The suberised layer is typical of such tissue found in other locations (the lenticel q.v., infection interface, part 6); the intercellular spaces are filled with electron dense material (plate 2.8), and the cell wall has two distinct bands which may or may not be equally developed (plates 2.9,2.10). Melanin granules may be present in the tonoplast as in the other suberising tissues studied; also groups of plastids are present in the cytoplasm; another feature in common with the other tissues and recorded in storage tubers of Helianthus tuberosus by Tulett et al. (1969), their peculiar nature can be seen in plate 2.11.

The wound periderm (plates 2.12,2.13) is similar to primary periderm, both have cell walls thinner than those of parenchyma. In the 14 day wound sample illustrated however, it is not well suberised; there are no intercellular spaces, and the cells are flattened anticlinally and arranged in ranks.

If complete suberisation depends on a combination of suberin encrustation of the cell wall by two bands and the intercellular space by a block, it is conceivable that both processes could be out of phase for a particular cell or group of cells, leading to the penetration of the suberin barrier. This event obviates the need for an enzyme system

attacking suberin for which there is no evidence, the suberin may be by-passed (plates 2.14,2.15,2.16) and the tissue disintegrated by pectolysis alone.

Since at the light microscope level it is not possible to distinguish between complete and incomplete suberisation, apart from experimental rotting, it is only by the use of the electron microscope that the efficacy of a given band of suberised cells as a barrier to bacterial infection can be predicted.

An experimental study of wound healing.

Experiment 2.1. to determine the environmental conditions which promote wound suberisation and periderm formation.

Materials and Methods.

Grade 1 King Edward tubers were washed in tap water to remove adhering soil, surface sterilised by soaking in 0.1% mercuric chloride solution for 10 minutes, and rinsed in sterile distilled water. A slice of periderm approximately 3 x 4 cm was cut from each using a razor sterilised by flaming after dipping in alcohol, then allowed to cool. The tubers were then placed in relative humidity chambers above various concentrations of sulphuric which held the relative humidity at either 100, 97.5, 93.9, 80.5, or 58.3% (Hodgman et al.,1962), then the chambers were kept in incubators at 5, 10, 20, or 30°C for 1 to 13 days. Samples were taken at intervals and

examined by taking hand sections which were stained with neutral red after treatment with 4% KOH, (Strugger, 1935) or with sudan III (Jensen, 1962) and mounted in glycerin.

Ester wax blocks were made of some of the samples to observe the formation of secondary periderm more closely.

Results and discussion.

Suberin was seen to be laid down in the cell walls of the outermost layers of intact cells. Periderm formation was seen as the development of periclinal walls in the storage parenchyma below; the periderm appeared lightly suberised at first, the cell walls were also thinner than those of the storage parenchyma which dedifferentiated into them, by a factor of 10 (plate 2.6). Suberisation appears earliest under conditions where temperature and relative humidity are both relatively high (Table 2.1), the same is true of secondary periderm formation, and it appears that suberisation is a necessary preliminary to the latter. Table 2.3 shows that the secondary periderm is also thickest at the highest temperatures and relative humidities, table 2.1 indicates that the same is true of the total depth of dead tissue, i.e. that depth of cells not responding to neutral red staining of the tonoplast (Strugger, 1935). Table 2.1 shows that cell death can occur without subsequent or prior suberisation, the possibility therefore arises that suberisation perhaps as a result of

Table 2.1 Suberisation and periderm formation of fresh wound surfaces of King Edward tubers. ^{mean of} (Sample of 4)

Key. _____ suberin present, _ _ _ _ periderm present, figures denote the mean thickness of dead tissue, including secondary periderm, at the wound surface, measured in mm, / result not recorded.

<u>Exposure (h)</u>	<u>Temp. (°C)</u>	<u>R.H. (%)</u>				
		53	60	94	97	100
24	5	.18	.21	.22	.21	.21
	10	.21	.21	.22	.19	.22
	20	.19	.20	.20	.20	.22
	30	.20	.20	.22	.23	.22
48	5	.21	.21	.21	.22	.23
	10	.21	.24	.23	.23	.24
	20	.23	.23	.23	.28	.29
	30	.28	.26	.24	.28	.29
72	5	.25	.31	.32	.32	.31
	10	.32	.32	.33	.33	.31
	20	.32	.36	.33	.35	.31
	30	.33	.35	.33	.42	.42
96	5	/	/	/	/	/
	10	.31	.31	.31	.34	.32
	20	.30	.31	.36	.33	.37
	30	.31	.31	.36	.66	.66

Table 2.1 cont.,

<u>Exposure (h)</u>	<u>Temp. (°C)</u>	<u>R.H. (%)</u>				
		<u>58</u>	<u>80</u>	<u>94</u>	<u>97</u>	<u>100</u>
144	5	.32	.31	.32	.31	.32
	10	.32	.31	.31	.36	.36
	20	.30	.33	.38	.38	.38
	30	.31	.35	.39	.61	.67
312	5	.32	.37	.32	.33	.31
	10	.30	.36	.32	.32	.33
	20	.31	.32	.42	.51	.49
	30	.33	.38	.68	.71	.72
480	5	.26	.30	.32	.32	.30
	10	.28	.32	.33	.40	.39
	20	/	.33	.38	.68	.68
	30	.30	.37	.66	.66	.72

Table 2.2 Number of parenchyma cell layers converted into secondary periderm. (Sample of ^{medium 1} 10)

<u>Exposure (h)</u>	<u>Temp. (°C)</u>	<u>R.H. (%)</u>				
		58	80	94	97	100
24	all	no cells converted.				
48	5	0	0	0	0	0
	10	0	0	0	0	0
	20	0	0	0	0	2
	30	0	0	0	0	2
72	5	0	0	0	0	0
	10	0	0	0	0	0
	20	0	0	0	3	3
	30	0	0	0	3	3
96	5	/	/	/	/	/
	10	0	0	0	0	0
	20	0	0	0	3	3
	30	0	0	5	5	5
144	5	0	0	0	0	0
	10	0	0	0	0	0
	20	0	0	3	3	3
	30	0	3	3	5	6

Table 2.2 cont.

Exposure (h)	Temp. (°C)	R.H. (%)				
		58	80	94	97	100
312	5	0	0	0	0	0
	10	0	0	0	0	0
	20	0	2	3	4	4
	30	0	3	6	6	6
480	5	0	0	0	0	0
	10	0	0	0	2	3
	20	/	2	3	6	6
	30	0	3	6	6	6

N.b. the tubers in experiment 2.1 were stored at 78% R.H.
and 5°C prior to their use experimentally.

an active metabolic process, causes cell death and not vice versa as suggested by Frey-Wyssling & Mühletaler (1965). The evidence of electron microscopy (q.v.) supports this theory.

The process by which the storage parenchyma dedifferentiates appears to involve an initial loss of starch grains, followed by elongation of the cell wall anticlinally then the formation of periclinal walls, rather than the formation of a small celled phellogen.

Rudd Jones(1948) reported a suberin infection barrier to be formed between the infected and intact cells. He indicated that in an infected system the suberin barrier was thickest at the lower temperatures and relative humidities, this appears contrary to the results expected from the above and those of other workers (Artschwager,1927; Priestley & Woffenden,1922), but was also found to be the case in the later part of this investigation (q.v. part 6). It was therefore felt desirable to determine whether the effect of environment on the behaviour of healthy cells covered by dead but uninfected cells resembled that reported by Rudd Jones. In order to simulate such a situation, two approaches were made. The first supposed the action of a traumatin type reaction (Bonner & English, 1938), potato tissue was ground up and placed on the cut surface, this was compared with indoleacetic acid soaked

cotton wool, sterile distilled water soaked cotton wool, together with a control. In the second, carborundum 180 grit powder was rubbed into the cut surface to give localised cell death above the healthy cells.

Experiment 2.2, to simulate the effect of the secretions of dead cells above the suberising surface.

Materials and methods.

Grade 1 dry brushed tubers var. King Edward were kept at

- i. 100% relative humidity and 10°C,
- ii. 100% relative humidity and 30°C,
- iii. 58% relative humidity and 10°C,
- iv. 58% relative humidity and 30°C,

and exposed to the following treatments :-

- a) control, sterile cut made with flamed razor previously dipped in alcohol, potato sterilised in 0.1% mercuric chloride as in experiment 2.1.
- b) 10 g crushed fresh potato was applied to the sterile cut for 24 h then removed.
- c) sterile cotton wool was soaked in 40 p.p.m. indoleacetic acid which was applied to the sterile cut for 24 h.
- d) sterile cotton wool was soaked in sterile distilled water then applied to the sterile cut for 24 h.

Hand razor sections were taken at intervals between 1 and 14 days and examined for suberisation and secondary periderm formation, and other related points.

Results and conclusions.

The results are given in tables 2.3,2.4. The indoleacetic acid and water treatments initiallyretarded both suberisation and secondary periderm formation but later stimulated them, particularly at low relative humidity. The crushed potato treatment appeared to retard suberisation, but the final expression was similar to the untreated control in all the attributes.

All the special treatments appeared to predispose the tubers to an odourless rot which was particularly severe at the higher temperatures and humidities. This rot indicated (R), made a sudden appearance over a large area of tuber.

Experiment.2.3.

To simulate the effect of dead cells above the healing surface of a wound, using carborundum grit to kill cells.

Materials and Methods

Dry brushed grade 1 King Edward tubers were treated by lightly rubbing 180 grit carborundum into:-

- a) a fresh wound surface b) an intact area of periderm and together with c) an untreated control, the tubers were kept in relative humidity chambers in incubators at
- 1) 100% R.H. and 10'C
 - 2) 100% R.H.and 30'C
 - 3) 58% R.H. and 10'C
 - 4) 58% R.H. and 30'C.
- for 1 to 14 days.

Samples were examined by taking hand razor sections.

Table 2.3 Suberisation and secondary periderm formation
 after treatment to simulate dead cells. (Sample 4)

Key. _____ suberin present _____ secondary periderm present
 figures denote the depth of dead cells, to nearest
 cell including secondary periderm, R unrecordable as rotted

<u>Exposure (h)</u>	<u>Temp. (°C)</u>	<u>Treatment</u>							
		<u>Control, Crushed potato, I.A.A., Water.</u>							
		<u>% R.H. 58</u>		<u>100</u>		<u>58</u>		<u>100</u>	
24	10	2	2	2	2	2	/	2	2
	30	2	2	2	2	/	2	2	2
48	10	2	2	2	2	2	2	2	2
	30	3	3	2	2	2	2	2	2
72	10	2	3	2	2	2	2	2	2
	30	3	4	2	2	2	2	2	2
96	10	3	3	2	3	2	2	2	2
	30	3	7	2	3	2	2	2	2
144	10	3	3	3	4	3	3	3	3
	30	3	7	4	5	4	4	4	4
240	10	3	3	3	4	3	3	3	3
	30	3	7	4	5	4	4	4	4
288	10	3	3	3	3	3	3	3	4
	30	3	7	R	R	4	R	4	7

Table 2.4 Number of parenchyma cells converted into secondary periderm, after treatment to simulate dead cells. (sample 10)

<u>Exposure (h)</u>	<u>Temp. (°C)</u>	<u>Treatment</u>									
		<u>Control, Crushed potato, I.A.A., Water.</u>									
		<u>% R.H.</u>		<u>58</u>		<u>100</u>		<u>58</u>		<u>100</u>	
24	10	0	0	0	0	0	0	0	0	0	0
	30	0	0	0	0	0	0	0	0	0	0
48	10	0	0	0	0	0	0	0	0	0	0
	30	0	2	0	0	0	0	0	0	0	0
72	10	0	0	0	0	0	0	0	0	0	0
	30	0	3	0	0	0	0	0	0	0	0
96	10	0		0	0	0	0	0	0	0	0
	30	0	5	0	0	0	0	0	0	0	0
144	10	0	0	0	0	0	0	0	0	0	0
	30	0	6	0	0	2	2	2	2	2	2
240	10	0	0	0	0	0	0	0	0	0	0
	30	0	6	R	R	4	R	4	R	4	7

N.b. R denotes that samples were not recordable because of the presence of bacterial rot.

Table 2.5 Suberisation and periderm formation after carborundum treatment to simulate bacterial attack. (Sample 7^{main} 12)

Key. ___ suberin present, ___ secondary periderm present, figures denote the depth of dead cells, to nearest cell, including secondary periderm, / no record.

<u>Exposure (h)</u>	<u>Temp. (°C)</u>	<u>Treatment</u>					
		<u>Control, Wound rubbed, Periderm rubbed.</u>					
		<u>% R.H.</u>	<u>58</u>	<u>100</u>	<u>58</u>	<u>100</u>	<u>58</u>
24	10	2	<u>2</u>	2	2	3	3
	30	<u>2</u>	<u>2</u>	2	2	3	3
48	10	<u>2</u>	<u>2</u>	<u>2</u>	<u>3</u>	3	3
	30	<u>3</u>	<u>3</u>	<u>3</u>	<u>3</u>	3	<u>3</u>
72	10	<u>2</u>	<u>3</u>	<u>3</u>	<u>3</u>	3	4
	30	<u>3</u>	<u>4</u>	<u>3</u>	<u>4</u>	3	<u>4</u>
96	10	<u>3</u>	<u>3</u>	<u>3</u>	<u>3</u>	3	<u>4</u>
	30	<u>3</u>	<u>6</u>	<u>3</u>	<u>4</u>	3	<u>4</u>
144	10	<u>3</u>	<u>3</u>	<u>3</u>	<u>4</u>	3	<u>4</u>
	30	<u>3</u>	<u>7</u>	<u>4</u>	<u>4</u>	3	<u>4</u>
240	10	<u>3</u>	<u>3</u>	<u>3</u>	<u>4</u>	4	<u>4</u>
	30	<u>3</u>	<u>7</u>	<u>4</u>	<u>4</u>	3	<u>4</u>
336	10	<u>3</u>	<u>3</u>	<u>4</u>	<u>4</u>	3	<u>4</u>
	30	<u>3</u>	<u>7</u>	<u>4</u>	/	3	/

Table 2.6 Mean number of cells suberised after carborundum treatment to simulate bacterial attack. (Samples ^{mean} 3 & 4)

<u>Exposure (h)</u>	<u>Temp. (°C)</u>	<u>Treatment</u>						
		<u>Control, Wound rubbed, Periderm rubbed.</u>						
		<u>° R.H.</u>	<u>58</u>	<u>100</u>	<u>58</u>	<u>100</u>	<u>58</u>	<u>100</u>
24	10		0	1	0	0	0	0
	30		1	1	0	0	0	0
48	10		1	1	0	2	0	0
	30		1	1	2	2	0	2
72	10		1	1	2	2	0	0
	30		1	2	2	2	0	2
96	10		1	1	2	2	0	2
	30		1	2	2	2	0	2
144	10		1	1	2	3	0	2
	30		1	2	2	2	0	2
240	10		1	1	2	3	0	2
	30		1	2	2	2	0	2
336	10		1	1	3	3	0	3
	30		1	2	3	/	0	/

Table 2.7 Mean number of parenchyma cells dedifferentiated into periderm after carborundum treatment. (Sample ^{no. 13} 4)

<u>Exposure (h)</u>	<u>Temp. (°C)</u>	<u>Treatment</u>						
		<u>Control, Wound rubbed, Periderm rubbed.</u>						
		<u>%R.H.</u>	<u>58</u>	<u>100</u>	<u>58</u>	<u>100</u>	<u>58</u>	<u>100</u>
24	10		0	0	0	0	0	0
	30		0	0	0	0	0	0
48	10		0	0	0	0	0	0
	30		0	2	0	0	0	1
72	10		0	0	0	0	0	0
	30		0	3	0	3	0	3
96	10		0	0	0	0	0	0
	30		0	4	0	3	0	3
144	10		0	0	0	0	0	3
	30		0	6	2	2	0	2
240	10		0	0	0	0	0	5
	30		0	6	2	3	0	3
336	10		0	0	0	0	0	4
	30		0	6	3	/	0	/

Results and conclusions.

The results are given in tables 2.5, 2.6, 2.7. It was concluded that where the intact periderm at low relative humidity was rubbed with carborundum suberisation was inhibited and new wound periderm was formed earlier than in the control. Perhaps the primary periderm acts in a similar way to the wound suberin barrier in the control even though it is not complete after the treatment.

Suberisation appeared slightly retarded after a cut wound surface was rubbed with carborundum powder, but secondary periderm formation was promoted especially at the lower relative humidity.

The total depth of dead cells is slightly less deep at high relative humidity compared to the control, but this could be explained by less cells being converted into wound periderm.

The differences between the effects of the rubbing treatment on the periderm and that on the fresh wounds, lies in the number of cells secondarily suberised at the surface or near it, at the higher relative humidity both were higher than the control, but at the lower relative humidity only the treated fresh wounds were highly suberised.

Discussion.

Neither experiment produced results in accordance with those described for an infection interface by Rudd Jones

(1948), as nowhere was suberisation greater at the lower relative humidity than at the higher. This evidence confirms the suggestion that the results obtained by Rudd Jones were due to low relative humidities affecting the progress of the bacterial rot rather than to a direct effect on the host itself.

In the field, the wound surface as caused by animal agency or other means may be wet for a considerable time. The following experiment was set up in order to simulate the state of affairs where the cut tuber surface is covered by a film of water.

Experiment 2.4.

To determine the effect of a film of water on suberisation and wound periderm formation.

Materials & Methods.

A half tuber of a grade 1 King Edward tuber, previously surface sterilised by immersion in 0.1% mercuric chloride, was sprayed with a fine mist of sterile distilled water at the rate of 48 cm³ per minute, through a 'sapphire' nozzle taken from a VS.625 spray-gun (Burgess Products Co. Ltd.) pumped by a sealed DY.mk.III pump (Charles Austen Pumps Ltd.), the tuber, nozzle and suction pipe were enclosed in a glass chamber. The experiment was at room temperature.

Samples were taken every 24 hours for five days; these were examined by means of hand razor and ester wax microtome sections.

Results

After 24 h there were no signs of suberisation, but there was considerable cell elongation in the fourth to sixth layers beneath the surface. These cells were lengthened anticlinally by up to 17.5% but narrowed periclinally by more than this. All the cells apart from the cut ones reacted positively to neutral red after 0.4% KOH indicating that they had an intact tonoplast and were presumably alive.

Forty-eight hours after the start of spraying there was no change but after 72h there was a trace of suberisation down to the third layer of cells; the situation was no different after 96h. After 5 days no tubers could be kept in the apparatus as they were macerated by an odourless bacterial rot, from which Bacillus cereus and Bs. megatherium were isolated. An interesting point in connection with this rot was the fact that where the tissue was sufficiently coherent for hand sections to be made, neutral red staining indicated that bacteria appeared to be present inside living cells; more will be said about this phenomenon in part 7. Ester wax sections also indicated bacteria inside the cytoplasm.

Conclusion

It was concluded that a fine film of water on a cut potato surface inhibits suberin production, and predisposes the tuber to a Bacillus rot, possibly both processes are promoted by a comparative lack of oxygen in the tuber.

General Discussion of Wound Healing Experiments.

It can be seen that suberisation and wound periderm formation of a fresh wound are promoted best by a combination of the higher temperatures (20-30°C) and relative humidities (above 80%) but free from surface water.

Although secondary periderm formation normally follows suberisation, it was found that if the healing surface was covered even by disrupted periderm, as in experiment 2.3, there was some promotion of the secondary periderm formation in the absence of a suberised layer. Priestley and Woffenden (1922) found that they could promote secondary periderm behind an unsuberised wound surface by covering the latter with Vaseline jelly.

It appears therefore that wound surface suberisation has the function of forming a watertight, sterile seal above the dedifferentiating parenchyma which forms the secondary periderm.

Because the effects of environment on healing beneath

the surface are similar to those on exposed surfaces, and dissimilar to those on suberisation at the soft-rot interface described by Rudd Jones (1948), it seems probable that the latter is due to the effect of the environment on the pathogen, not the host.

Following from this study of the events leading from the healing of a fresh wounded surface and its transformation into resistant periderm, the question arises, when is the precise point in time that the fresh wound is no longer susceptible; is it when the suberisation of the wound is finished, when the secondary periderm is complete or some other time?. The following experiment answers this point.

Experiment 2.5.

To investigate when a healing wound is efficient as a barrier to infection.

Materials and Methods.

A 42 h culture of Erwinia carotovora var. atroseptica in nutrient broth was centrifuged at 3,000 r.p.m. into 0.1 ml aliquots containing approximately 2.5×10^{10} cells per ml. These were inoculated into 7mm diameter wells made in the cut wound surface of half tubers which had been left at relative humidities of 100%, 97%, 80% and 58%, and 5°C

Table 2.8 Effect of length of exposure time after wounding on rotting following subsequent inoculation.

Key. R, rotting in all wells; -, no rot.

<u>Temp. (°C)</u>	<u>R.H.(%)</u>	<u>Exposure time (h)</u>					
		<u>0</u>	<u>18</u>	<u>24</u>	<u>40</u>	<u>137</u>	<u>238</u>
25	100	R	-	-	-	-	-
	97	R	-	-	-	-	-
	80	R	R	-	-	-	-
	58	R	R	R	-	-	-
5	100	R	R	R	R	-	-
	97	R	R	R	R	-	-
	80	R	R	R	R	R	R
	58	R	R	R	R	R	R

Table 2.9 Effect of length of exposure time after wounding on suberisation and secondary periderm formation below the cut surface.

Key. S, suberisation; P, periderm formation; - no. S or P.

<u>Temp. (°C)</u>	<u>R.H. (%)</u>	<u>Exposure time (h)</u>					
		0	18	24	40	137	238
25	100	--	s-	s-	sp	sp	sp
	97	--	s-	s-	s-	sp	sp
	80	--	--	s-	s-	s-	sp
	58	--	--	s-	s-	s-	s-
5	100	--	--	s-	s-	s-	s-
	97	--	--	s-	s-	s-	s-
	80	--	--	--	--	s-	s-
	58	--	--	--	--	s-	s-

or 25°C for 0, 18, 24, 40, 137 and 238 h to heal in enclosed glass chambers. Each tuber had six such wells and after 80h incubation at 100% R.H. and 25°C the wounds were examined for the presence of rotted tissue by immersing them in a 0.1% solution of 2:3:5 - triphenyl-tetrazolium chloride which indicates the presence of bacterial cell bound dehydrogenase. At the same time hand razor sections were taken both to confirm this and examine for the presence of suberin or wound periderm.

Results.

The results are given in Table 2.8.

Conclusions

It was concluded that the absence of a suberised layer of cells allowed a **soft rot** to be initiated, however the presence of suberin does not preclude infection in all cases. For example, at low relative humidity (58% R.H.), healing for 40 h results in a suberised layer of cells, but this is not effective as a barrier against the pathogen.

This incompleteness of suberin as a barrier is explained by its mode of formation as the electron microscope study of wound healing shows.

Discussion of wound surfaces as channels of infection.

It appears evident that fresh wound surfaces act as channels of infection until complete suberisation prevents

bacterial entry by the formation of an intimate seal at the cellular level followed by the conversion of parenchyma to secondary periderm.

Infection interface suberisation appears to be affected largely by the reaction of the environment with the pathogen, rather than with the host as in wound suberisation.

Suberisation is important in determining the efficacy or otherwise of another channel of entry of infection; the lenticel, which has the importance of being ubiquitous, which is not always true of wounds on tubers.

4. The Lenticel.

Many workers have mentioned bacterial entry via the lenticel including Ruele (1940), Smith & Ramsey (1947), Davidson (1948), Logan (1964), Bétencourt & Prunier (1965) and Pérombelon (1969). It is recognised that the lenticel may be 'proliferated' under conditions of heavy rainfall allowing bacterial entry, and that a secondary periderm may be present under the lenticel. Pérombelon (1969) has compared records of bacterial soft rot and rainfall and suggested that infection was prevalent after periods of heavy rainfall. He found that a time lag of several weeks might elapse between the two events. From this a hypothesis involving mother tuber rotting was put forward, a heavy period of rainfall promoting both mother tuber

rotting and lenticel proliferation in the daughter tubers

The object of this section of the present investigation was to compare the effects of various relative humidities on the state of the lenticel and its efficiency as a channel for infection before and after inoculation. At present there appears to be a gulf between observations of the environment of the potato crop in the field and its subsequent behaviour in store. As a particular crop would have experienced a variety of conditions before storage it was decided to standardize the conditions of a sample by storing the potatoes under constant conditions, exposing them to a variety of relative humidities, and inoculating before or after such exposure in order to determine the effect of environmental conditions at these different stages in the history of the sample. In this way it was hoped that data could be obtained permitting a grower knowing both the past history of the crop before harvesting, and the conditions at which the tubers were stored, to predict and control the 'spontaneous' rots which devastate potato stores at present. The possibility that the so called 'latent' infection of tubers is due to the interaction of environmental factors before and after harvesting is discussed, as the early stages of lenticel infection are completely undetectable by eye compared to fresh wound infection.

The Structure of the lenticel.

Introduction.

The techniques employed were the same as described earlier for light and electron microscopy of infected potato tissue.

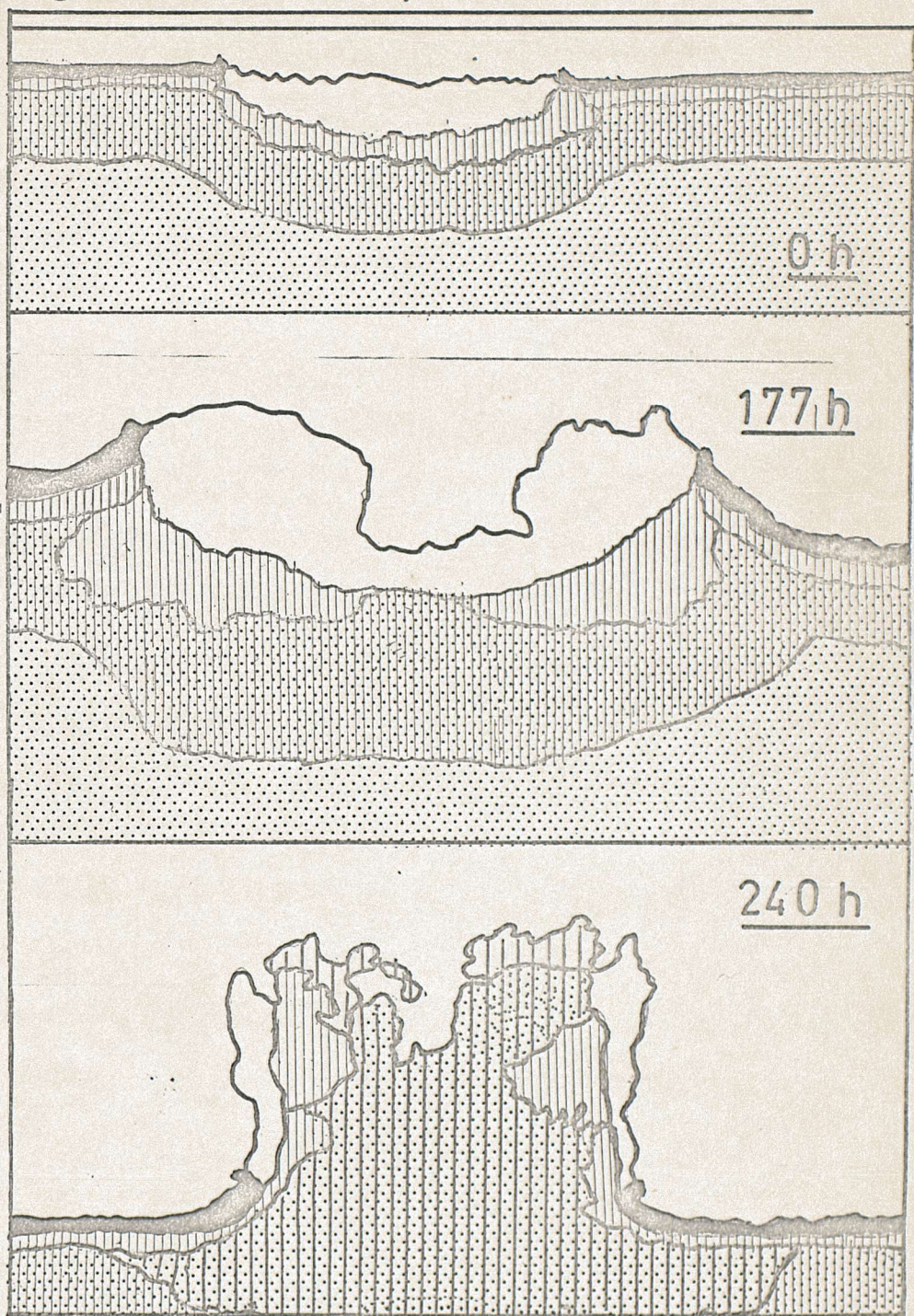
i) Light Microscopy.








Artschwager (1924) described the formation of lenticels beneath the stomata in stolon tips. Their subsequent development during the growth and maturation of the tubers is one of development in size rather than development of new structures, (plates 2.17 & 2.18).

However, lenticels of mature tubers kept under different relative humidities for long periods may have quite different anatomical arrangements, which are only recognisable in the extreme cases (plate 2.20), but which have a profound influence on the extent to which the lenticels act as channels for bacterial entry.

The lenticels illustrated in plates 2.21- 2.23 show the effects of development at 58.3% and 80.5% R.H., and with liquid water on the tuber surface. At 58.3% R.H. a secondary periderm develops beneath the dead suberised cells of the filling tissue, there is no distinct meristematic zone, and the periderm is adjacent to cortex. Storage at 80.5% R.H. results in a more typical lenticel with a distinct filling tissue, lying above a dispersed

Fig. 2.2 Lenticel proliferation. [x84]



Key.  filling cells,  meristem,  phelloderm,
 dead cells,  periderm,  proliferating cells,
 cortex.

meristem and phelloderm, which in turn is above the cortex. The gap between the lenticel filling tissue and the periderm appears to be a point of weakness as seen in plate 2.24 -2.25 where bacterial colonisation has spread between the meristematic tissue of both into the cortex. If the surface of a potato tuber is kept moist by being wrapped in sphagnum, absorbant tissue or cotton wool or occasionally even when stored above water, 'proliferation' of the lenticel occurs, to form a patch of exposed cells on the potato surface resembling a cauliflower; the 'choufleur' lenticels referred to by Bétancourt and Prunier (1965) which they found in the field after heavy rain. These workers found inoculation of such lenticels uniformly successful after wetting with bacterial suspension. As the structure of the 'proliferated' lenticel has not been described it was decided to section ester wax and araldite embedded specimens. The cells protruding from the lenticel can be seen under light (plate 2.2), and electron microscopy (plate 2.26) to be large unsubsided cells lacking in starch which appear to be continuous with the phelloderm. The meristem appears to be reduced to a ring around the lenticel, and the filling tissue pushed up and outwards (fig.2.1). The early stages of proliferation appear similar to the type of lenticel produced by storage under high humidity and

and under 100% R.H., fully developed proliferation may sometimes occur without liquid water. It appears that under liquid water the 'proliferated' cells are not suberised, this corresponds to the results of the mist experiment described earlier, experiment 2.4. in which fresh wounds were sprayed with a fine mist.

ii) Electron microscopy of the Lenticel.

Lenticels blocked by a secondary periderm have an ultrastructure similar to that of wound periderm except that the suberin layer is derived from filling tissue.

Lenticels kept at relative humidities of at least 80.5% show a differentiation into suberised filling tissue, which merges through a zone of suberising cells into meristem, and cortex (plate 2.22). The filling tissue is suberised both between the cells and in the intercellular spaces (plates 2.27, 2.28; this pattern is established in the suberising cells (plates 2.29 and 2.30). At increased relative humidity the filling tissue is composed of more living component cells than dead tissue. These living cells in the filling tissue have intact organelles (plate 2.31), nuclei, and tonoplasts, the latter often containing melanin granules which may be stained with Lillie's reagents.

In the proliferated lenticels the unsuberised cells which appear continuous with the phelloderm have an intact

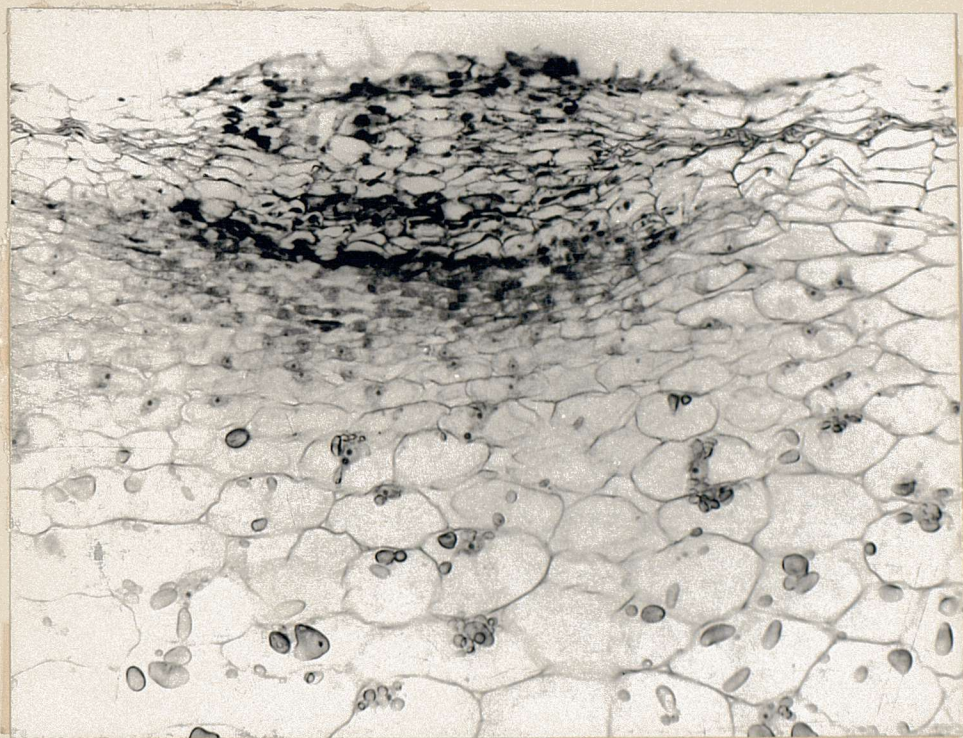


Plate 2.17

(x 160, light micrograph

Immature lenticel from 3 cm diameter tuber.

(Heidenhain's haematoxylin.)



Plate 2.18

(x 160, light micrograph)

Immature lenticel from 5 cm diameter tuber.

(Heidenhain's haematoxylin.)



Plate 2.19 (x3)

Potato tuber surface showing lenticels after storage
at 80.5% R.H.



Plate 2.20 (x3)

Potato tuber surface showing proliferated lenticels after storage in damp sphagnum. The emergence of a new proliferated lenticel, next to one which has remained blocked, is arrowed.

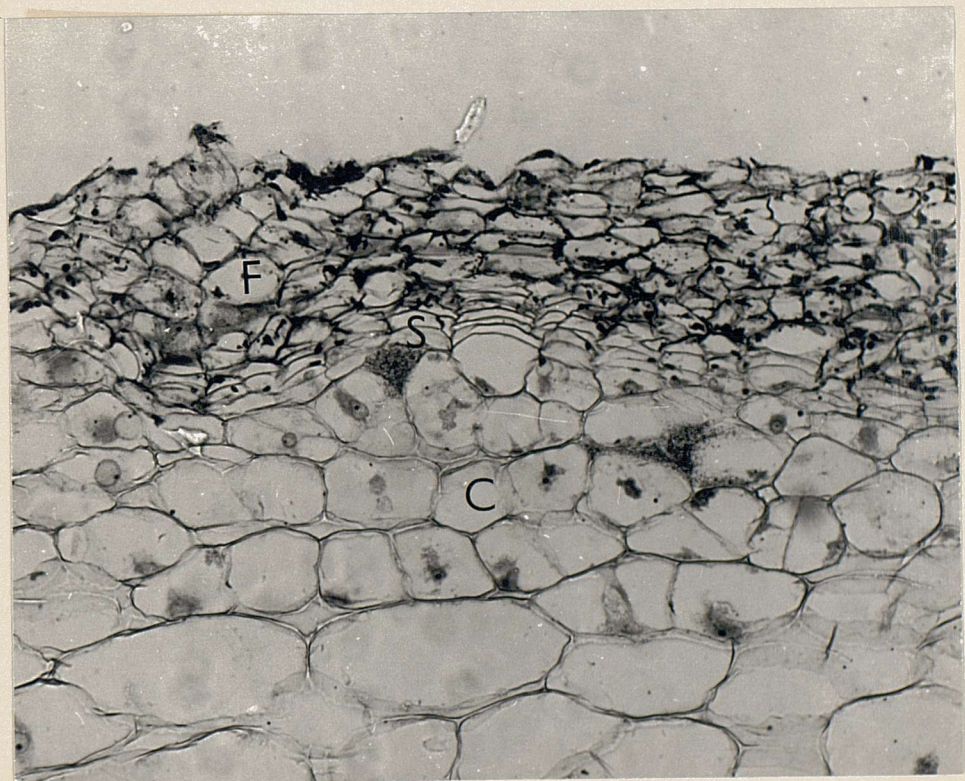


Plate 2.21

(x 252, light micrograph)

Section through lenticel of tuber pretreated at 58.3%

R.H. S, secondary periderm; F, filling tissue; C, cortex.

(Heidenhain's haemotoxylin.)

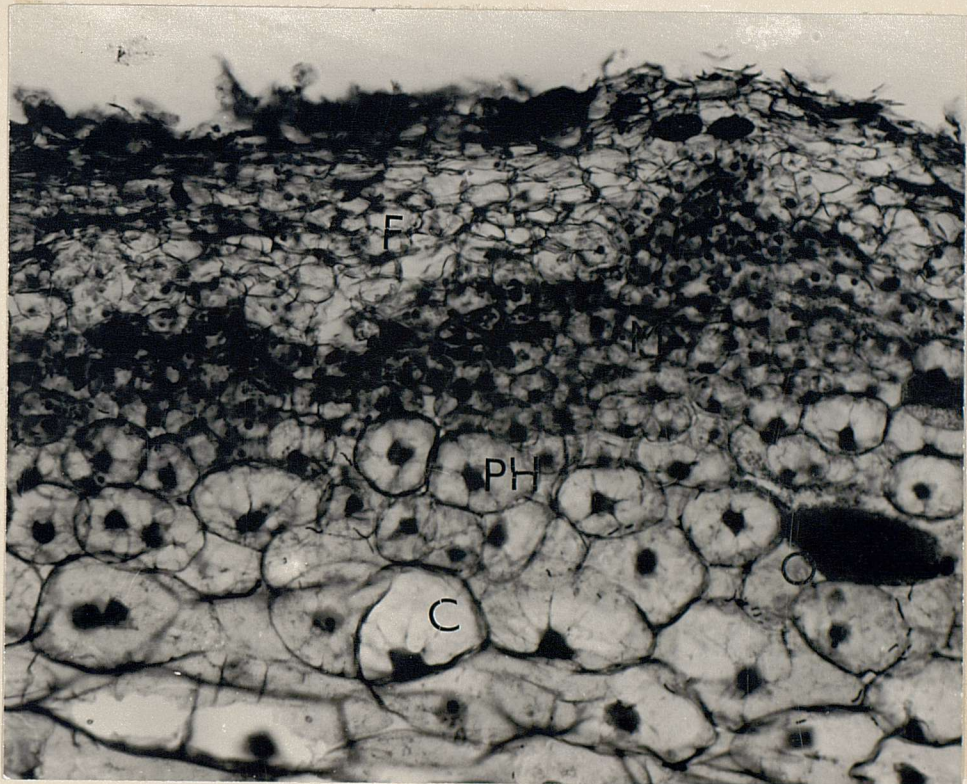


Plate 2.22

(x 160, light micrograph)

Lenticel of the 'normal type' from tuber stored at 80.5% R.H.

F, filling tissue; M, meristematic zone; PH, phelloderm;

C, cortex; O, calcium oxalate cell.

Plate 2.23

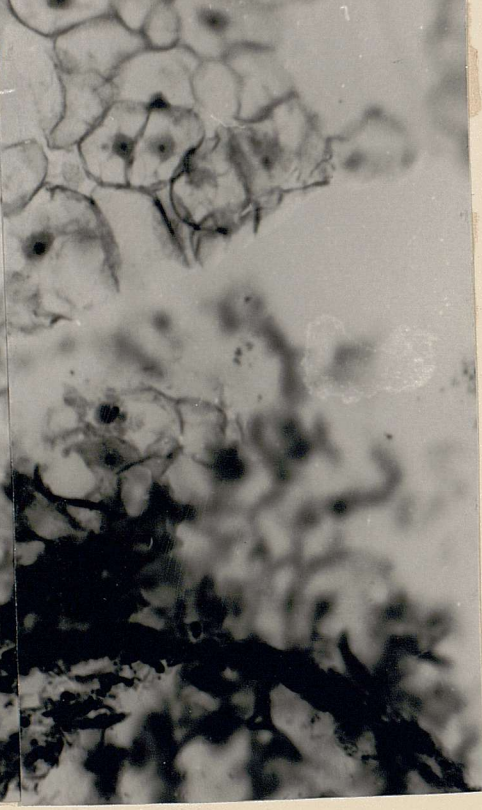
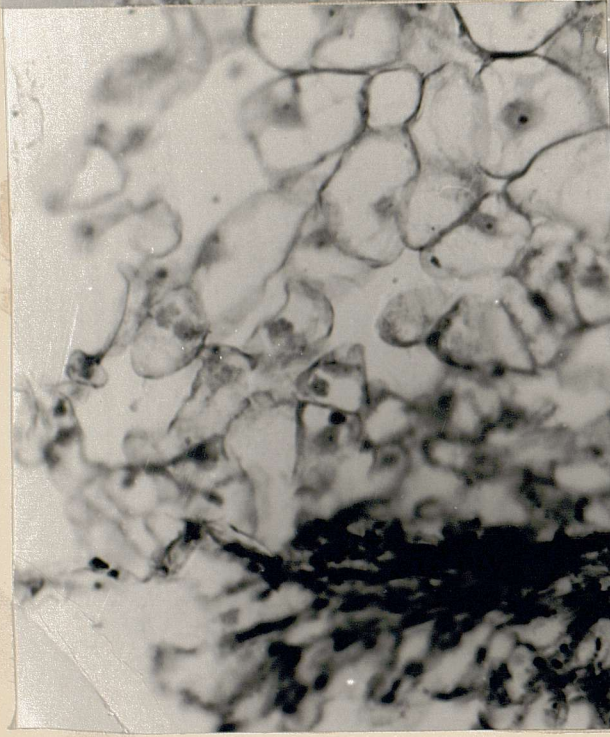
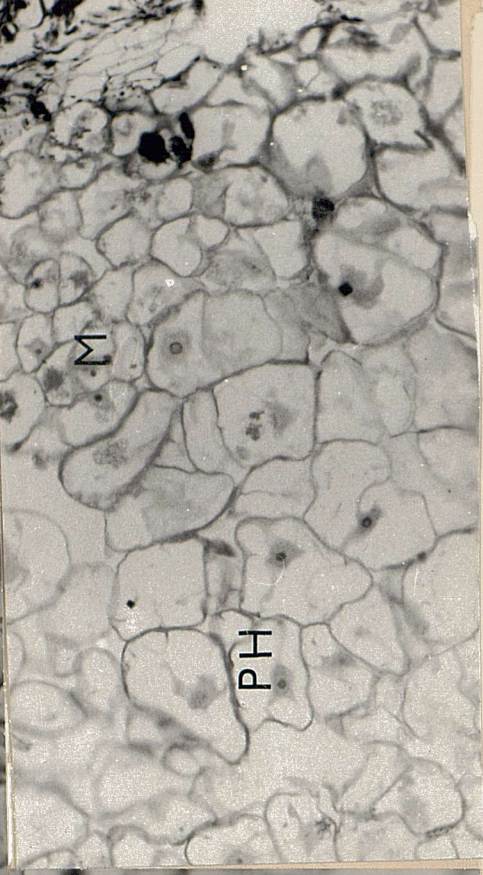
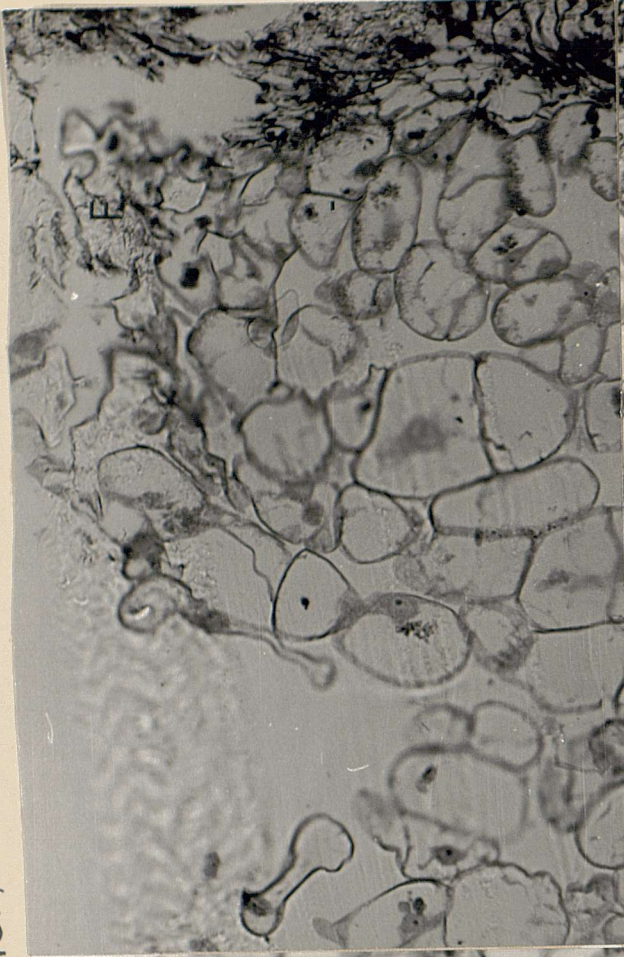
(x 252, light micrograph)

OVERLEAF,

'Proliferated' lenticel from tuber stored in damp sphagnum.

F, filling tissue; M, meristematic zone; PH, phelloderm.

EXTERIOR (TOP)



INTERIOR

(BOTTOM)



Plate 2.24

T.T.C. stained tangential sections beneath lenticels stored under different pretreatments before inoculation. Left to right, top; 0, 58.3, 80.5, bottom; 93.9, 97.5, 100 % R.H. N.b. 'ring' of infection at 80.5% R.H.

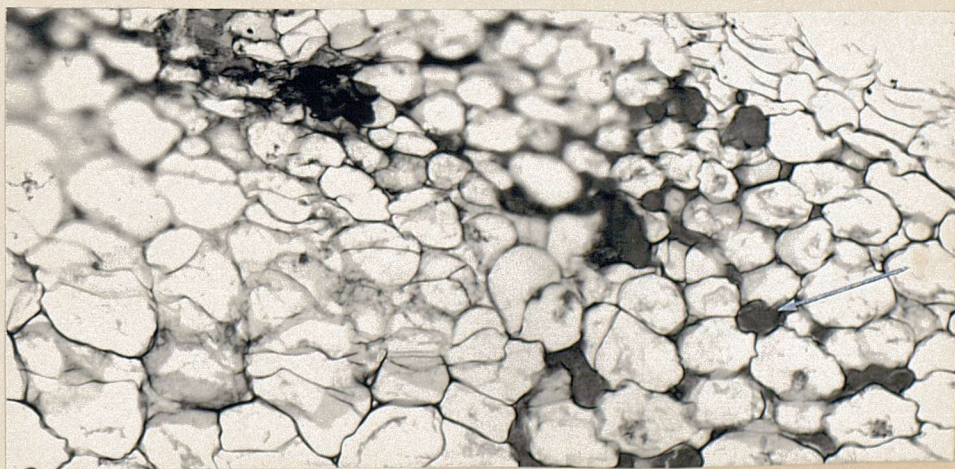


Plate 2.25

(x 120, light micrograph)

Section through 'ring' of infection between phelloderm of filling tissue and primary periderm, tuber pretreated at 80.5% R.H., bacterial colonies arrowed.

(carbol fuchsin, fast green.)

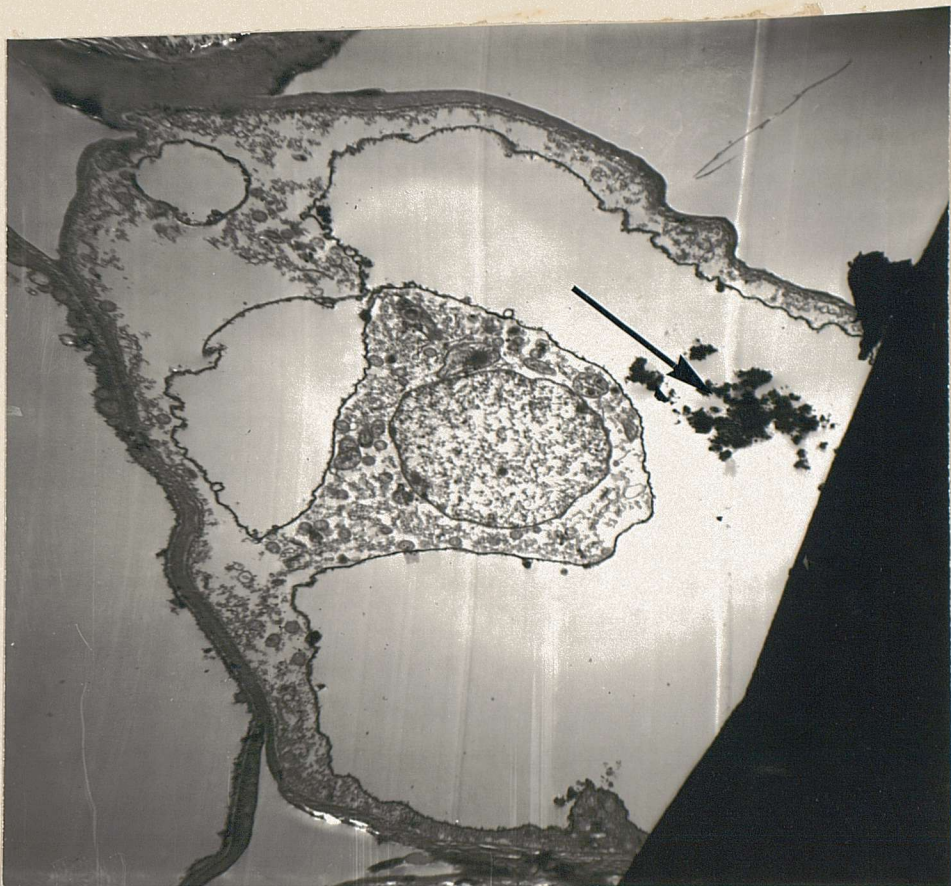


Plate 2.26 a.

(x 3,000)

Cell protruded from proliferated lenticel, showing large area of vacuole, lack of starch, melanin (arrowed) and small length of contact with neighbouring cells.

(osmic acid, lead citrate.)

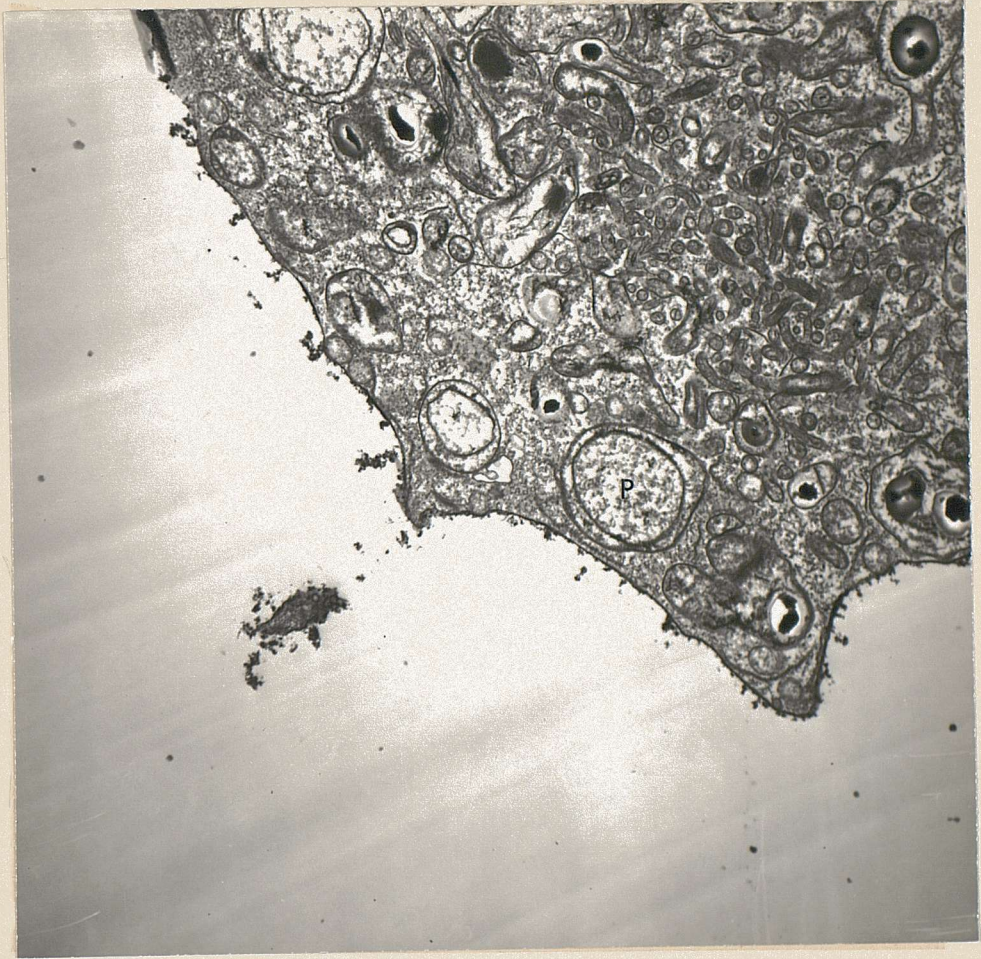


Plate 2,26 b.

(x 6,000)

p

Proliferated cell cytoplasm with abundant plastids.

(osmic acid, lead citrate.)

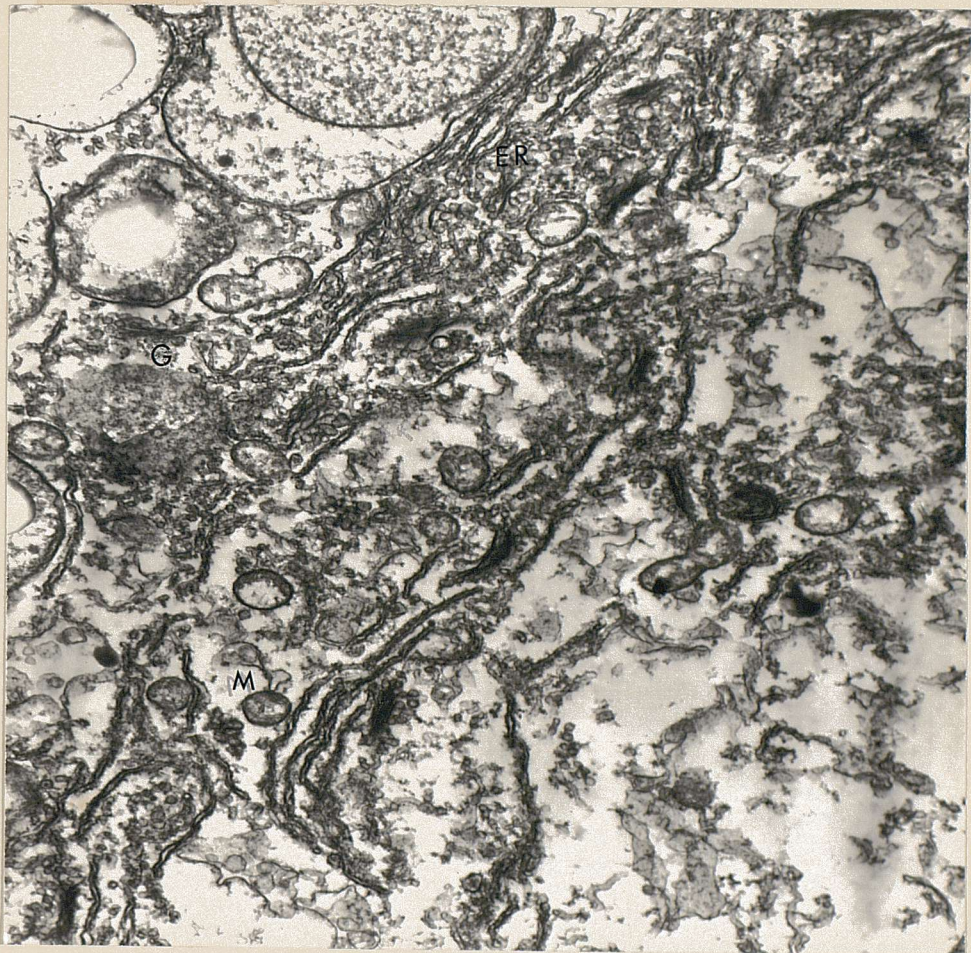


Plate 2.26 c.

(x 15,000)

Cytoplasm of proliferated cell, relatively intact, showing
endoplasmic reticulum, golgi bodies, and mitochondria.

(osmic acid, lead citrate.)



Plate 2.27

(x 8,250, electron micrograph)

Section through filling tissue cells of a lenticel
stored at 80.5% R.H., showing blockage of the inter-
cellular spaces, (unaged).

(osmic acid, lead citrate.)



Plate 2.28

(x 30,000, electron micrograph)

Filling tissue cell intercellular space with complete
plug of suberin.

(osmic acid, lead citrate.)



Plate 2.29 a.

(x 2,500, electron micrograph)

Early stages of suberisation in cells below the filling
tissue.

(osmic acid, lead citrate.)

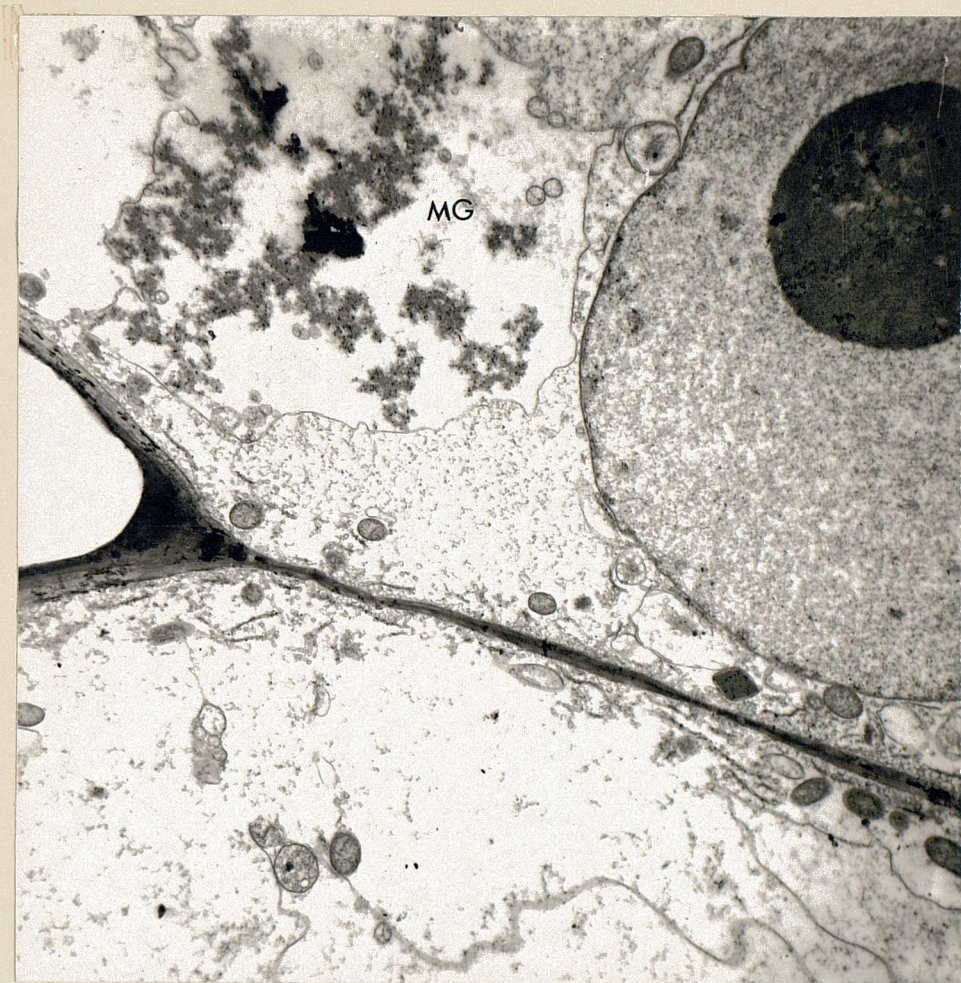


Plate 2.29 b.

(x 8,250, electron micrograph)

The vacuoles of the suberising cells beneath the lenticel
contain melanin granules, *MG*.

(osmic acid, lead citrate.)

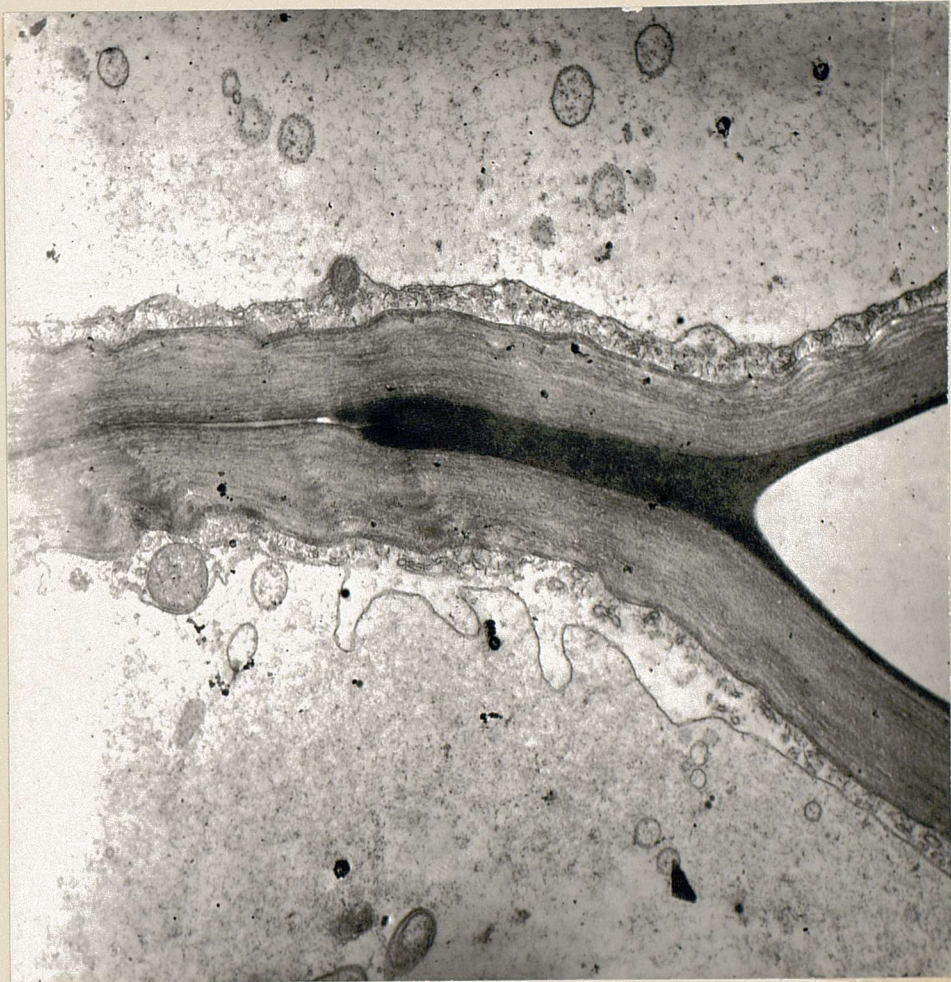


Plate 2.30

(x 25,000, electron micrograph)

An early stage of suberisation, at the intercellular space of a cell beneath the filling tissue.

(osmic acid, lead citrate.)

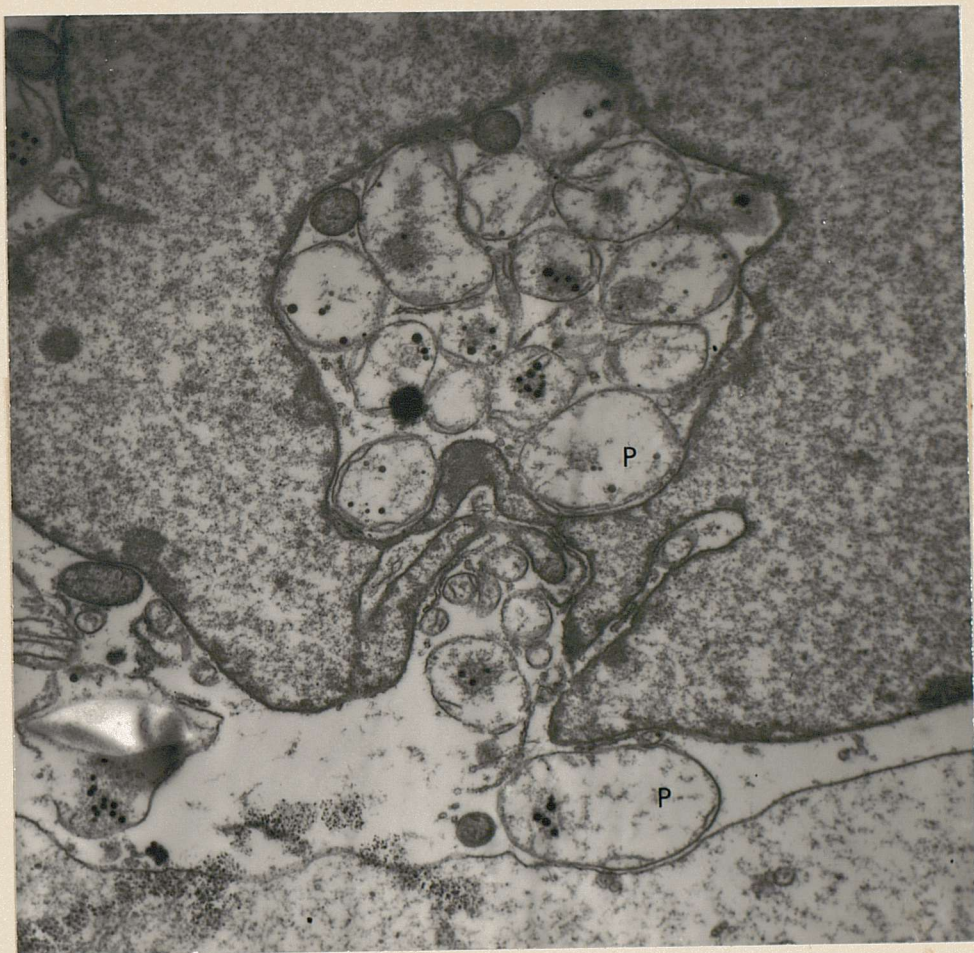


Plate 2.31

(x 8,500, electron micrograph)

The suberising cells below the filling tissue contain numerous plastids¹ clustered near the nucleus.

(osmic acid, lead citrate.)

cytoplasm (plate 2.26), nuclei, tonoplasts sometimes with melanin, and active plastids, though some may be rather disorganised cytoplasmically and the cell wall may be deformed, possibly due to lack of turgor pressure suggesting cell death.

Experiments 2.6-2.8.

Investigations into the effect of different relative humidity and temperature regimes, before and after inoculation, on the anatomy of the lenticel and its efficiency as a channel of infection.

Materials and Methods.

Grade 1 dry brushed King Edward tubers were placed under constant conditions for 3 weeks or more in enclosed glass relative humidity chambers containing dilute sulphuric acid of various concentrations, kept in incubators, inoculated by submersion in a suspension of bacteria for 10 mins, then replaced under controlled conditions before harvesting. On harvesting the lenticels were cut out using a 7 mm. cork borer and treated as follows:-

i) The lenticels, 10 of each chosen at random, were cut tangentially, so that the cells beneath the filling tissue were exposed; the slices were then placed in 1% 2:3:5 - triphenyl-tetrazolium chloride. If infection had occurred the dehydrogenase bound to the bacteria

was stained bright red.

ii) The lenticels were fixed in C.R.A.F., and ester wax embedded for light microscopy.

iii) The lenticels were fixed in 1% osmic acid in Kellenberger buffer with 5% sucrose, and embedded in araldite for light and electron microscopy. Some lenticels were treated for pectin by Albersheim's method.

iv) hand razor sections of the lenticels were cut and stained in neutral red or sudan III in order to observe cell death and suberisation.

Results. Experiment 2.6.

In this preliminary experiment tubers were stored for 22 days at 100% or 0% R.H. at 25°C. Inoculation by submersion in a 21 hour old culture containing approximately 8.96×10^8 cells per ml for 10 min was followed by further storage at 0% and 100% R.H. and 25°C. Harvests were taken after 2, 22 and 40 h. Infection, confirmed by methods i) ii) and iii) occurred only where initial storage at 100% R.H. was followed by subsequent storage at 100% R.H. Infection could be detected by the T.T.C. method only in the 22 and 40h samples where it was visible to the naked eye as a patch of approximately 3 mm diameter around the lenticel. The results at 40 h are given in Table 2.10.

Experiment 2.7.

Relative humidities of 100%, 97.5%, 93.9%, 80.5% 58.3%

Table 2.10

T.T.C. reactions at 40 h harvest of inoculated lenticels (25°C).

<u>% R.H.</u>		<u>T.T.C. reaction</u>				<u>Anatomy of lenticel.</u>
<u>Pre-</u>	<u>Post-</u>	<u>0</u>	<u>1</u>	<u>2</u>	<u>3</u>	
<u>inoculation.</u>						
0	0	10	-	-	-	Secondary periderm under dead tissue.
0	100	10	-	-	-	Secondary periderm.(c.f. plate 2.21)
100	0	10	-	-	-	Dead cells below filling tissue no soft rot damage apparent.
100	100	-	-	-	10	Soft rot damage evident under filling tissue to 1800 μ below surface.

Table 2.11

T.T.C. reactions at 50 h harvest of inoculated lenticels postinoculation incubation at 100% R.H. (25°C).

<u>% R.H.</u>		<u>T.T.C. reaction</u>				<u>Anatomy of lenticel.</u>
<u>Preinoculation</u>		<u>0</u>	<u>1</u>	<u>2</u>	<u>3</u>	
100		-	-	-	10	Normal division into filling tissue, meristem, phelloderm.
97		-	-	5	5	Normal.(c.f. plate 2.22)
94		-	4	6	-	Normal.
80		1	9	-	-	Normal.
58		9	1	-	-	Secondary periderm.(c.f. plate 2.21)
0		10	-	-	-	Secondary periderm.

Key. to tables 2.10-2.12. T.T.C.reactions; 0, no infection detectable, 1, 'ring' of infection (plates 2.4,2.5); 2, complete infection around the lenticel; 3, infection beyond lenticel.

and 0% were employed in preinoculation treatment lasting 28 days, the chambers were stored at 25°C. Inoculation was with a 23 hour old culture containing approximately 1.152×10^9 cells /ml, and after inoculation the tubers were placed at 25°C and 100% R.H then harvested after 2, 26 and 50 h.

The results of T.T.C. treatment showed a gradation of area of potato colonised according to the pretreatment; plate 2.24 and table 2.11 show the range at 50 h. After 50 h at 100% R.H there was extensive colonisation by bacteria, the affected area around the lenticels being 5 mm diameter; this was true also of the 97.5% and most of the 93.9% R.H. samples, but 4 of the latter displayed the same reaction as most tubers stored at 80.5% R.H. namely invasion confined to a ring of colonisation around the filling tissue. Light micrographs of this ring of infection showed that bacteria were present in the gap between the filling tissue and the primary periderm, (plate 2.25), suggesting that the filling tissue is impermeable to bacterial entry after 28 days at 80.5% R.H. a state of affairs in accordance with the results of the electron microscope study of the lenticel described earlier. After 58.3% R.H. or 0% R.H. for 28 days the lenticels were not infected in any way. The state of affairs after 22 hours similar to the

distribution of the types of infection, but their extent at the higher R.H.s was slightly less pronounced being approximately 3mm diameter; after 2 h there was not sufficient infection to be visible after T.T.C. treatment.

When the anatomy of the samples was examined under the light microscope using fresh and araldite sections the 100%, 97.5%, 93.9% and 80.5% R.H. pretreatment specimens were apparently identical, but secondary periderm was evident at 58.3% and 0% relative humidity, beneath the filling tissue.

Experiment 2.8.

In this complementary experiment, tubers were pretreated by storage for 28 days at 25°C and 100% R.H. followed by inoculation by submersion in a 31 hour culture containing approximately 1.185×10^9 cells/ml. The tubers were then stored at 25°C at 58.3%, 80.5%, 93.9% and 97.5% R.H., and harvested after $3\frac{1}{2}$, 44, and 71 h.

After $3\frac{1}{2}$ h there was slight infection in all the 97.5% R.H. posttreatment samples, two showing complete lenticel colonisation, and four of the 93.9% R.H. sample also showed slight colonisation. There was no infection evident in any of the other samples.

The situation after 44 h is shown in Table 2.12.

Table 2.12

T.T.C. reaction at 44 h harvest of inoculated lenticels
preinoculation incubation at 100% R.H. (25°C):

<u>% R.H.</u>	<u>T.T.C. reaction</u>				<u>Anatomy</u>
<u>postinoculation.</u>	<u>0</u>	<u>1</u>	<u>2</u>	<u>3</u>	
97	-	-	10	-	Normal.(c.f.plate 2.22)
94	4	-	6	-	Normal.
80	7	-	3	-	Normal.
58	10	-	-	-	Normal.

After 71 h there was extensive colonisation of lenticels at all relative humidities, the zone of infection in each case being greater than the diameter of the lenticel, i.e. over 3 mm. When 120 h had passed all the lenticels were so far infected as to have been depressed below the general surface of the tuber.

Experiment 2.9.

In this experiment, tubers were kept at 5,10,20 and 25°C for 29 days at 100% R.H., were inoculated by submersion in a 36 h culture containing approximately 1.547×10^9 cells/ml., then kept at either 55 or 25°C for 2, 29 or 72 h at 100% R.H.

T.T.C. treatment demonstrated that after 2 h there were traces of infection in all samples. In 29 h and 72 h samples this was extensive, having spread outside the lenticels, to about 5mm in the latter. The lenticel anatomy of all samples was of the 'normal' type.

Conclusions.

It was concluded that entry through the lenticel depended on the nature of its internal anatomy, and in some cases ultrastructure, which appeared to be controlled by the ambient relative humidity. In the first experiment, where the relative humidities of storage before inoculation varied resulting in the formation of secondary periderm at lower humidities,

infection was only possible where this barrier was absent. The resistance due to the secondary periderm was not broken by the passage of time. At 80.5% relative humidity pretreatment it appears that the filling tissue itself is a block to infection, but the bacteria may enter between this and the primary periderm.

Where there was pretreatment at 0% R.H. and 58.3% before inoculation, infection appeared to be curtailed by the development of a secondary periderm under the filling tissue, this appeared similar to primary and wound periderm described earlier. It is evident that even at very low humidities a periderm sufficient to block bacterial entry can be formed over a period of weeks if under suberised filling tissue.

Pretreatment at 100% R.H. before inoculation appears to favour the orthodox production of filling tissue, which appears to be less of a barrier to infection than the secondary periderm developed at lower relative humidities.

When the relative humidities were varied after inoculation only temporary inhibition of infection occurred at relative humidities below 80.5% R.H. After 120 h all of the lenticels kept at different post-treatments were rotted sufficiently for their depressions to be visible to the naked eye.

It appears that posttreatment at 0% R.H. may prevent subsequent infection by drying up the inoculum. There is also the possibility that the death of the cells below the filling tissue could prevent infection by releasing the chlorogenic and caffeic acid present in their tonoplasts; infection does not however appear to be checked by a special suberin barrier.

The effect of temperature, if any is of a much lower magnitude, and probably affects the tuber generally rather than the lenticel in particular.

Differences in temperature and posttreatment did not strikingly affect the anatomy of the lenticels, and it appears that the temporary check on bacterial entry caused by low R.H. after inoculation is a direct one on the pathogen possibly caused by the drying up of the inoculum.

PART 3. Initial colonisation of the potato tuber.

Part 3. Initial Colonisation of the potato tuber.

Introduction.

Once the bacteria have entered the potato tuber there is a period of initial colonisation before the cells are completely macerated. This period is of interest as it is here that the resistance induced in the host begins to take effect.

Tubers were inoculated by submersion in a bacterial suspension as this method avoids the complications due to the inoculum drying up, and provides an amount of inoculum large enough to be non-limiting; comparative measurements were made of the depth beneath fresh wounds and lenticels that bacterial colonies were formed. In this way it was hoped the innate morphological and other differences between these two channels and their effect on the initial stages of colonisation would be established. The effects of temperature on these initial stages were established.

The relative efficiency of these channels for the entry of a known limited amount of inoculum, in a situation where drying out of inoculum was important was also investigated.

The results of the two methods, one involving a comparison between a finite inoculum and the other a

practically infinite one are compared and discussed in relation to situations occurring in the field.

Experiment 3.1.

To calculate the rates of initial colonisation through fresh wounds and lenticels.

Materials and methods.

Grade 1 dry brushed King Edward tubers were washed, surface sterilised in 0.1% mercuric chloride solution, then washed in sterile distilled water, dried, then inoculated by submersion in a 24 h culture either whole or in slices. After a period of inoculation of up to 10 days, these were removed from the suspension, fixed in Randolph's C,R.A.F., embedded in Ester Wax (1947), and stained in carbol fuchsin and fast green. Using a micrometer eyepiece, the perpendicular distances from the surface of the furthest bacterial colonies next to apparently healthy tissue were measured. In the case of lenticels the area covered lay between perpendiculars dropped beneath the filling tissue. From these perpendicular distances the rates of colonisation were calculated for lenticels and fresh wounds at different temperatures.

Results.

The results expressed in Table 3.1.- graph 3.1. show that the rate of colonisation varies at different

Table 3.1

Fresh wound channel of entry;

Mean distance colonised (μ) at different temperatures.

<u>Temp. ($^{\circ}$C)</u>	<u>Exposure to inoculum (h)</u>						
	<u>24</u>	<u>25</u>	<u>36</u>	<u>48</u>	<u>121</u>	<u>143</u>	<u>165</u>
5	354.8	362.1	508.6	534.3	768.0	1033.0	/
10	329.8	396.6	554.7	547.5	1295.1	1592.0	1437.3
20	302.0	431.0	/	777.6	1575.2	1392.0	1427.2
25	447.4	349.5	892.6	1135.1	1231.0	1048.0	1556.0

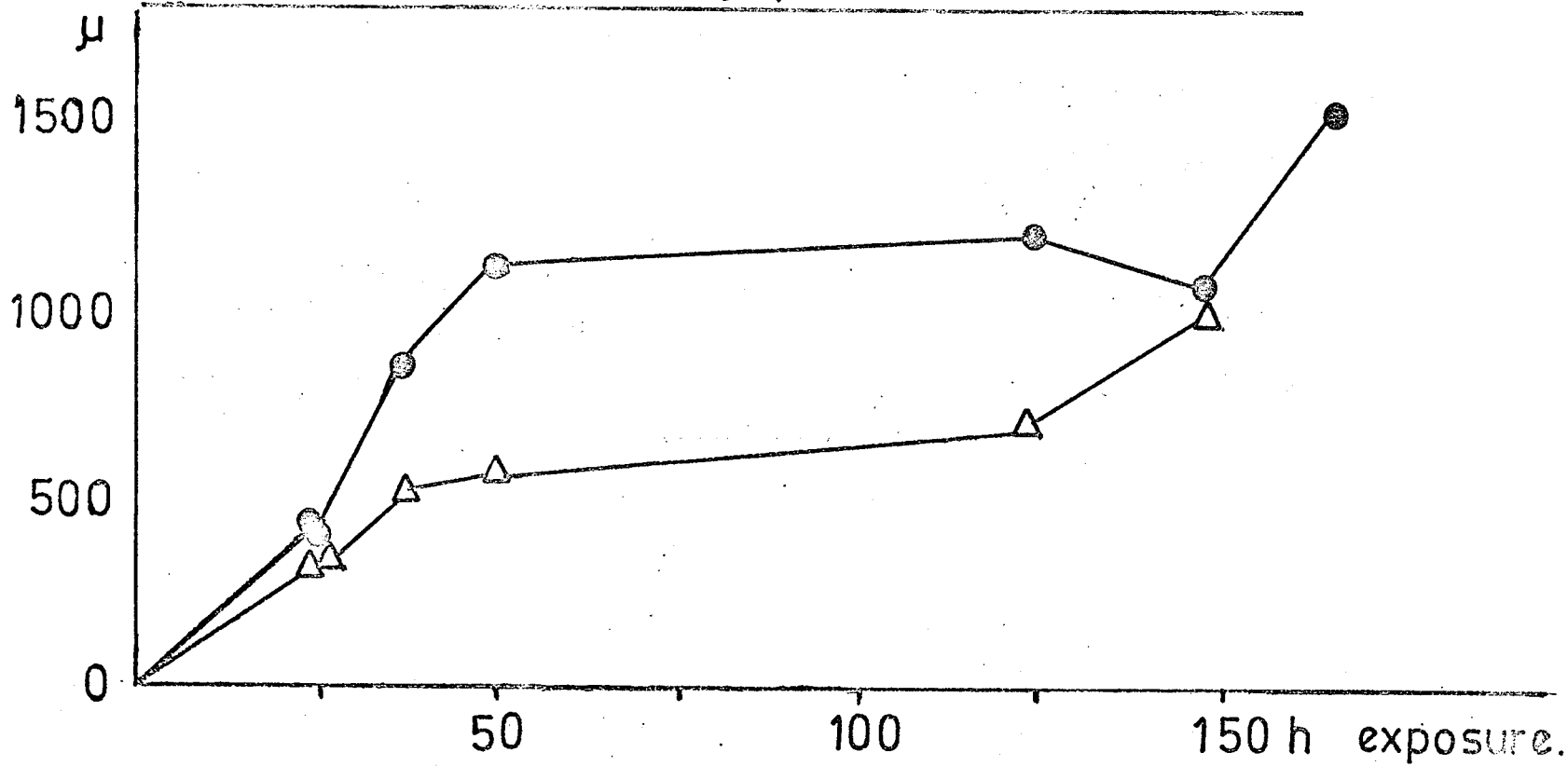
Table 3.2

Fresh wounds and lenticels;

Mean distances colonised (μ)

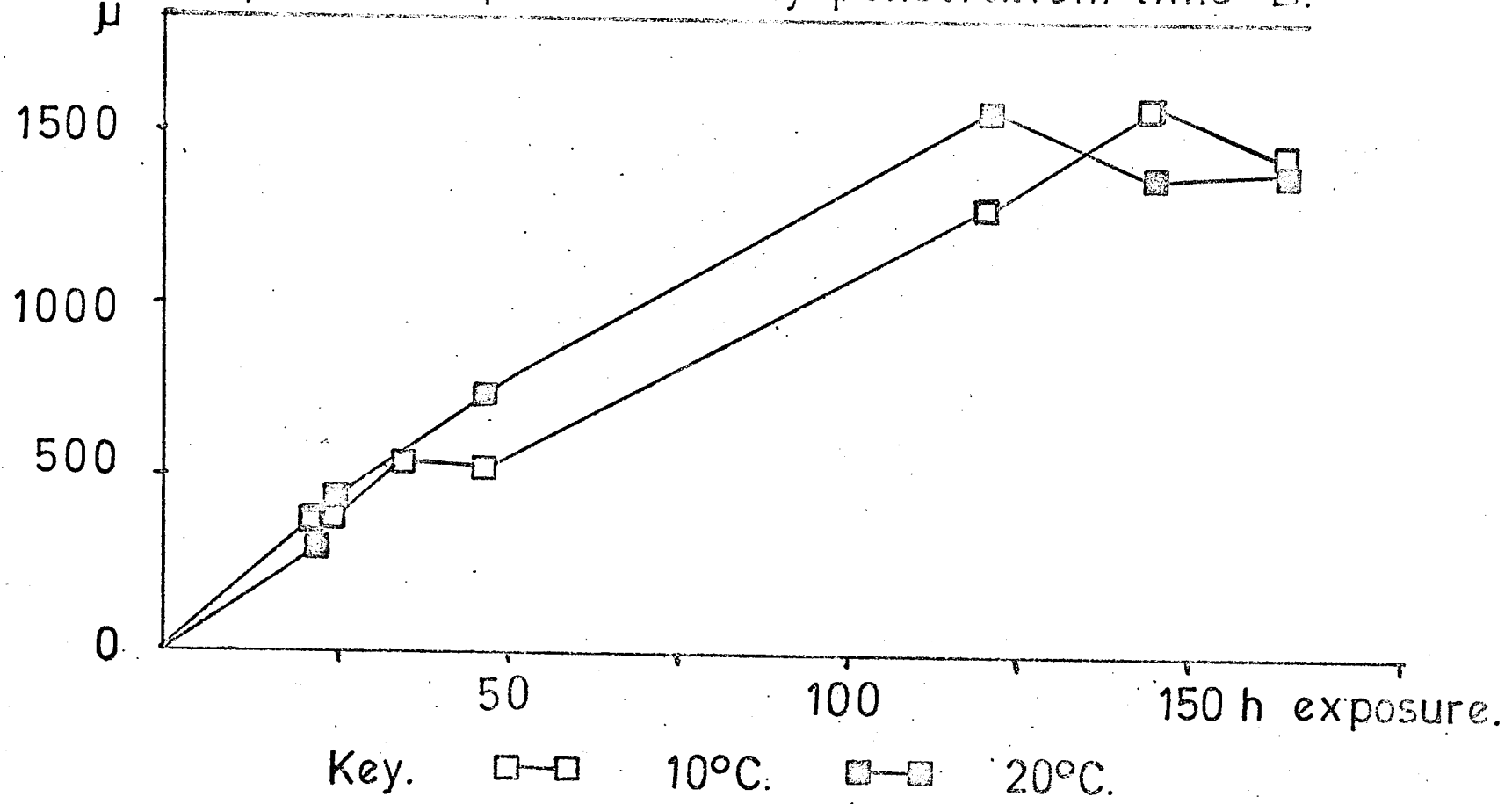
	<u>Exposure to inoculum (h)</u>									
	<u>11</u>	<u>19</u>	<u>21</u>	<u>24</u>	<u>28</u>	<u>39</u>	<u>41</u>	<u>46</u>	<u>48</u>	<u>62</u>
<u>Fresh wounds</u>										
	39	123	241	291	408	468	529	524	529	711
<u>Lenticels</u>										
	253	344	264	424	578	543	360	649	464	614

Graph 3.1 Depth of colony penetration/time. A.



Key. $\triangle-\triangle$ 5°C. $\bullet-\bullet$ 25°C.

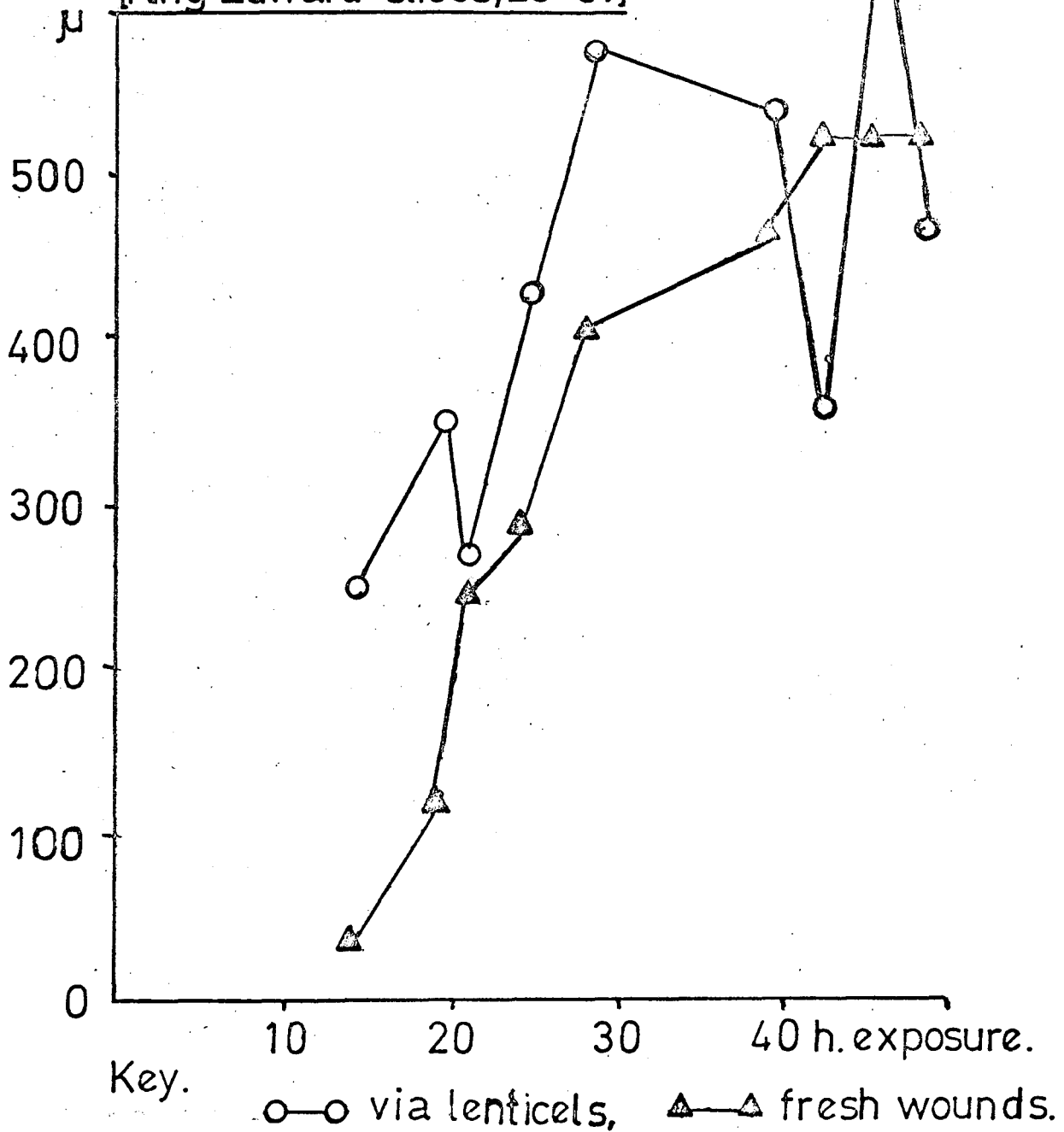
Graph 3.1 Depth of colony penetration/time B.



Graph 3.2 A.

Depth of penetration of colonies.

[King Edward slices, 25°C.]



Graph 3.2 B. Rates of colonisation/time.
[King Edward slices, 25°C]



temperatures. At lower temperatures the colonisation does not appear to be checked as early as at 25°C where the rate falls off after 60 h.

When the mean distances of colonisation through fresh wounds are compared with those through the lenticels (table 3.2), and graphs of the rates drawn (graph 3.2), there appears to be an initial difference in rates of colonisation which later disappears. The nature of the two channels is sufficient to explain this difference in rates, the larger intercellular spaces around the lenticels allowing a higher rate at first which falls off as the tissue encountered becomes more typical parenchyma.

Both the average rate of colonisation through the lenticels and that through the fresh wounds, $16.15 \mu\text{m}/\text{h}$. and $11.34 \mu\text{m}/\text{h}$. respectively, are far slower than the rate of movement of the bacterium diffusing through a liquid. Adler & Dahl (1966), using glucose found that ~~it~~ diffused at $1730 \mu\text{m}/\text{h}$., whereas Escherichia coli moved at over $700 \mu\text{m}/\text{h}$. measured by the same method, which involved movement over in a capillary tube, using a microscope and clock, the distance moved was over $10,000 \mu\text{m}/\text{h}$., Ogiuti calculated the latter as $9,000 \mu\text{m}/\text{h}$. From this it appears that the passage of bacteria through the tuber is unlikely to be unrestricted diffusion.

The actual distance that the bacteria travel intercellularly is likely to be a factor of approximately $\pi D/2$ more than the distances quoted above, where D is the sum of the diameters of the cells traversed, as the path taken by colonising bacteria must be between, rather than directly through, the host cells. This increased figure, of course does not bring the rate equal to that explicable by unrestricted diffusion.

The next series of experiments were to determine how efficient the different channels are quantitatively in allowing inoculum to enter the tuber.

Experiment 3.2.

To determine the relative amount of inoculum entering through different channels of infection.

Method.

Dry brushed grade 1 King Edward tubers from a store at 5°C and 78% R.H., were washed in tap water, surface sterilised in 0.1% mercuric chloride, washed in sterile distilled water, then dried. Van Tieghem rings were then stuck to surface of the tuber with ester wax over various types of areas, such as periderm with no lenticels, the eye region, area with lenticel, fresh wounds etc.. Other types of adhesive, including vaseline, which melted away, and 'depex' which was toxic to the tuber, were tried without success and were

discarded in favour of ester wax. The 'wells' formed by the van Tieghem rings were inoculated with 0.5 ml of an 18 h culture containing 4.32×10^5 cells per ml washed off a nutrient agar slope culture with sterile distilled water. The tubers were placed in a relative humidity chamber at 100% R.H. and 25°C.

Results.

After five days the mean amounts of inoculum left in the well were as follows.

- i) periderm with no lenticels. 0.20 ml.
- ii) periderm with one lenticel. 0.13 ml.
- iii) periderm with two lenticels. 0.09 ml.
- iv) wound, two days old. no inoculum present.
- v) fresh wound, no inoculum present.
- vi) eye region with leaf scar. 0.01 ml.
- vii) glass slide control. 0.5 ml.

Conclusions.

It appears that the liquid component of the inoculum may enter the tuber readily at fresh and recently healed wounds, almost as readily at the eyes, and more readily through the periderm where two lenticels are present than one, and more where one is present than none. These results confirm the findings of the gentian violet experiment described earlier, but the high rate of absorption here may be due to active uptake of water.

The initial colonisation of the tubers in the field may well depend upon active uptake of inoculum by the host. In the case of gentian violet, particles only entered through fresh wounds and the lenticels, so that even if active uptake does operate to draw water into the tuber then this can only initiate infection if large enough gaps are available for bacterial entry.

Discussion.

Among other factors it appears that the initial rate of colonisation depends on the channel of infection, the temperature; and perhaps the ability of the underlying tissue to take up water. There is no single relationship between the rate of this apparent active up-take of inoculum liquid by the different channels of infection and the bacterial colonisation of the underlying tissue. For example, both the periderm and the eye region appear to take up inoculum, but neither show bacteria on sectioning, presumably since they can absorb water but not bacteria.

Initial colonisation is a stage in potato soft rot that deserves further study as the early stages must take place in the absence of defence systems which develop after infection.

, The part played by the host in taking up inoculum appears to be an important factor under field conditions

where organisms may be scattered through the soil water. This facet of infection requires further investigation, as does the incidence of Erwinia in soils under all conditions especially when the inoculum is very low.

PART 4. Spread inside the tuber.

Part 4

Spread inside the tuber.

Introduction.

The invasion of the interior of the potato by the bacterium appears to follow a common course regardless of the mode of entry.

Once inside the tuber the bacterium appears to be confined to the intercellular spaces during the early stages of infection. Infection is not uniform in any area of tissue more than a few cells in extent. This is visible both at the cellular and subcellular levels. This was found when the effect of temperature on cytoplasmic damage in apparently uniform tissue, at given levels in the tuber from the infection front was then investigated. Different states of cytoplasmic damage could be seen in adjacent cells at any one level. An intercellular space colonised by bacteria is surrounded by adjacent intercellular spaces and primary cell walls which show signs of rotting without bacteria being present, therefore the number of bacteria present in an electron micrograph may not reflect the extent of cytoplasmic damage in the particular level being examined.

Colonisation of the intercellular spaces progresses in a well defined way; the intercellular spaces become

enlarged and portions of the primary cell wall leading from them are dissolved, permitting the bacterium to move into the newly created space. This can be seen in light micrographs where the bacteria can also be seen to be aligned in one direction (plate 4.1).

At no time do the Erwinia cells enter the host cells, except in the case of the cells containing calcium oxalate discussed later. In the final stages of rotting, Bacillus may enter the host cells and form spores (see part 7).

Spread through vascular tissue and parenchyma ; an assessment of relative importance and mechanisms.

Vascular tissue.

As well as the steady spread of soft rot through the cortex, storage parenchyma and pith cells of the tuber, there may be narrow 'horns' of rot extending up the vascular strands. Using light and electron microscope techniques, the nature of this rot was studied; the vascular parenchyma can be seen to be much better preserved than the storage parenchyma which is macerated and shows cytoplasmic damage (plate 4.3a). This was confirmed by using the same material for electron microscopy (plates 4.3 b & c). Some phloem sieve plates in this rotted vascular tissue was blocked with callose (plate 4.4), which appears similar to that described by

Esau and Cheadle (1965) in electron micrographs of Cucurbita, healthy tissue. Callose may also be seen in healthy tuber phloem of potato (plate 4.5).

The effects of the vascular rot on the storage parenchyma are localised and the structure of the cytoplasm is not particularly badly damaged even at 25°C, as little as 250 µm away from the vascular tissue (plates 4.6 & 4.7).

Staining with ammoniacal gentian violet reveals that the extent of the suberisation is more extensive in the vascular tissue than in the storage parenchyma (plate 4.8); osmic acid staining as well as showing suberin also shows melanin granule containing cells which may or may not be abundant (plate 4.9) in the storage parenchyma; their occurrence accounts for the dark colour of the vascular rots. The melanin reacts to both Lillie's and Gomori's tests the former being diagnostic. (Lillie,1957; Gomori;1952).

Conclusions.

It appears that the spread of the pathogen through the vascular tissue is through the sieve tubes and vessels, but is confined to them by suberin and perhaps polyphenol oxidation products, the former in the vascular parenchyma and the latter in the storage parenchyma. Pectolytic activity is not so confined and

may cause localised rotting of the adjacent storage parenchyma, but with less than complete cytotoxic damage. If healthy vascular issue is sectioned, under the electron microscope it can be seen that vascular parenchyma is small-celled with no intercellular spaces and appears to be suberised as is some of the adjacent storage parenchyma (plates 4.10a & b, and 4.11). This is confirmed by sections stained with ammoniacal gentian violet; hand sections stained with Hoepfner-Vorsatz reagents indicate a locally high concentration of cells containing polyphenols in their vacuoles, but melanin although present is rare. It is evident from this that the infection is not contained due to a host reaction barrier but rather to the normal anatomical structure of the vascular tissue. Vascular tissue does not appear to be the major point of spread in the tuber, but rather is a secondary effect of the spread through the parenchyma.

The Parenchyma.

In contrast to the localised spread of inoculum through the vascular tissue by rapid intracellular movement, the majority of the bacteria spread intercellularly through the storage parenchyma. This spread is at first entirely confined to the intercellular spaces with very little inoculum between. If the advancing edge of a 121 h old infection is sectioned, young intercellular colonies are

found(plate 4.12). The bacteria appear to occupy a central zone (1) which is heterogeneous, with some of the material contiguous with the outer homogeneous zone (2) which is in turn enclosed inside the cell wall in the newly created space to one side of the original intercellular space (3). Whether this zonation is an artifact due to the reaction of the original structure with the fixative, or due to shrinkage during dehydration and embedding, or is a natural structure, perhaps due to the zonation of breakdown products of pectolysis, is not conclusively determined. The former seems unlikely however, due to the good preservation of the individual cells which as pointed out by Kellenberger are very sensitive to fixation and embedding techniques. In the adjacent secondary sites of rotting (plate 4.13 a,b.), where there are no bacteria present, there is no central zone, only zones 2 and 3 are present.

After the rot has begun to macerate the potato storage parenchyma tissue as in the 36 day old sample, taken approximately 5mm behind the advancing edge, where the tissue is still coherent, this discrete zoned nature of the small colony has disappeared (plates 4.14 a,b,& c). Whether only the discrete colony is active pectolytically, and is surrounded by the zoned products of pectolysis, which are absent when the primary cell wall has been

completely utilised, or whether the discrete colony is broken up by staling product poisoning in the older infection has not been determined. In later stages of infection, the bacteria may occupy the former position of the primary cell wall which has been etched away, as well as the old position of the intercellular space.

In the terminal stages of infection Bacillus cells may be seen inside the cells but this behaviour is not characteristic of Erwinia except in the vascular tissue and in the calcium oxalate cells of the cortex. Both of these exceptions are described later in more detail.

Conclusions.

From the evidence it appears that the spread of infection through the tuber is primarily intercellular through the parenchyma, but vascular infection does occur without much attendant bacterial spread, due to the nature of the vascular anatomy.

Intercellular spread through the parenchyma is of at least two phases, the first comprising small compact zoned colonies, the latter is dispersed and unzoned; the former is associated with active spread, the latter with the terminal stages of maceration.

The processes of tissue degradation are fully discussed in the next section, but it appears evident that the extent of the effects of the rot at any one level are

not homogeneous; each colony has secondary effects on adjacent cell intercellular spaces, and cytoplasmic damage also radiates from foci.

Calcium oxalate containing cells and their colonisation, and possible relevance to individual tuber susceptibility.

Introduction.

Erwinia is in general intercellular, not intracellular in the potato, as already noted in the early stages of infection, but Paton (1969) observed that certain potato tuber cells become internally colonised. In these cells, about 1% of the total in the tissue, the pathogen appeared to multiply rapidly and to be extremely motile; the cells were opaque in hand section. This description fits the cells (plates 2.22 & 4.15) found between the phellem and cortex of the tuber, which are the only cells to be entered during the early stages of infection (plate 4.16). These cells are most common beneath the lenticel and may be seen independent of variety, season or storage pretreatment of the tuber. A tangential cut beneath the periderm reveals these cells as white, evenly spaced particles easily visible to the naked eye. Under oil immersion the cell contents can be seen to be 'heart shaped' crystals 2-10 um long; apparently embedded in mucilage. When treated with

corallin in 4% sodium carbonate, the crystals are negatively stained against a pink background indicating mucilage is present (McLean & Ivimey-Cook 1952). When quinalizarin in ammonical solution is used in a dye-lake reaction a blue stain, characteristic of calcium oxalate, (Broda, 1939) results. Using a polarising microscope and oils of different refractive indices, the refractive indices of the crystals placed on a glass slide were found to be $x = 1.515 - 1.532$, $y = 1.501 - 1.620$, $z = 1.626 - 1.657$; these fall very near those of calcium oxalate monohydrate ($x = 1.491$, $y = 1.551$, $z = 1.645$). The crystals are also vitreous, pearly, yellowish, soluble in 1 N HCl and H₂SO₄ but not in distilled water in common with calcium oxalate monohydrate (Winchell, 1954). If crystals on a glass slide are coated with carbon, shadowed with Pt/Pd floated off from N/10 HCl and examined under the electron microscope the shape of the crystal is also that described for calcium oxalate monohydrate, i.e. (plate 4.19), simple prismatic or orthorhombic, the faces being irregularly developed. When crystals or sections are scanned using an X ray spectrometer, calcium is found to be present in the crystals and in the sections where crystals are present. X ray diffraction studies were marred by the presence of starch in the

samples, which obscured the pattern.

Pathology of the Calcium oxalate containing cells.

As Erwinia does enter these cells (plate 4.16), and the view has been expressed that free calcium is important to bacterial spread in the potato (Paton,1969) a survey of bacterial colonisation in relation to the abundance of the cells in a particular lenticel appeared essential. This seemed especially important as there is some individual variation in rate of penetration through this channel of infection during its early stages compared to fresh wound entry (graph 3.2) in samples of similar anatomical disposition. The survey was made on Randolph's CRAF fixed tubers inoculated for various lengths of time by submersion in an 18 h Erwinia suspension at 25°C embedded in 1947 Ester Wax, and examined as 12 um sections stained in carbol fuchsin and light green; this staining procedure was adopted as the bacteria stain green and the calcium oxalate crystals become intense yellow. Two points were investigated:

i) do the calcium oxalate cells provide a source of energy for the bacteria and if so is the number of 'crystal cells' diminished by colonisation of the lenticel?.

ii) is the number of 'crystal cells' a factor in

determining the number of bacterial colonies present or the depth at which these are found vertically beneath the lenticel?. The maximum depth of entry was defined as the vertical distance between the potato surface and a bacterial colony, one developed sufficiently to be seen under the oil immersion objective (x 900), within a few cells of the infection interface. Sections through the middle of lenticels submerged for 14-28 h and 39-62 h were compared, a sample of 39 in the former and 35 in the latter.

The mean numbers of calcium oxalate cells present were found to be 1.8 and 2.2 cells/section respectively. It was therefore assumed that although the surrounding cells were macerated and their contents completely destroyed this was not true of the calcium oxalate cells.

There was no significant correlation between the number of calcium oxalate cells and the number of bacterial colonies or their depth of entry even at $P < 0.10$, as judged by a t-test on 19 hour submersion infected lenticels. This confirms the view that the colonisation of the calcium oxalate cells is possibly due to the nature of the cell wall rather than that of the contents. Under the oil immersion there did appear to be a thinner cell wall round the calcium oxalate

cells than the surrounding cortex, but this could not be more critically proved as the crystals locally blunted the glass knives used on the ultratome, making thin sections of these cells impossible to obtain.

The possibility remained that the calcium oxalate cells could promote bacterial growth by increasing the concentration of available calcium but it was assumed that the decrease in the pH necessary for this event would not permit the bacteria to survive in the vicinity which they evidently do in the lenticel (plate 4.16).

In cortical cells of the potato tuber isolated crystals may be found inside microbodies in the cytoplasm and these survive soft rotting. There is no proof that these are calcium oxalate crystals but it appears that cells which contain large quantities of calcium oxalate are surrounded by cells which contain this substance in the form of individual crystals. Plates 4.17 & 4.18, show these crystals in healthy and rotted tissue respectively and their shape is compatible with the replicas in plate 4.19.

Conclusion.

It was concluded that calcium oxalate cells play no great part in Erwinia colonisation and cannot explain the individual irregularities in the rate of entry

through the lenticel which must be due to the influence of an unknown factor. The complex nature of the state of suberisation of the cells of the filling tissue, phellogen, and the area between them and the primary periderm and the effects of the environment on this, have been discussed earlier and may be this factor could be an expression of the different histories of different tubers within a single batch rather than something more tangible.

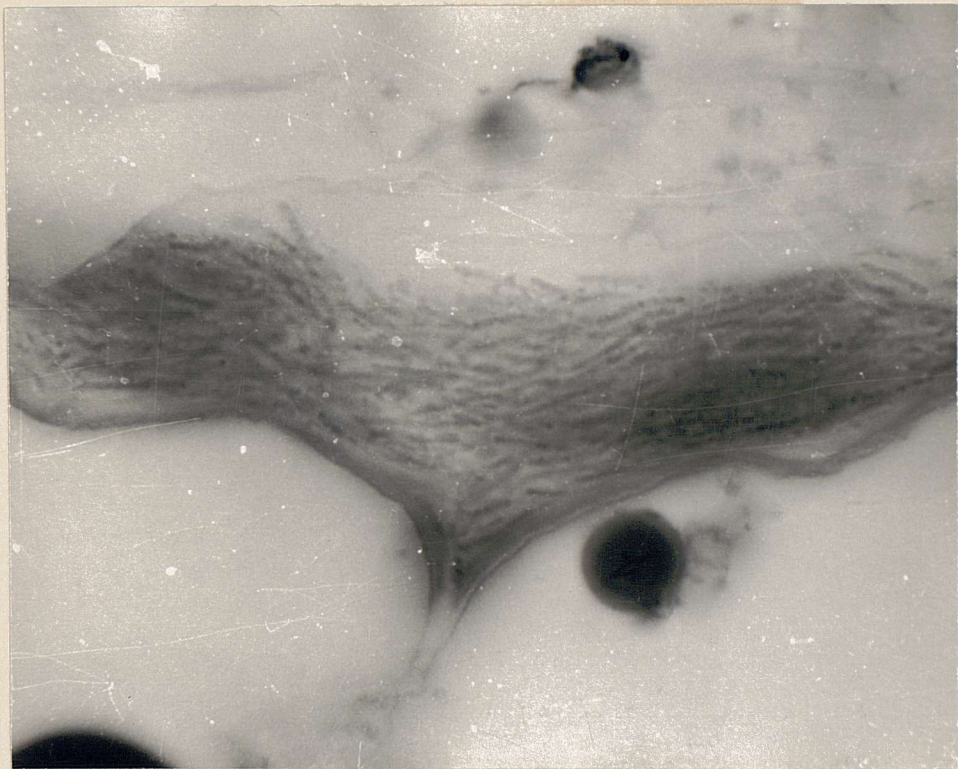


Plate 4.1

(x 1,500, light micrograph)

Longitudinal section through bacterial colony in soft rotted tissue, the individual Erwinia cells are generally aligned in the same direction.

(carbol fuchsin, fast green.)

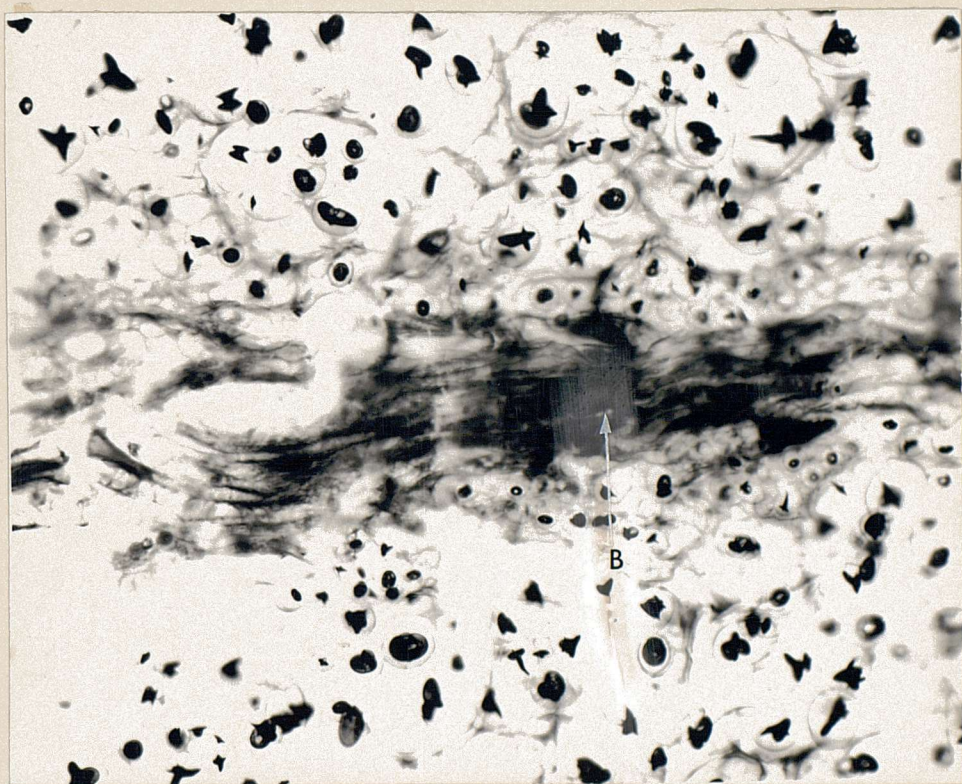


Plate 4.2 a.

(x 120, light micrograph)

Longitudinal section through infected vascular tissue
showing bacterial colonies in the xylem and phloem elements.

(carbol fuchsin, fast green.)



Plate 4.2 b.

(x 6,000, electron micrograph)

Transverse section through an infected secondary thickened
xylem element, δ .

(osmic acid, lead citrate).

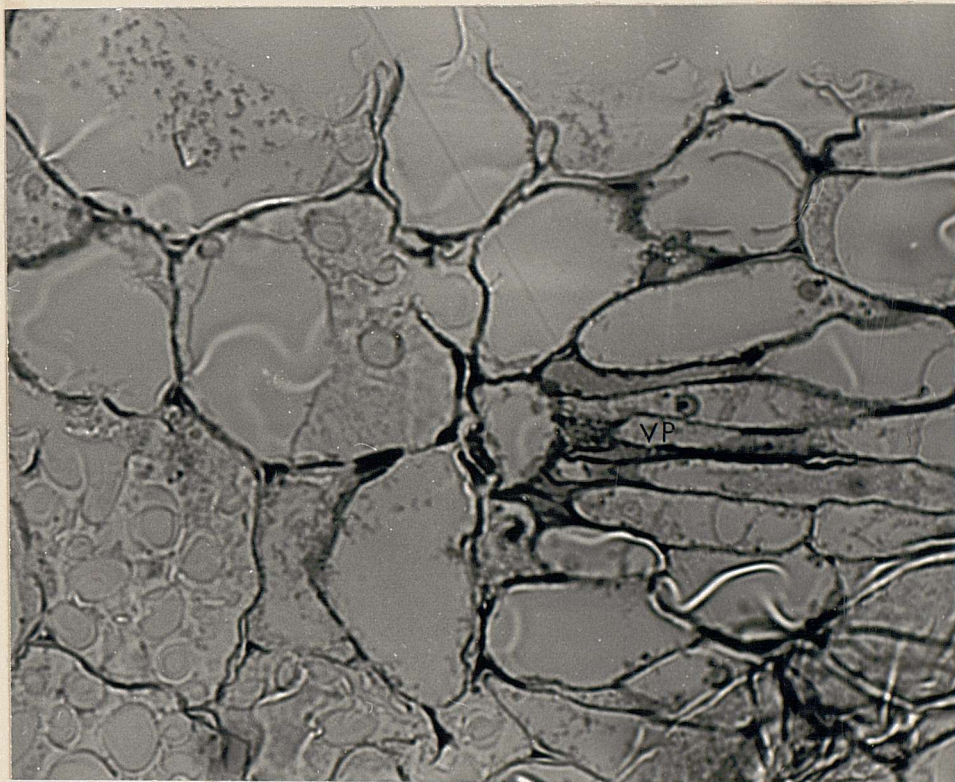


Plate 4.3 a.

(x 252, light microscope)

VP

Infected vascular tissue showing intact vascular parenchyma.

(phase contrast.)

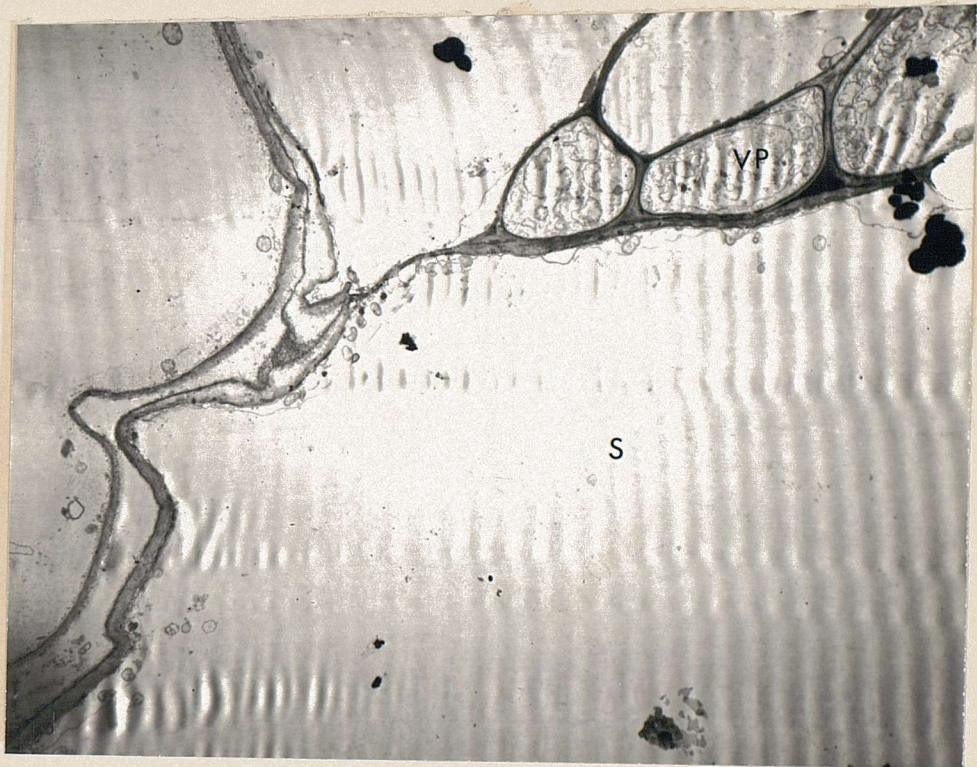


Plate 4.3 b.

(x 1,500, electron micrograph)

Transverse section through infected vascular tissue showing
soft rotted storage parenchyma^S but relatively intact
vascular parenchyma, VP

(osmic acid, lead citrate.)

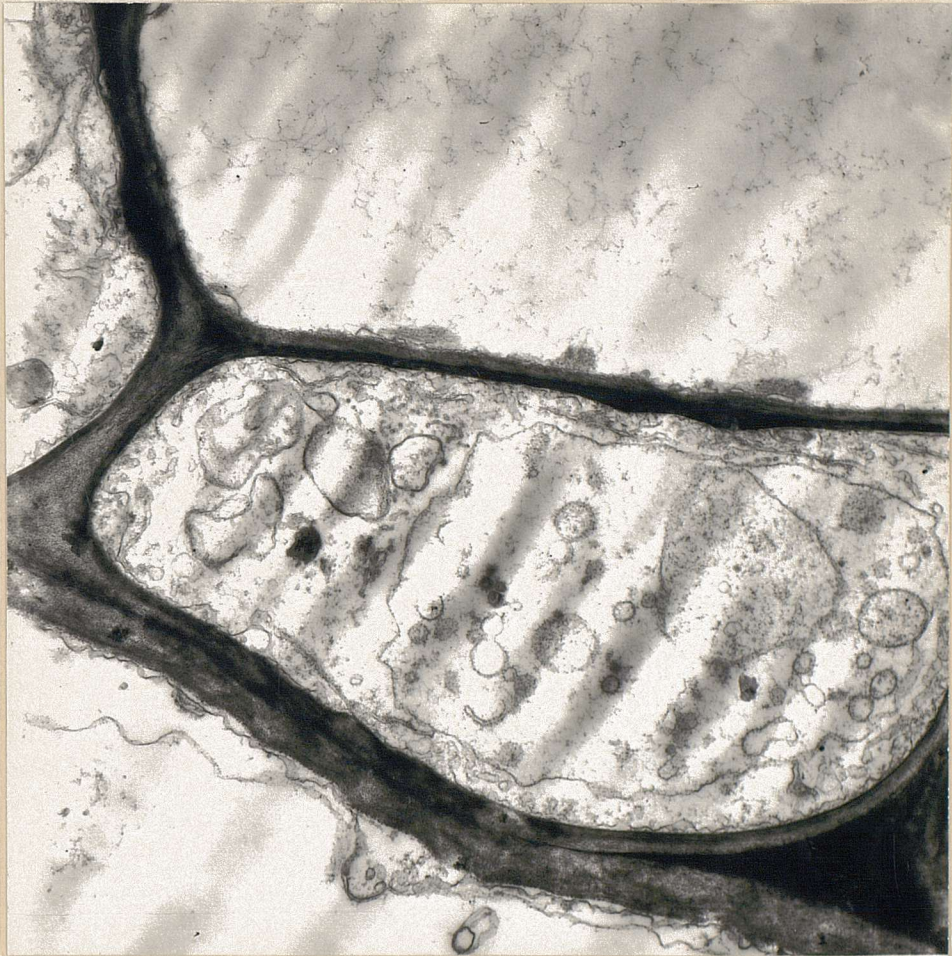


Plate 4.3 c.

(x 15,000, electron micrograph)

Vascular parenchyma near infected vascular tissue showing the relatively intact cytoplasm and suberin in the intercellular spaces and between the cell wall.

(osmic acid, lead citrate.)



Plate 4.4

(x 6,000, electron micrograph)

Phloem from infected vascular tissue, blocked with callose.^c

(osmic acid, lead citrate.)

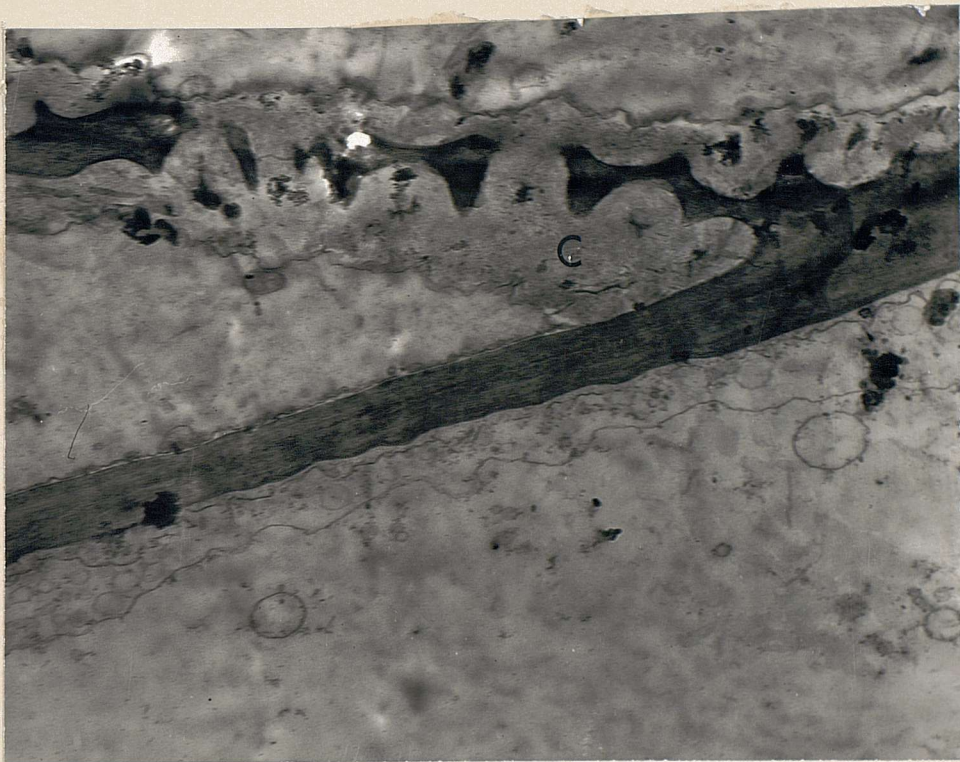


Plate 4.5

(x 15,000, electron micrograph)

Phloem from healthy tissue blocked with callose. ^c

(osmic acid, lead citrate.)

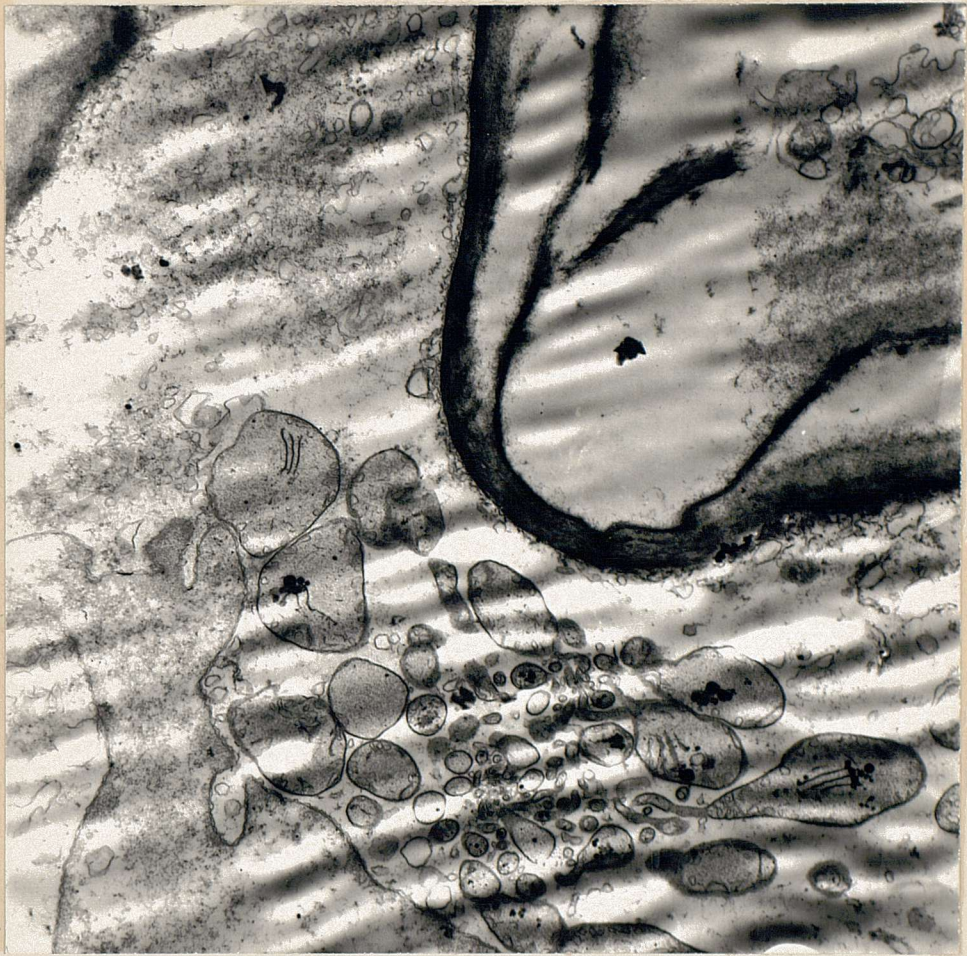


Plate 4.6

(x 15,000, electron micrograph)

The cytoplasm of the storage parenchyma is not damaged
as much as the cell wall, in tissue near vascular infection.

(osmic acid, lead citrate.)



Plate 4.7

(x 40,000, electron micrograph)

The cytoplasm of storage parenchyma near infected vascular tissue, has fairly intact mitochondria^M despite a disintegrating cell wall.

(osmic acid, lead citrate.)

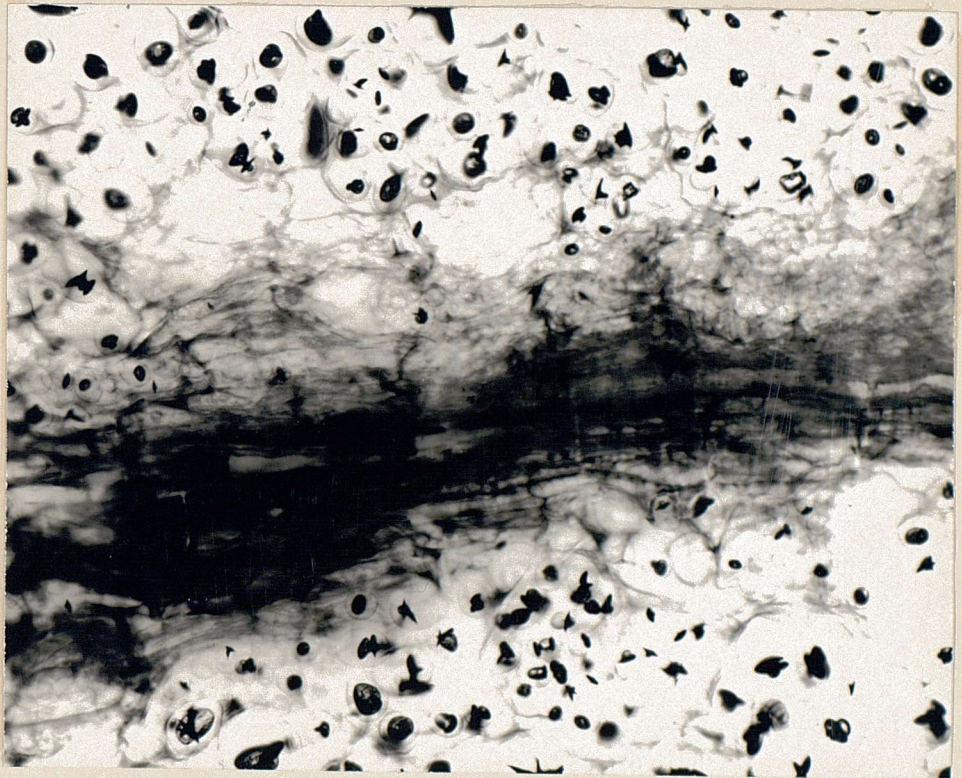


Plate 4.3

(x 120, light micrograph)

Longitudinal section through infected vascular tissue stained to show suberin, indicating that the vascular parenchyma and some of the storage parenchyma is suberised.

(ammoniacal gentian violet.)

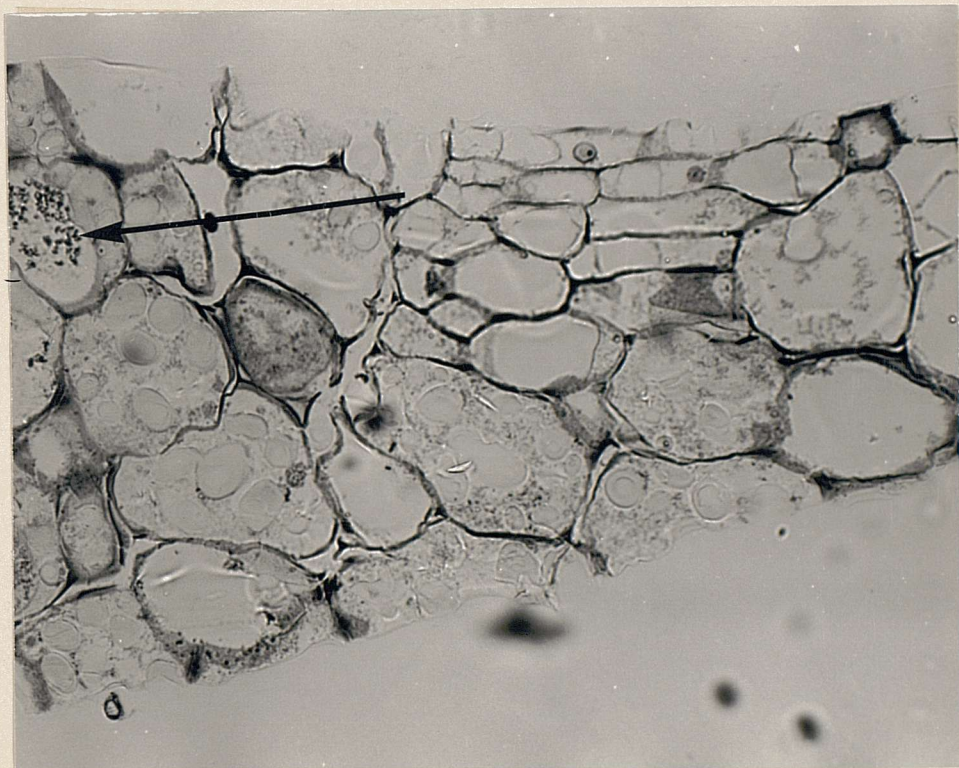


Plate 4.

(x 120, light micrograph)

Longitudinal section through infected vascular tissue showing rotted cells and melanin granules (arrowed) present in the tonoplasts of the surrounding storage parenchyma cells.

(osmic acid, lead citrate.)

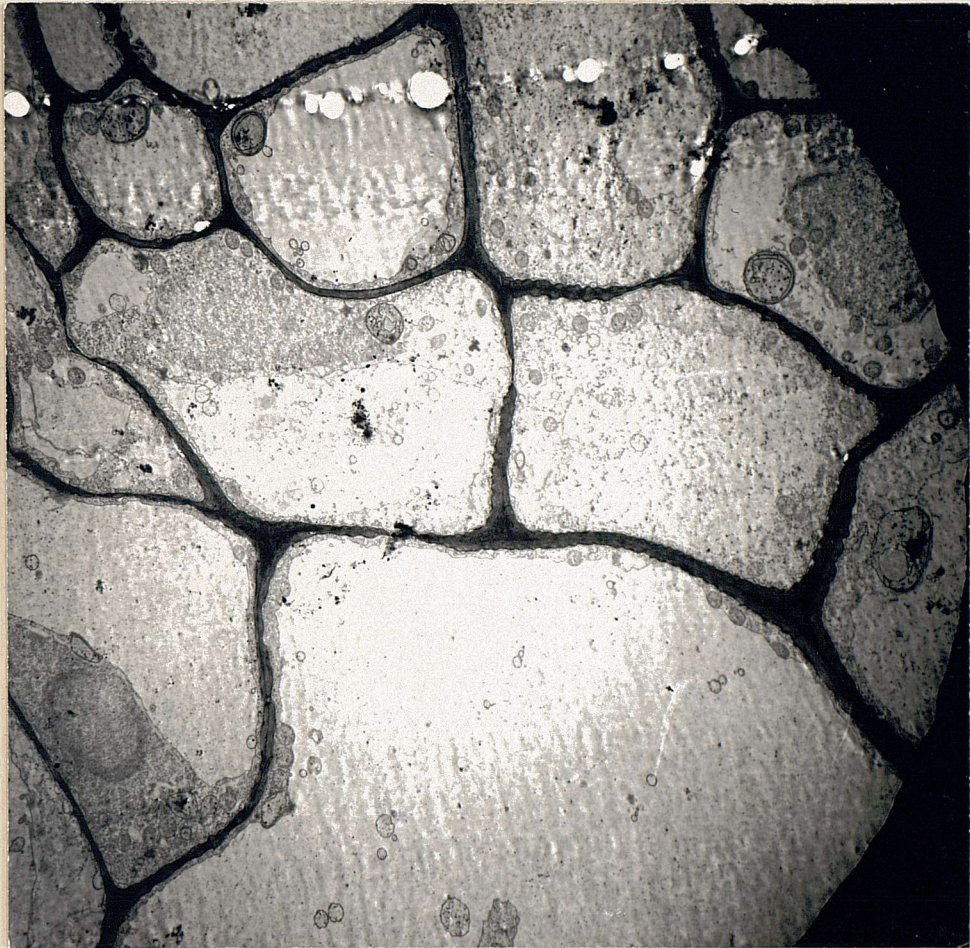


Plate 4.10 a.

(x 3,000, electron micrograph)

Transverse section through vascular parenchyma of healthy
tuber.

(osmic acid, lead citrate.)

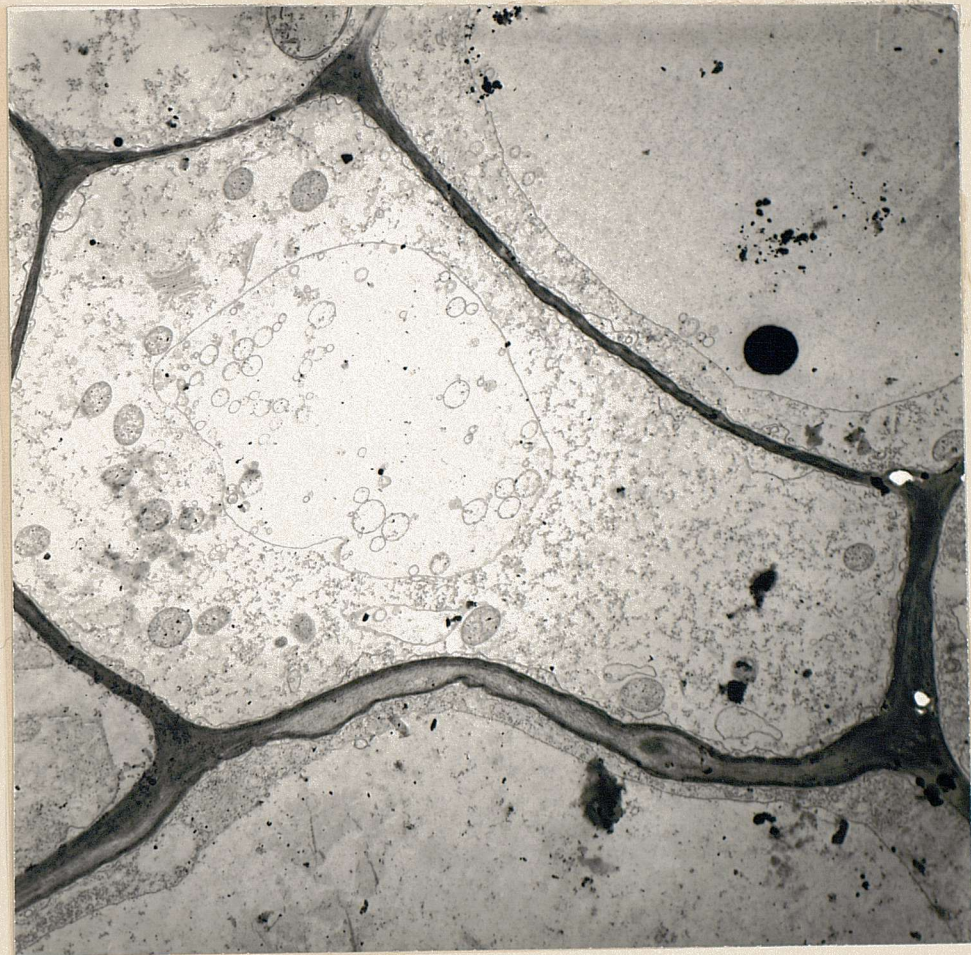


Plate 4.13 b.

(10,000, electron micrograph)

Transverse section through vascular parenchyma cell from
healthy tuber showing cytoplasm and suberised cell walls.

(osmic acid, lead citrate)

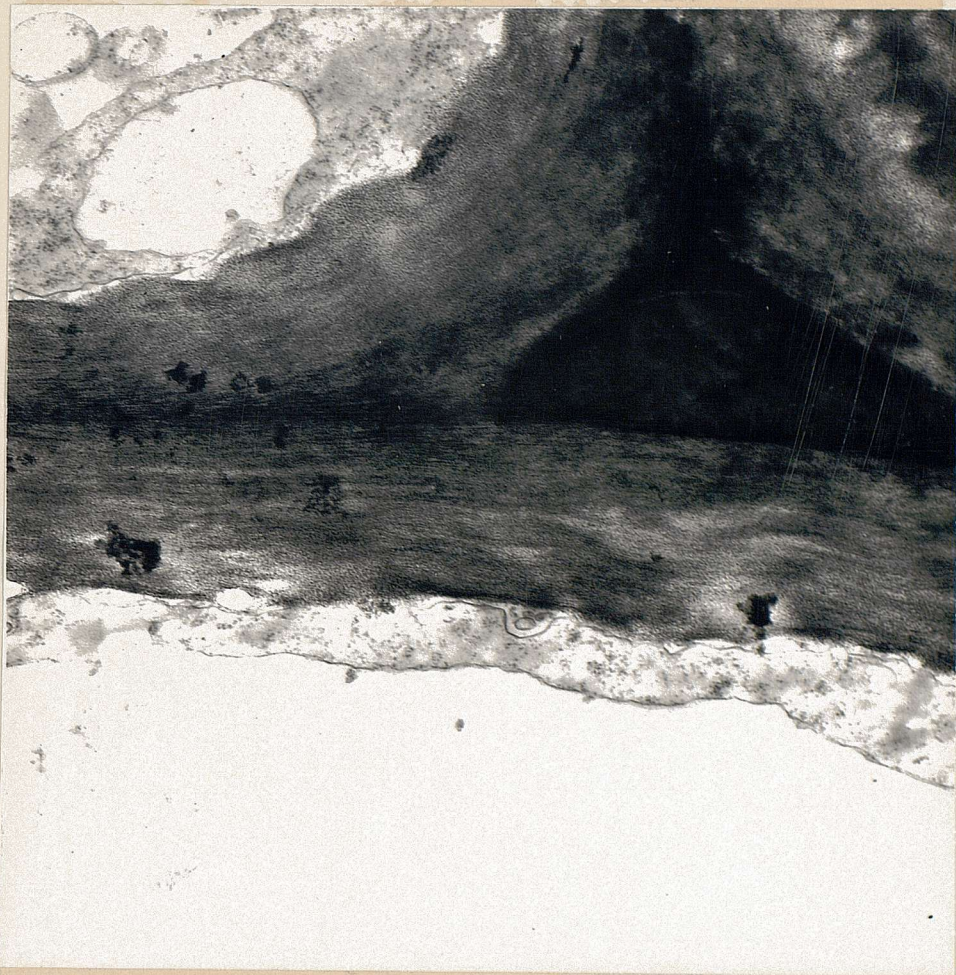


Plate 4.11

(x 20,000, electron micrograph)

Suberin plug in the intercellular space of storage paren-
chyma near vascular tissue from healthy tuber.

(osmic acid, lead citrate)



Plate 4.12 a.

(x 3,750, electron micrograph)

Early colony of Erwinia showing zonation. The bacteria are confined to the central zone (1), around this is the homogeneous zone (2), and the space occupied by the original intercellular space,⁽³⁾.

(osmic acid, lead citrate.)

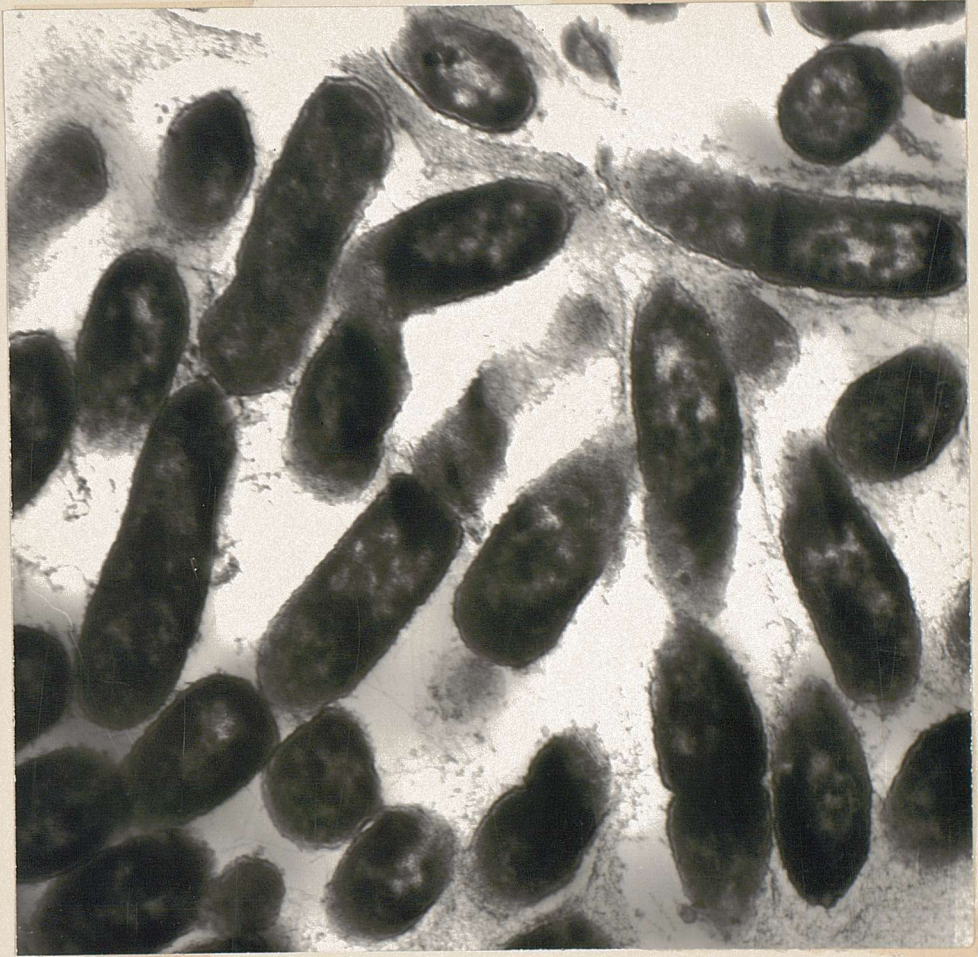


Plate 4.12 b.

(x 37,500, electron micrograph)

Section through central zone (1) of an early Erwinia colony.

(osmic acid, lead citrate.)



Plate 4.12 c.

(x 80,000, electron micrograph)

Section through early Erwinia colony showing the fine-
structure of zones (1), (2), and (3) of plate 4.12 a.

(osmic acid, lead citrate.)



Plate 4.13 a.

(x 15,000, electron micrograph)

Intercellular space of subsidiary infection near bacterial colony, showing zones (2) and (3) only. Stored at 10°C, note fairly intact cytoplasm.

(osmic acid, lead citrate.)

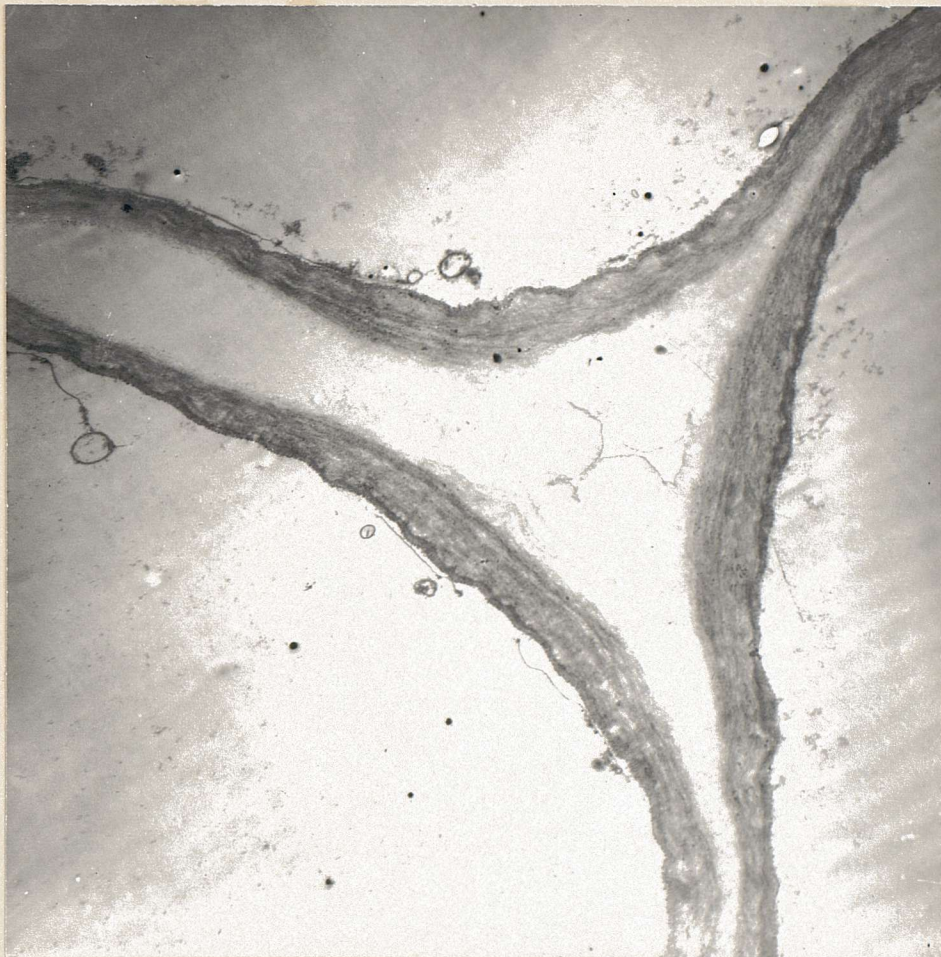


Plate 4.13 b.

(x 6,000, electron micrograph)

Subsidiary intercellular space rot near bacterial colony.

Stored at 20°C note more cytoplasmic damage than plate 4.13 a.

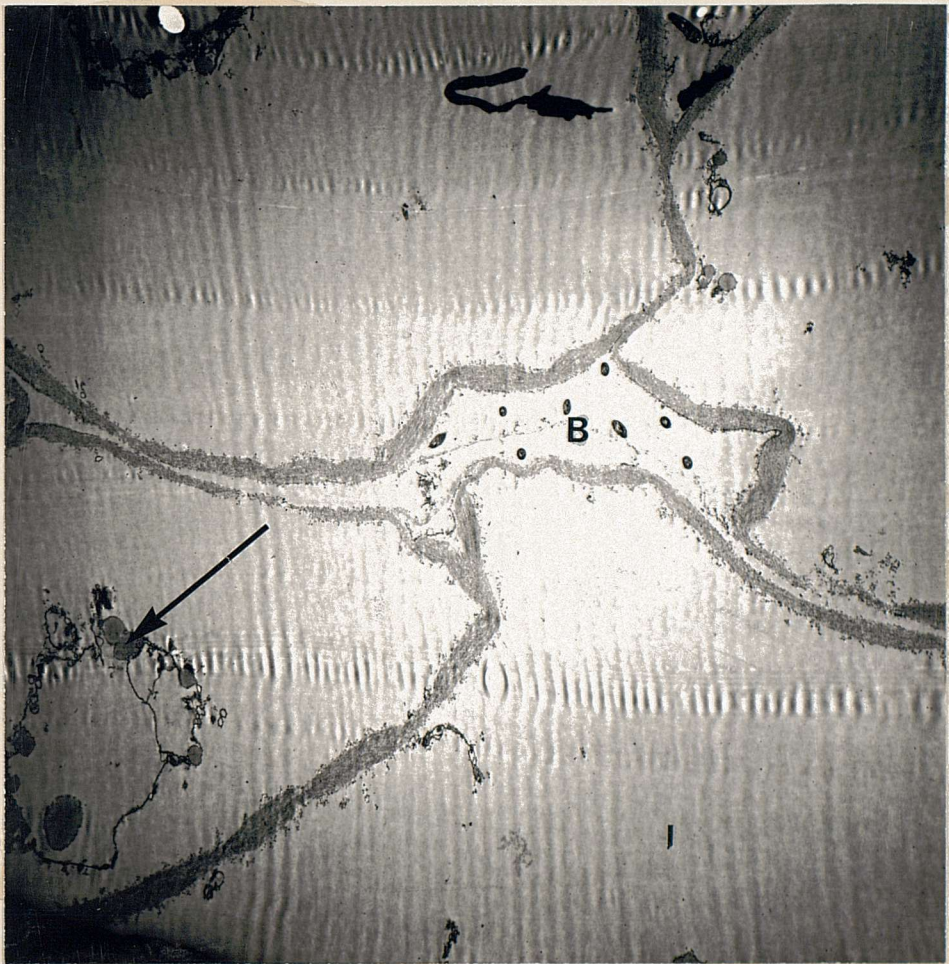


Plate 4.14 a.

(x 3,000, electron micrograph)

Older infection showing the absence of zoned colonies,
the bacteria^B are loosely spread between the cells.

The microbodies are enlarged (arrowed) and all that is
left of the cytoplasm apart from the plasma membrane.

(osmic acid, lead citrate.)

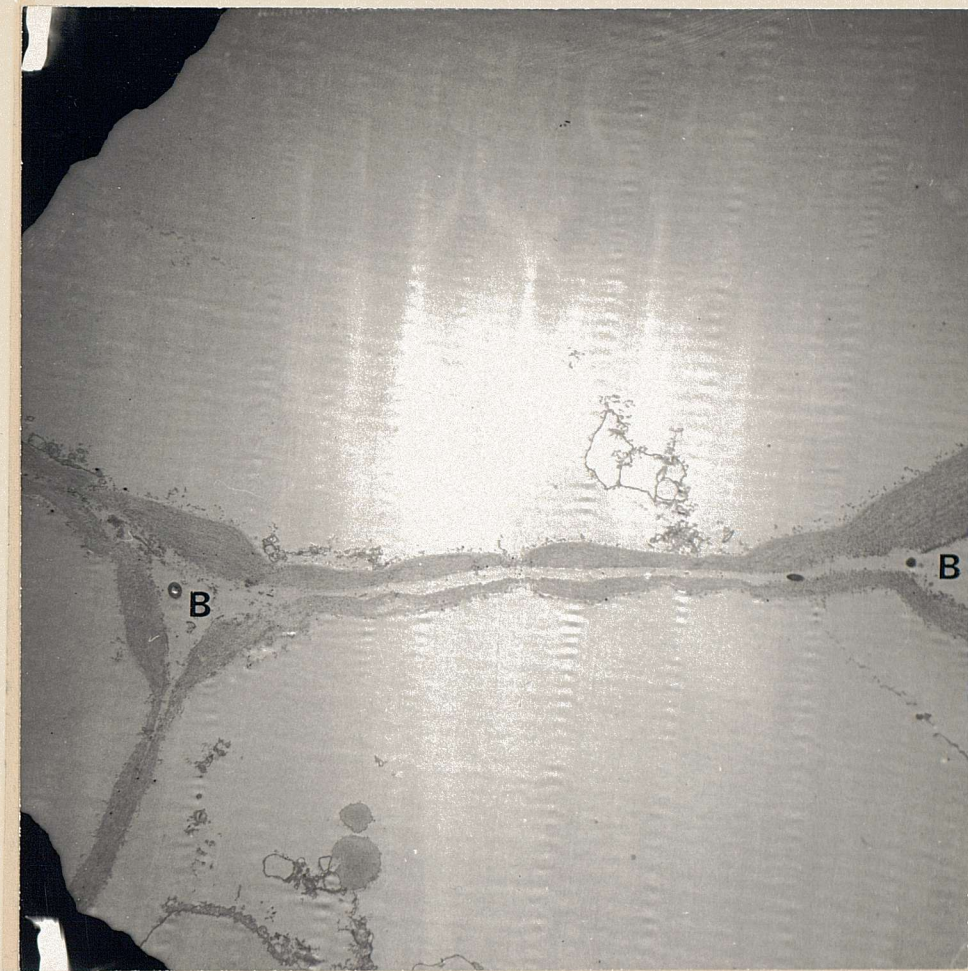


Plate 4.14 b.

(x 7,500, electron micrograph)

Completely macerated cells of old infection, showing
bacteria^B confined to the spaces between the cells.

(osmic acid, lead citrate.)

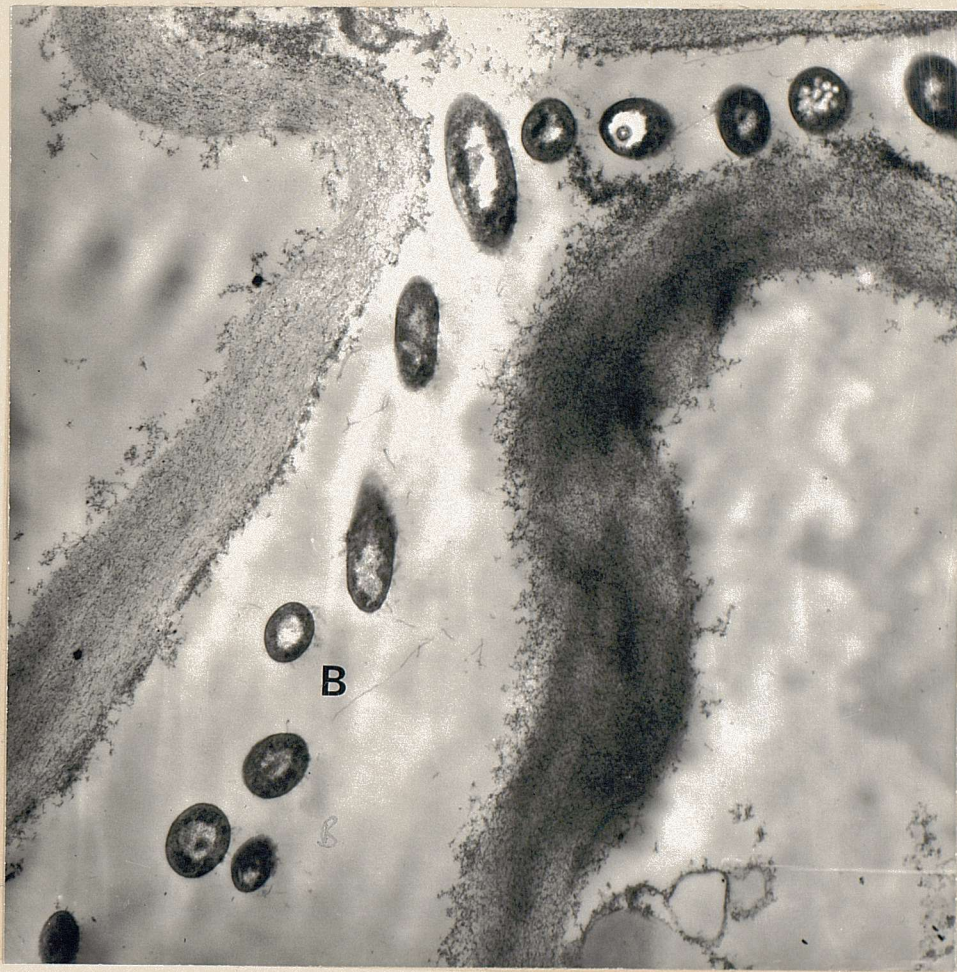


Plate 4.14 c.

(x 25,000, electron micrograph)

The bacteria⁶ are contained in the enlarged gap between the cells in old infection, the cell wall itself is swollen and the fibrils separated.

(osmic acid, lead citrate.)

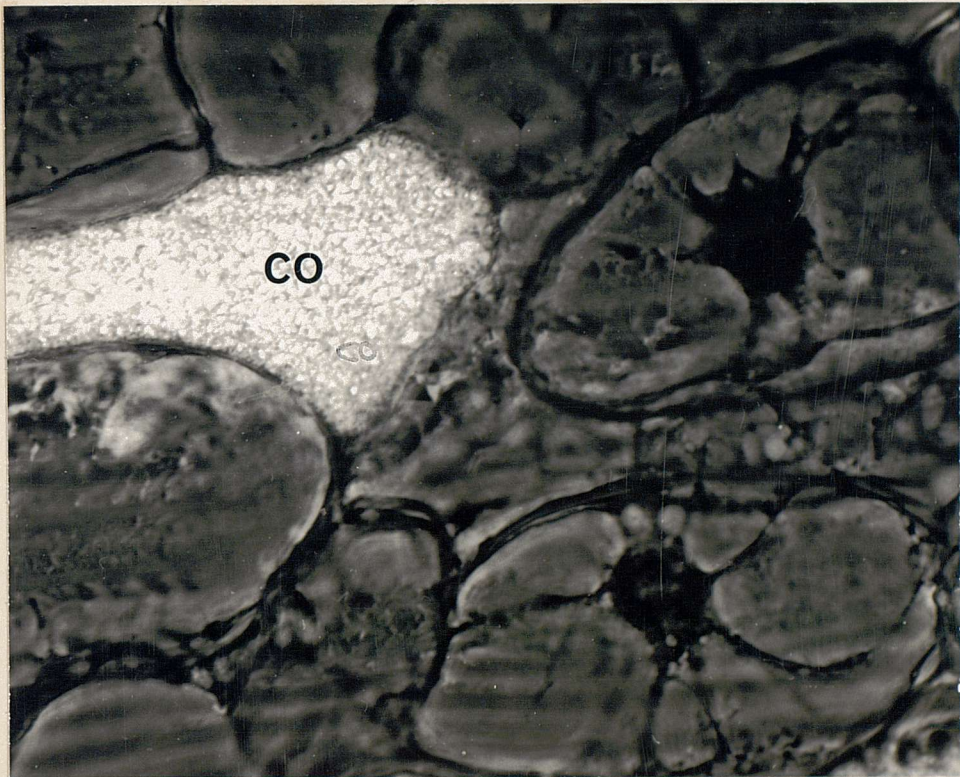


Plate 4.15

6

(x 980, light micrograph)

Calcium oxalate cell beneath the lenticel.

(phase contrast.)



Plate 4.16

(x 252, light micrograph)

Infection of calcium oxalate cells by bacterial colonies
(arrowed).

(carbol fuchsin, fast green)

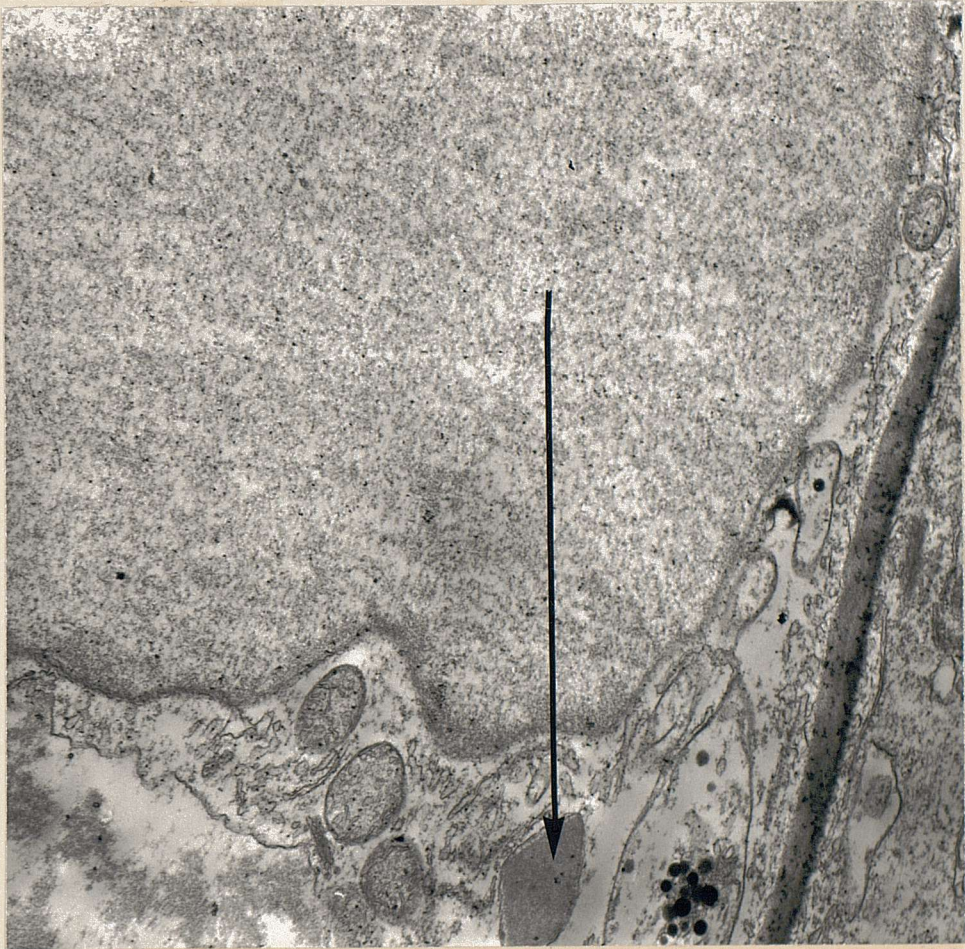


Plate 4.17

(x 20,000, electron micrograph)

Crystal in microbody (arrowed) in cell beneath the lenticel.

(osmic acid, lead acetate)

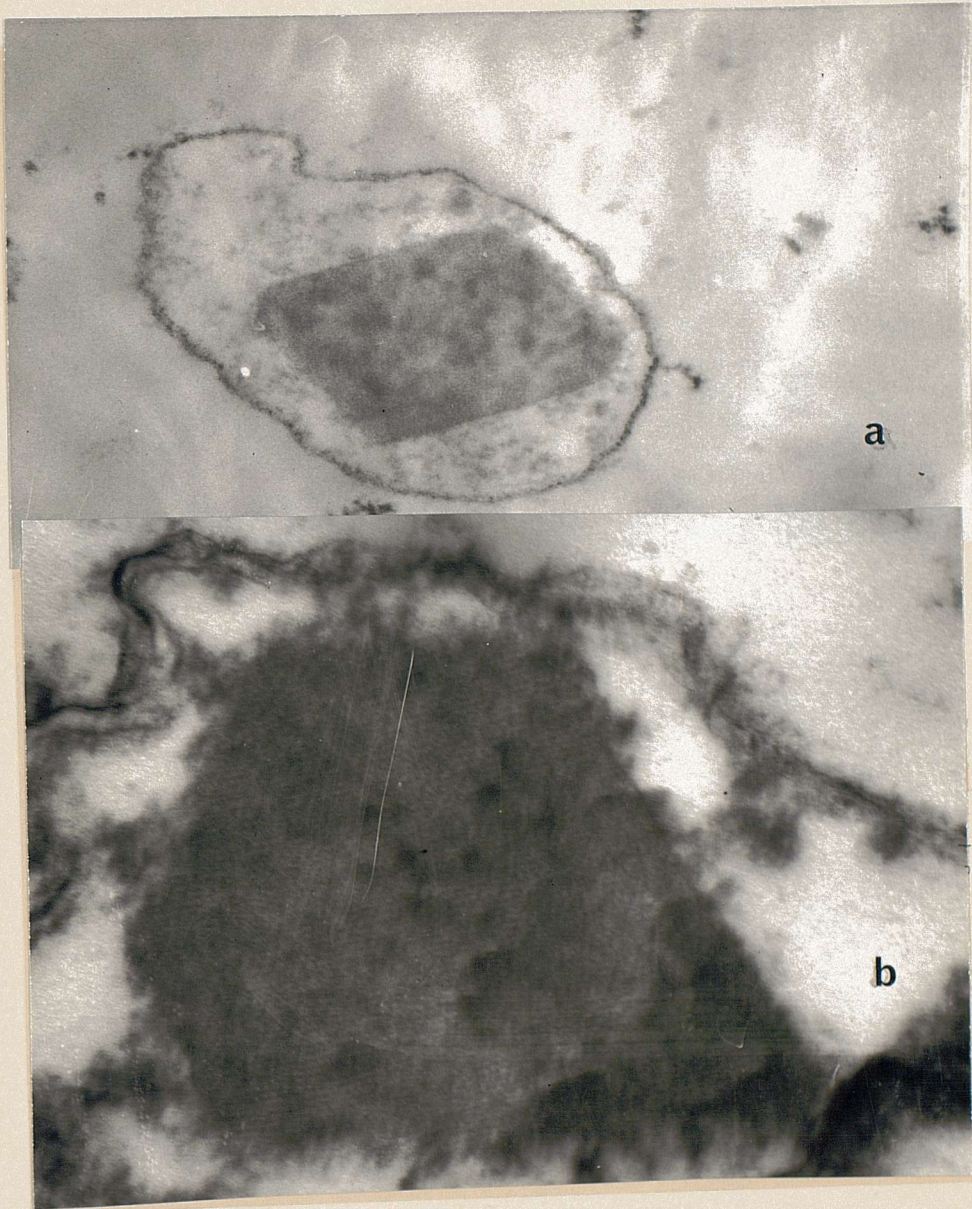


Plate 4.18. a.

(x 40,000, electron micrograph)

b.

(x 80,000)

Crystals in cytoplasm of soft rotted cells.

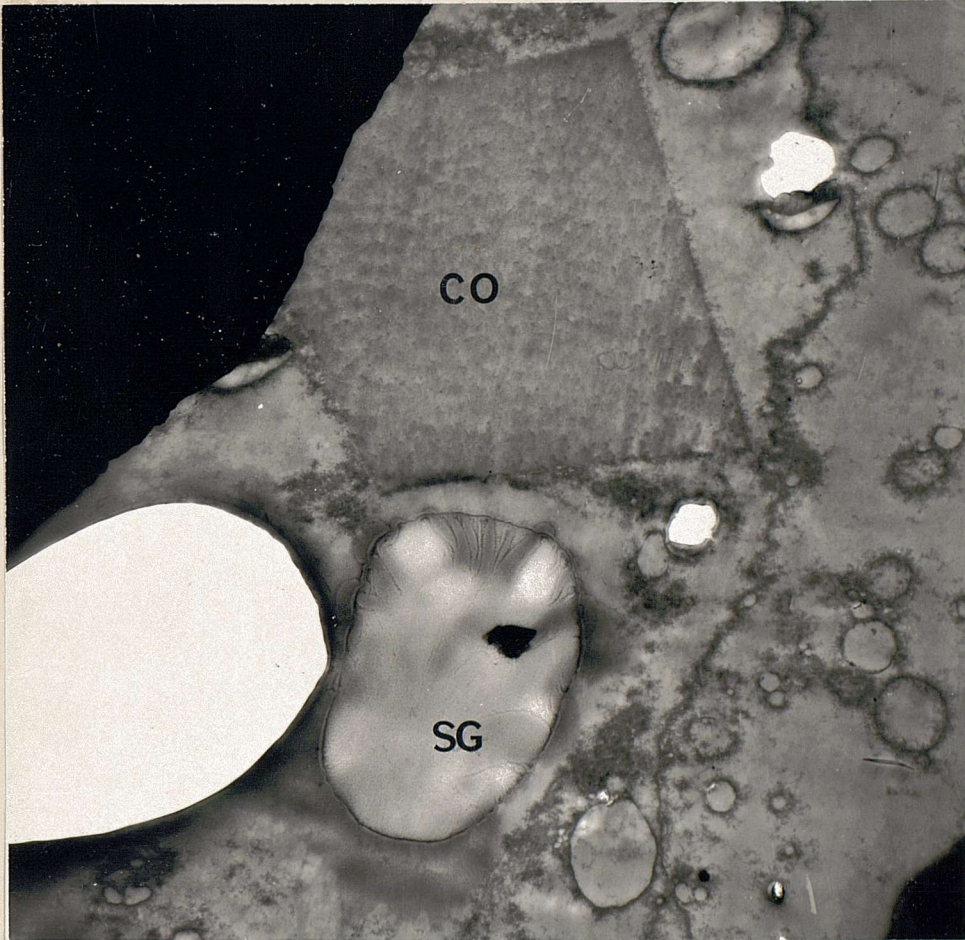


Plate 4.18 c.

(x 30,000, electron micrograph)

Crystal^{CO} in cytoplasm of soft rotted cell, starch grains^{SG}
also remain unaltered.

(osmic acid, lead citrate)

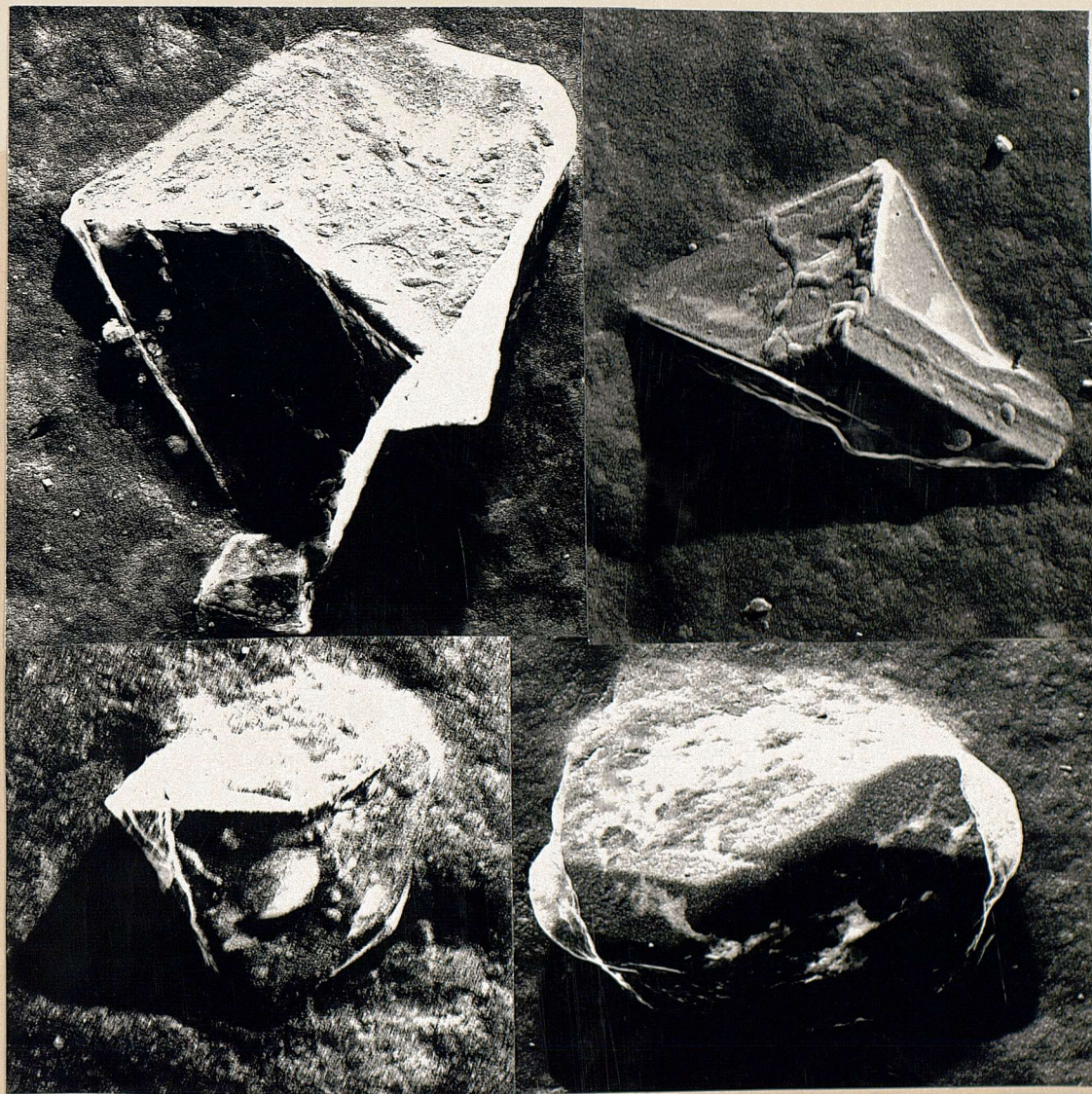


Plate 4.19: a. (x30,000) b. (x40,000) c. (x40,000)

d. (x20,000, electron micrographs)

Pd/Pt shadowed replicas of crystals from calcium oxalate cells.

PART 5. Tissue disintegration.

Part 5

Tissue degradation.

Introduction.

Among the symptoms of soft rot described by other workers using light microscopy have been maceration, cytotoxicity, osmotic damage, and changes in the nuclear size (Wood, 1967). None of these host reactions has been studied at the electron microscope level and most of the workers have studied only a single symptom. Many of the symptoms are interconnected and the way in which they combine to form a picture ~~a picture~~ of tissue degradation appears to have economic relevance. It is the rot visible to the naked eye which offends; rotting may often be advanced at the cellular level at low temperature but not be evident on visual inspection of the tubers.

The effects of the pectolytic enzymes produced by Erwinia on the the primary cell wall have been extensively studied from a biochemical viewpoint (Wood, 1967). Although the precise distribution of the substrate for each enzyme isolated has yet to be elucidated, the work of Calonge, Fielding, Byrde, & Akinrofen (1969) is of this type but on fungal extracellular enzymes. Less emphasis has been placed on the cytotoxic effects of soft rot; Pitt ^{& Coswiler} (1969) has reported an increase in the size of the potato cell lysosomes in the region which

has been soft rotted, compared with those in healthy tissue. Butler & Jones (1949) refer to an increase in size of the nucleus of turnip root tissue during soft rot but little other reference to this phenomenon has been made, although Allen (1933) observed similar nuclear swelling in wheat after infection by *Puccinia graminis tritici* .

The **different** aspects of tissue degradation have been split into cell wall, plasmodesmatal, cytoplasmic and nuclear damage; Where points of similarity occur these are discussed.

1. Cell wall damage.

Even at the earliest stages of soft rot infection visible to the naked eye, there is evidence of maceration of the tissues, but before this there is little sign of damage even at the highest power of the light microscope. If, as suggested by enzymological studies there is little evidence of cellulose degradation by *Erwinia in vivo*, even though in vitro degradation may occur (Amman, 1952), the relatively extensive removal of pectic substances would be almost undetectable by light microscopy.

The electron microscopy of this thin area can provide further information into the process of maceration. Albersheim's method of staining can be employed to determine the distribution of methoxyl groups (giving

evidence of pectinates) in the cell wall (Albersheim, Mühletaler, and Frey-Wyssling, 1960). As well as the histochemical staining the electron microscopy of soft rotted tissue has the advantage over light microscopy in that the part played by the plasmodesmata in holding together otherwise macerated tissue can be determined.

Experiment 5.1

To determine the extent of the damage to the cell wall pectin by Erwinia rot using Albersheim's method.

Materials and methods.

King Edward tubers were inoculated with approximately ¹¹ 4×10^8 cells/ml of inoculum by means of a well bored into the surface of a half tuber and incubated at 25°C and 100% R.H. until a zone of well defined rot had developed. Transsects across the diameter of the tuber were then made and samples along each transect were taken and fixed in 5% formaldehyde. These were then stained by Albersheim's method and after dehydration embedded in araldite. Sections were cut at 2 μ m and 50 nm, ~~and~~ used for both phase contrast and electron microscopy, and compared with similar preparations from uninfected storage parenchyma.

Results.

Plate 5.1 shows the results of staining on uninfected tissue. In a. a section through storage parenchyma

cell wall away from the intercellular spaces; the pectin is widely distributed across the central area; where the section is nearer the intercellular space, the pectin is more centralised. In 5.1.b. where an intercellular space is sectioned, the process is even further advanced, with pectin coating the intercellular space. Using a chromoscan 1005 aperture and 490 lamp and filter, a scan showed that across plate 5.1 (b) over 60% of the pectin is in the middle 10% of the cell wall; nearer the intercellular space the concentration of pectin in the middle of the cell wall is even greater.

Pectin is apparently absent from tissue which is thoroughly rotted and the cell wall is rather 'ragged' to the inside where it has split apart (plate 5.2.u.) Where the tissue is less rotted (plate 5.2 (b)) the nature of the process by which pectin is removed can be examined. In the latter the intercellular space appears to be enlarged by the removal of pectin from around the inside of the space as well as from between the cell walls; here there is not the 'raggedness' seen where the pectin was removed from the cell wall away from the intercellular space. Again, this suggests that the pectin is not so broadly spread through the cell wall in the neighbourhood of intercellular space or elsewhere.

~~When~~ These results ^{may be} ~~are~~ compared with the pictures of tissue fixed in 1% osmic acid in Kellenberger buffer with 5% sucrose; ~~in which~~ the whole cell wall can be seen in greater detail from the preparation stained by Albersheim's method, ~~are confirmed~~. Plate 5.3 a, shows a 143 h old rot in which maceration is more advanced at the bottom right than it is at the top left; at the top left the centre of the cell wall is intact, the centre is then removed, and the final stage involves destruction of the remainder of the cell wall from the inside to the outside with a corresponding increase in thickness. Plate 5.3 b, shows the preferential removal of the centre of the cell wall, plate 5.3 c, shows where the remains of the plasmodemata hold together neighbouring parts of a cell wall which has reached the stage where the disintegration has extended beyond the central zone. Fibrillar or lamellar material may be found between the cell walls which have been macerated (plate 5.3 d.). Whether this is a component of the cell wall which has been exposed or is a by-product of bacterial metabolism is not known.

When well rotted tissue is examined it can be seen that although the cell wall is much increased in thickness, this swelling is irregular with constrictions

at the plasmodesmata where the cell walls are held together. (Plates 5.4 a & b.) Whether there is a change in the overall surface area of the cell walls or not is difficult to ascertain but it is significant that the mean gap round the central microtubule canals in plasmodesmata of healthy storage parenchyma is 91 n.m. but after soft rotting it may be increased as much as 231 n.m., plates 5.5 a-b. It is not clear whether this indicates a general increase in the surface area due to a loosening of the fibrillar structure of the cell wall in its matrix or the reverse, or neither. The increase in the thickness of the cell wall suggests the former is true.

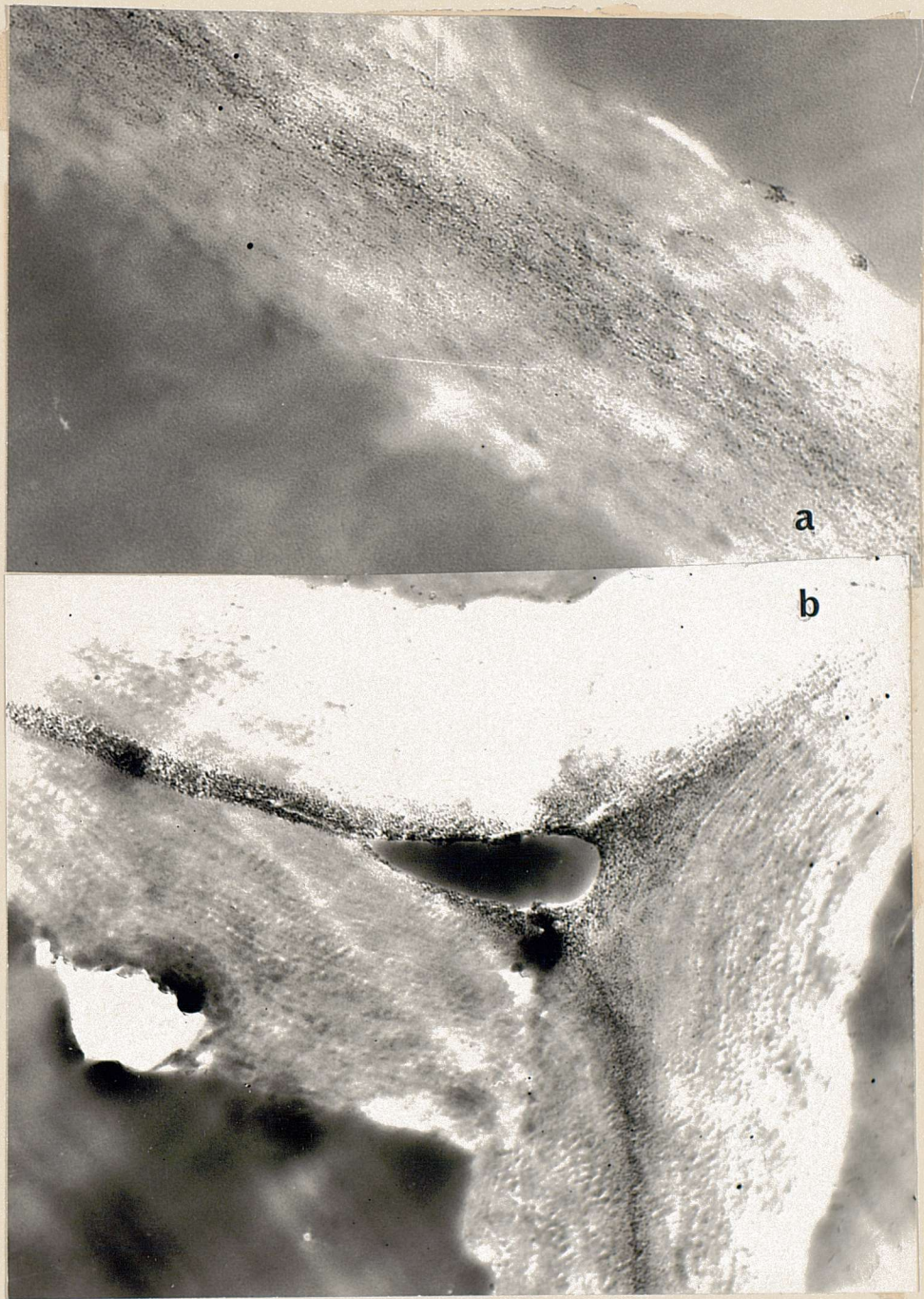
2. The plasmodesmata.

The fine structure of higher plant plasmodesmata has recently been described by O'Brien^{+ Thimann} (1967) and Robards (1968). The plasmodesmata of the potato storage cells conform to the type found in oat coleoptiles by the former workers. The possibility that bacteria in the vegetative state could pass through them is unlikely, as both workers have calculated that the width of the connecting microtubules would prohibit even macromolecule passage.

Plate 5.6 illustrates the structure of the plasmodesmata in potato storage parenchyma. These appear to be

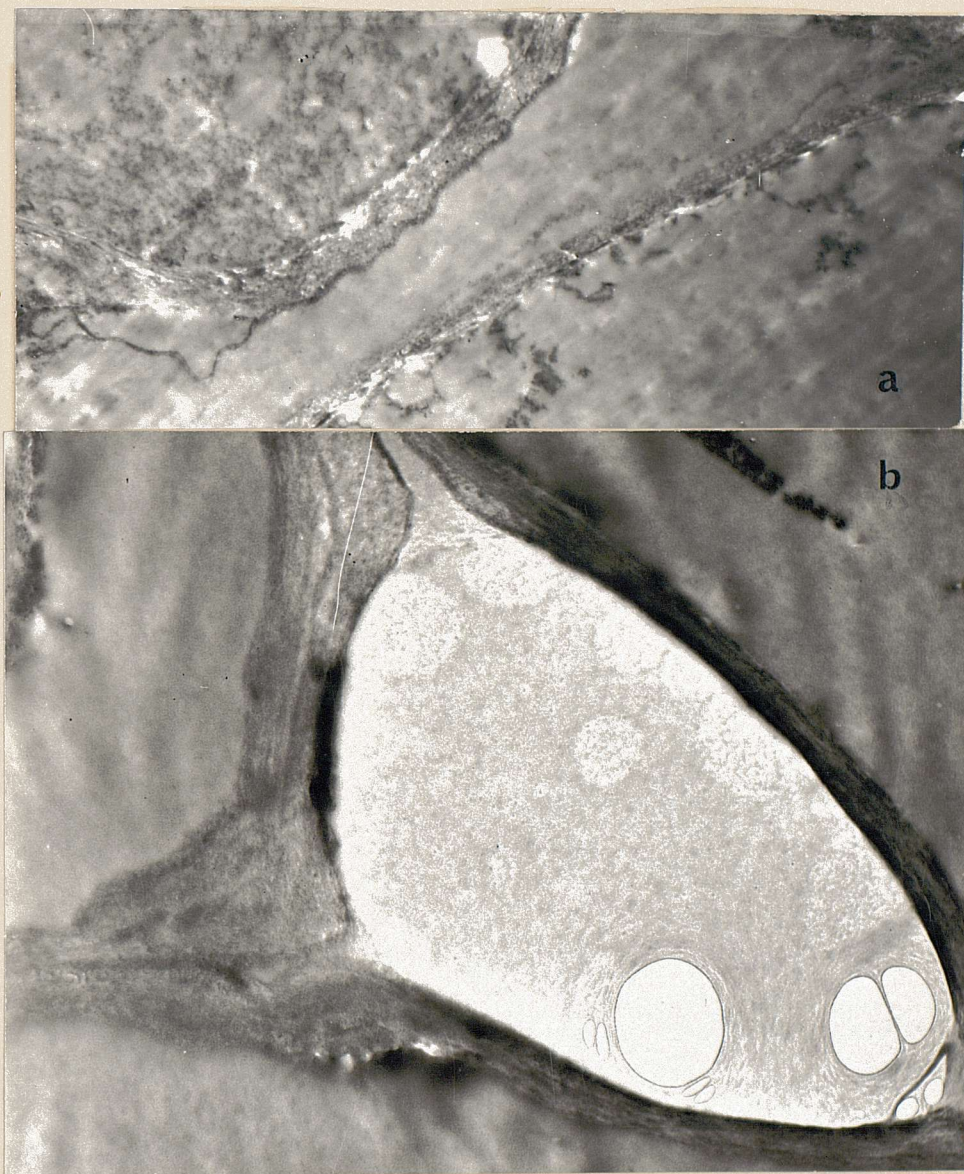
unbranched, and there appears to be no intercellular continuity unless the plasmalemma lines the plasmodesmatal canal, but this may be a different membrane (plate 5.6c). The centre of the plasmodesma is traversed by a desmotubule, and from the sections it appears unlikely that there is space enough for any other structure, for example endoplasmic reticulum. Between the two cells there may be a central cavity as described in oat coleoptile, making the concept of direct cytoplasmic continuity unlikely. There appears to be some convoluted plasmodesmatal tubules in this central cavity. The plasmodesmata or part of them, the tubules may be attached to the cytoplasm in old infections similar to the results of plasmolysis described by Sitte (1963) in Elodea cells.

It appears unlikely that when a cell wall is macerated ~~that~~ severance of the plasmodesmata in the region of the middle lamella would disrupt any cytoplasmic continuity between the cells macerated. In fact the plasmodesmata are the last section of the cell wall to become macerated plate 5.4, and the transverse microtubules appear to hold the cell wall together such tissue appears coherent to the naked eye. When the plasmodesmatal connection breaks as it does in older infections, a hole is made which could allow

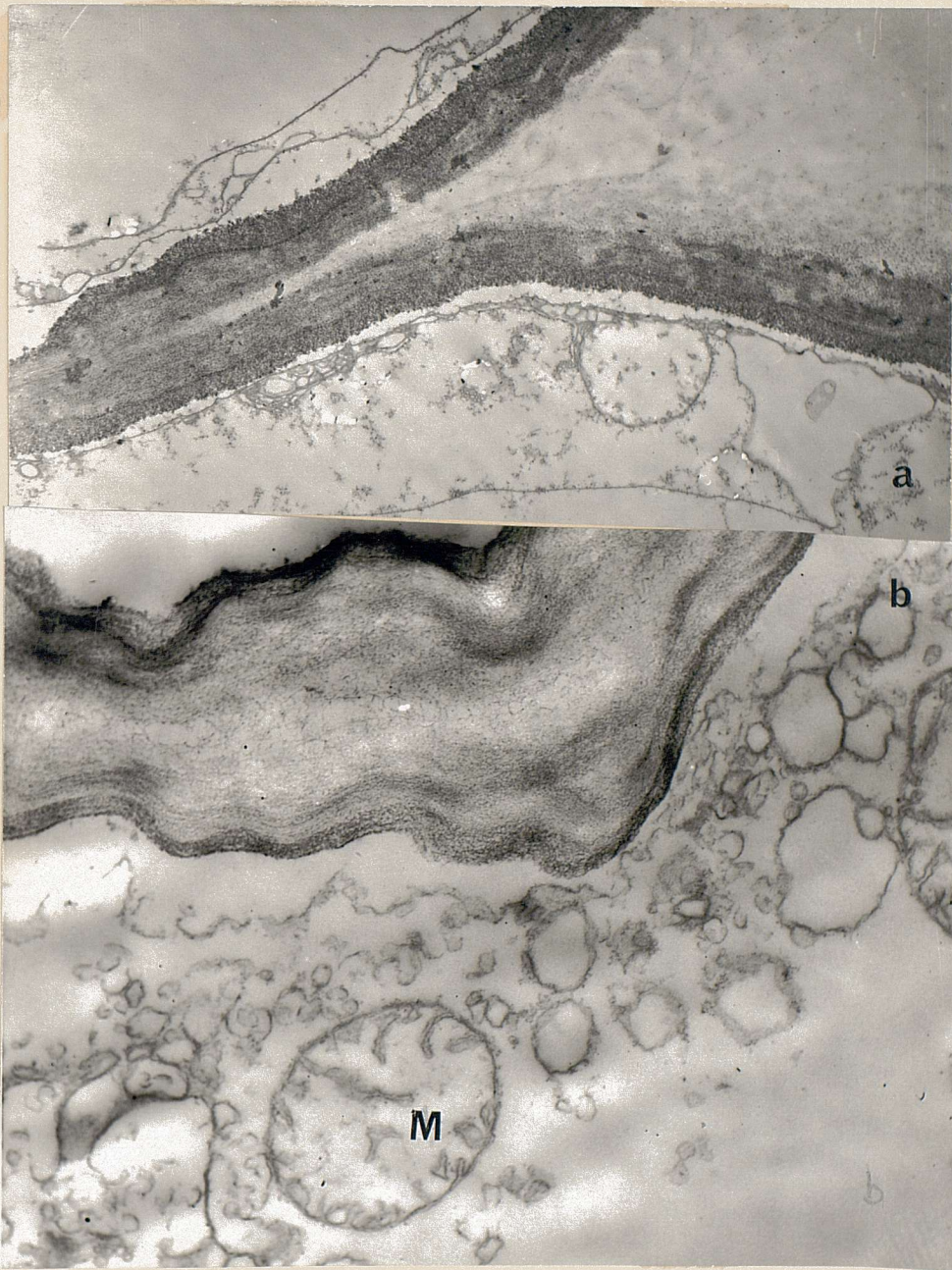


Plates 5.1 a.(x60,000), b. (x20,000)

Electron micrograph of a., section through storage parenchyma cell wall away from intercellular space, and b. at intercellular space, stained to show pectin. (Albersheim's reagents).



Plates 5.2 a. (x 6,000) b. (x 8,000) Electron micrographs.
a. Storage parenchyma cell wall after soft rotting, stained
to show pectin, none detectable; b. intercellular space of
the same. (Albersheim's reagents).



Plates 5.3 a. (x 15,000) b. (x 30,000) Electron micrograph.
Degradation of the cell wall. Note intact mitochondrion in b.
(osmic acid, lead citrate.)

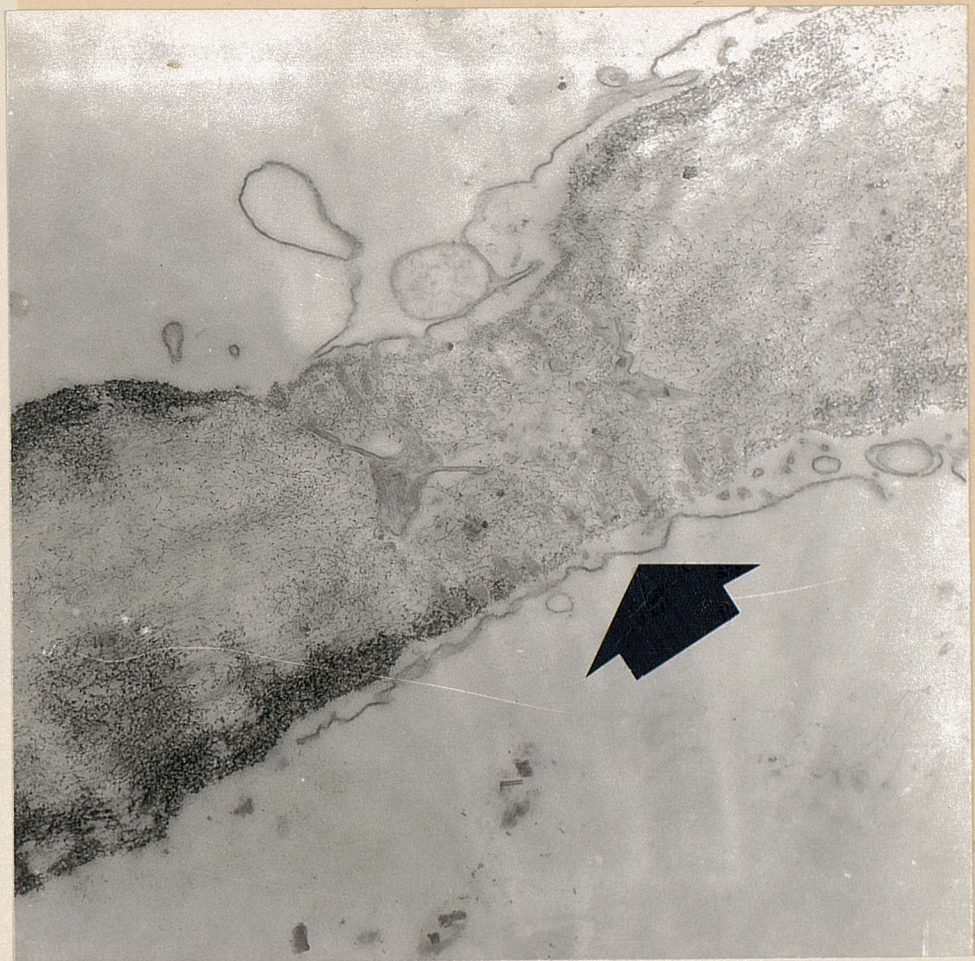


Plate 5.3 c.

(x 30,000)

Late stage of cell wall dissolution showing the plasmodesma
embedded in the separated fibrils, *arrowed*.

(osmic acid, lead citrate)

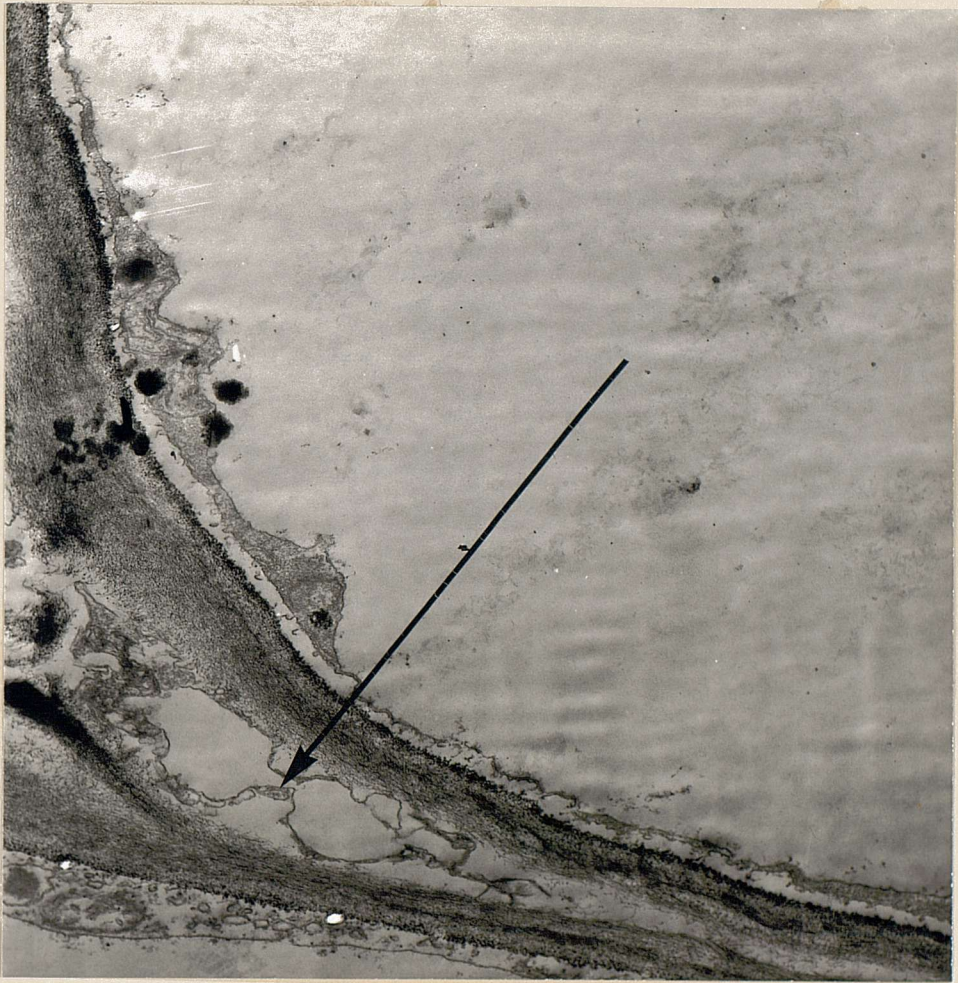
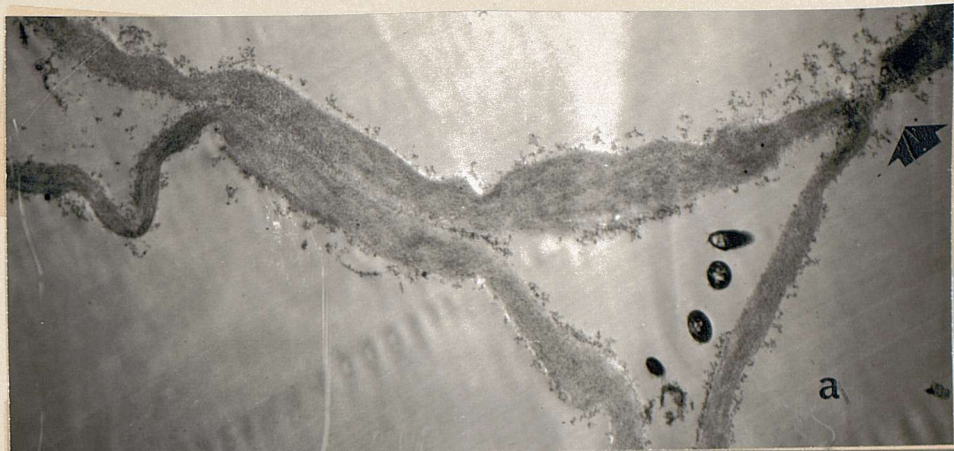


Plate 5.3 d.

(x 20,000, electron micrograph)

Section through early stage of maceration of the cell wall showing lamellar or fibrillar material in the newly created space (arrowed).

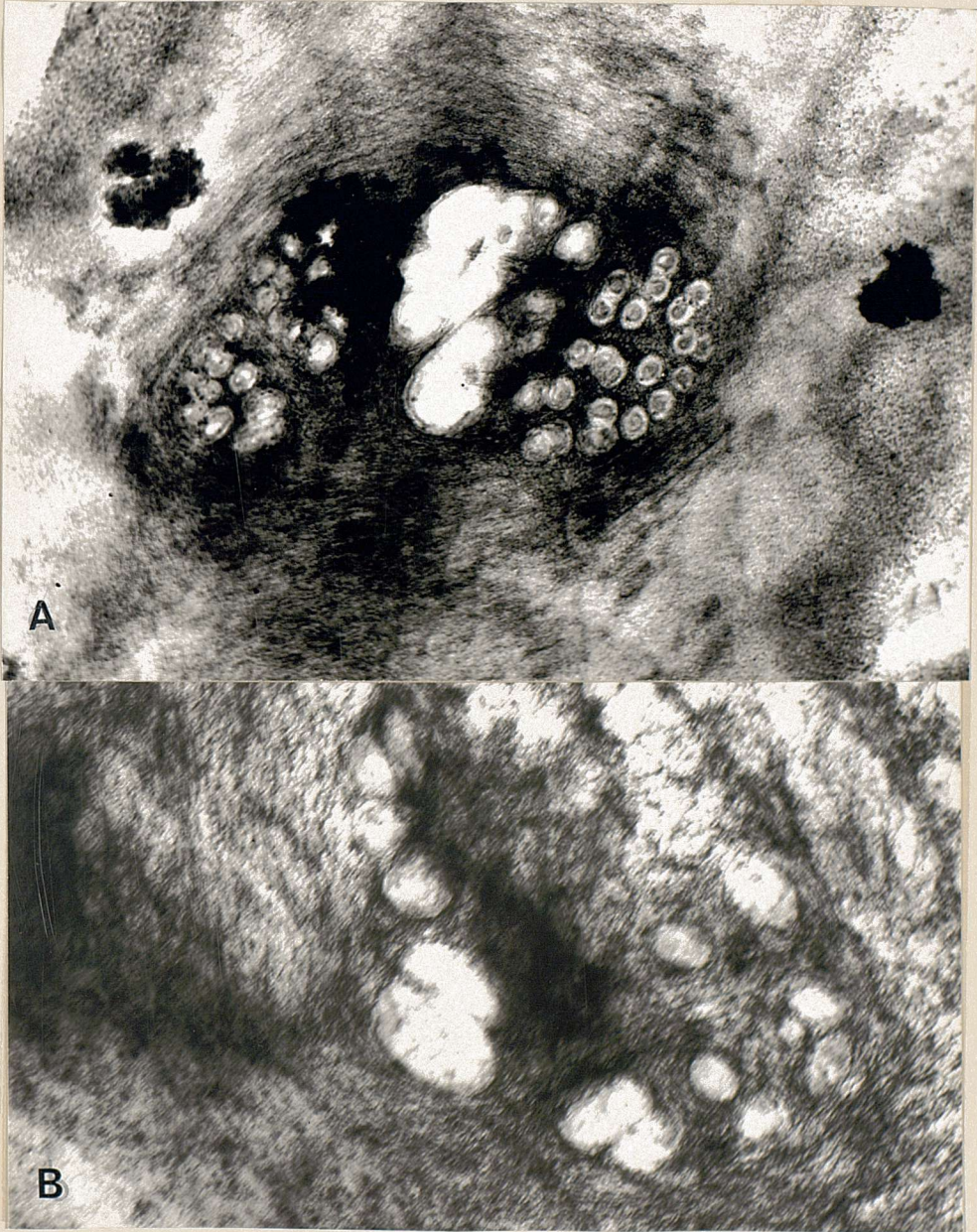
(osmic acid, lead citrate)



Plates 5.4 a. (x6,000) b. (x 15,000)

The increase in thickness of the cell wall on soft rotting is not uniform, a. shows constriction at the plasmodesma, arrowed.

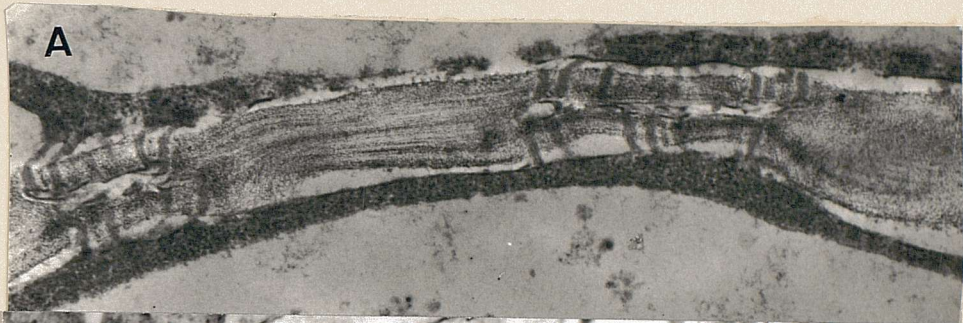
(osmic acid, lead citrate)



Plates 5.5 a. & b. (x 40,000, electron micrograph)

Sections through plasmodesmata, transverse to canals,
a. from healthy tuber b. after soft rot.

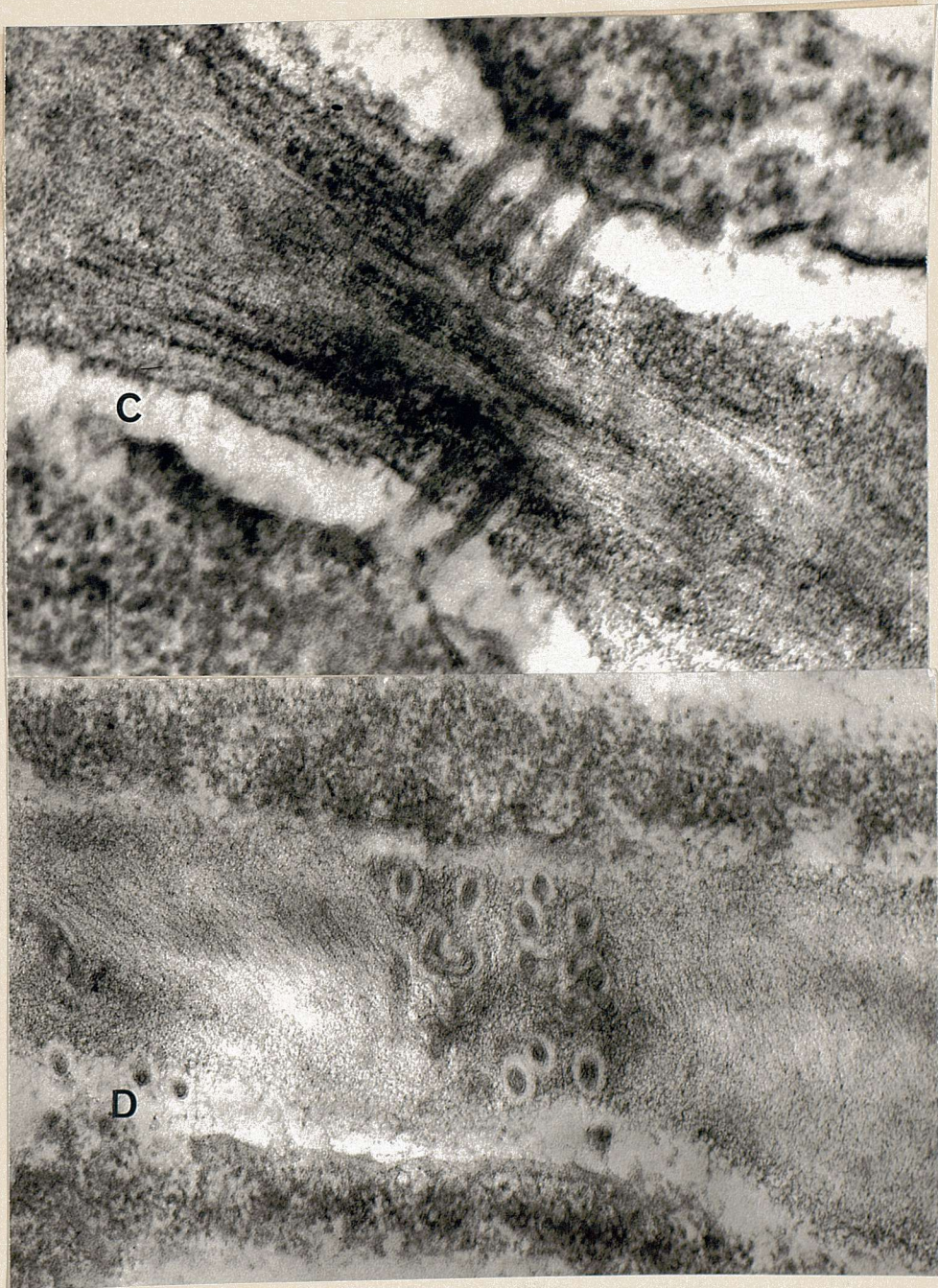
(osmic acid, lead citrate.)



Plates 5.6 a. (x 20,000) b. (x 30,000)

Plasmodesmata from healthy tubers showing the structure.

(osmic acid, lead citrate.)



Plates 5.6 c. (x 120,000) d. (x 80,000) Electron micrographs.
Details of plasmodesmatal structure, desmotubules arrowed.
(osmic acid, lead citrate.)

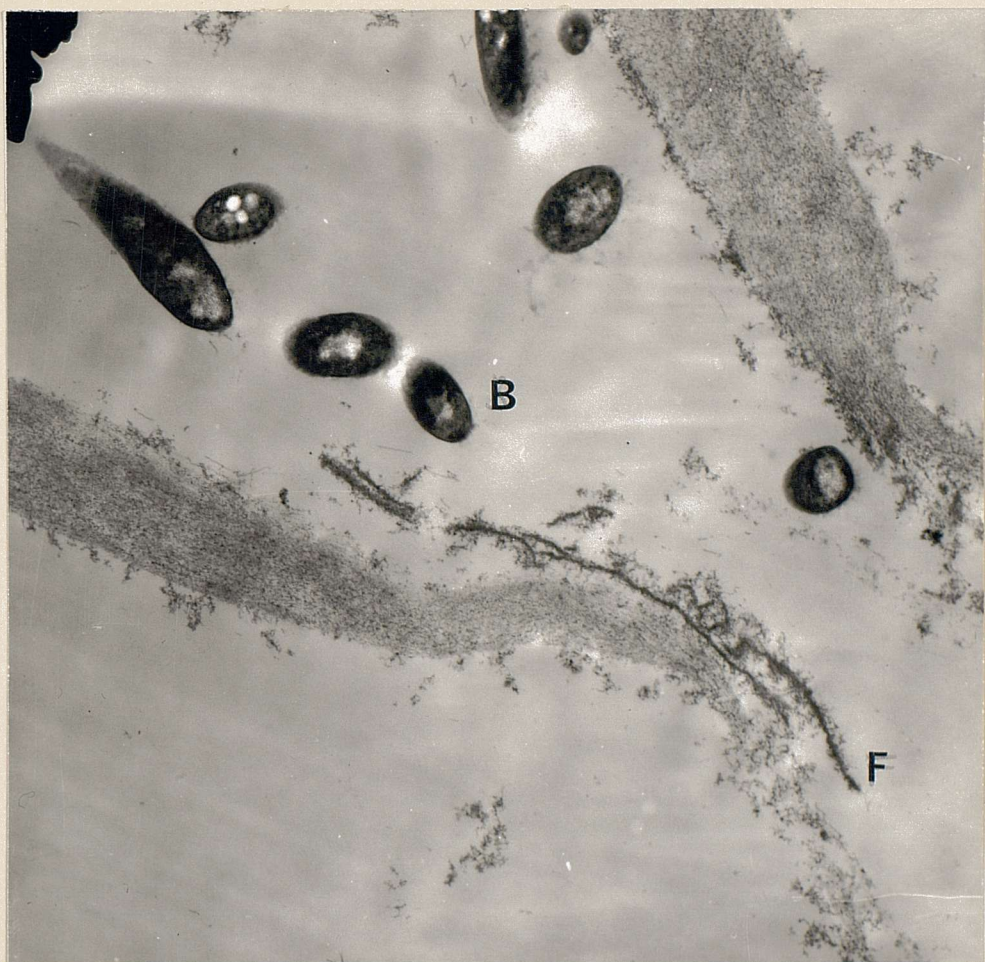
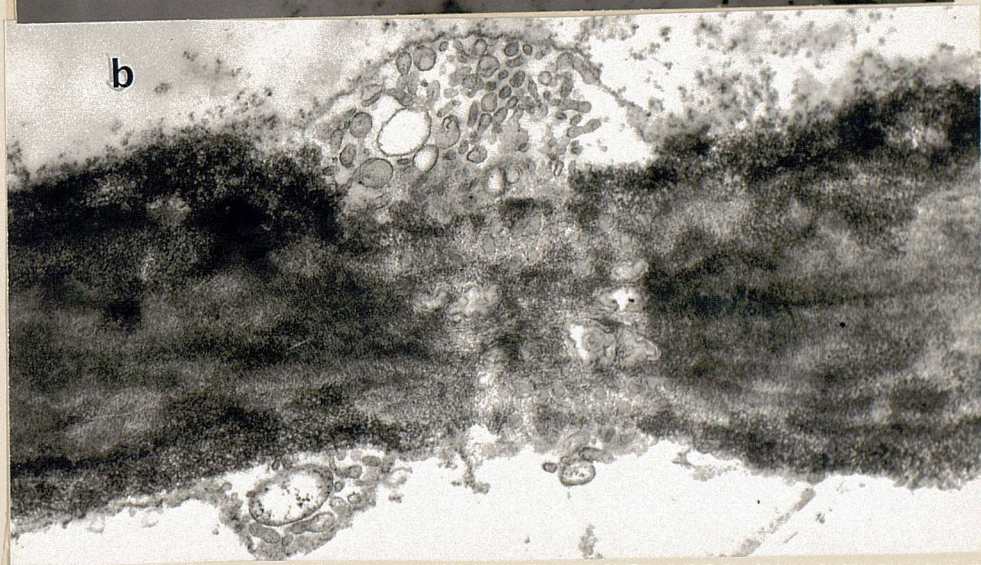
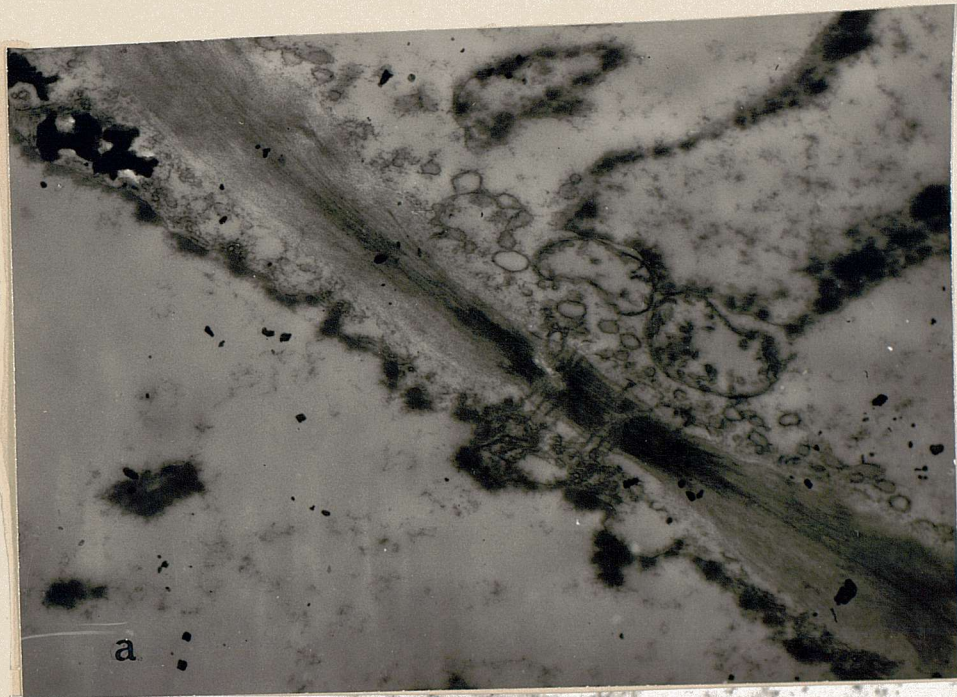


Plate 5.6 a.

(x 20,000, electron micrograph)

Break in the cell wall at the position of decayed plasmodesma, in terminal stage of maceration. Note bacteria^B and lamellar or fibrillar material.^F

(osmic acid, lead citrate.)



Plates 5.7 a. (x15,000) b. (x 30,000) **Electron micrographs.**
The plasmodesmata remain attached to the cytoplasm in old
infection. (osmic acid, lead citrate.)

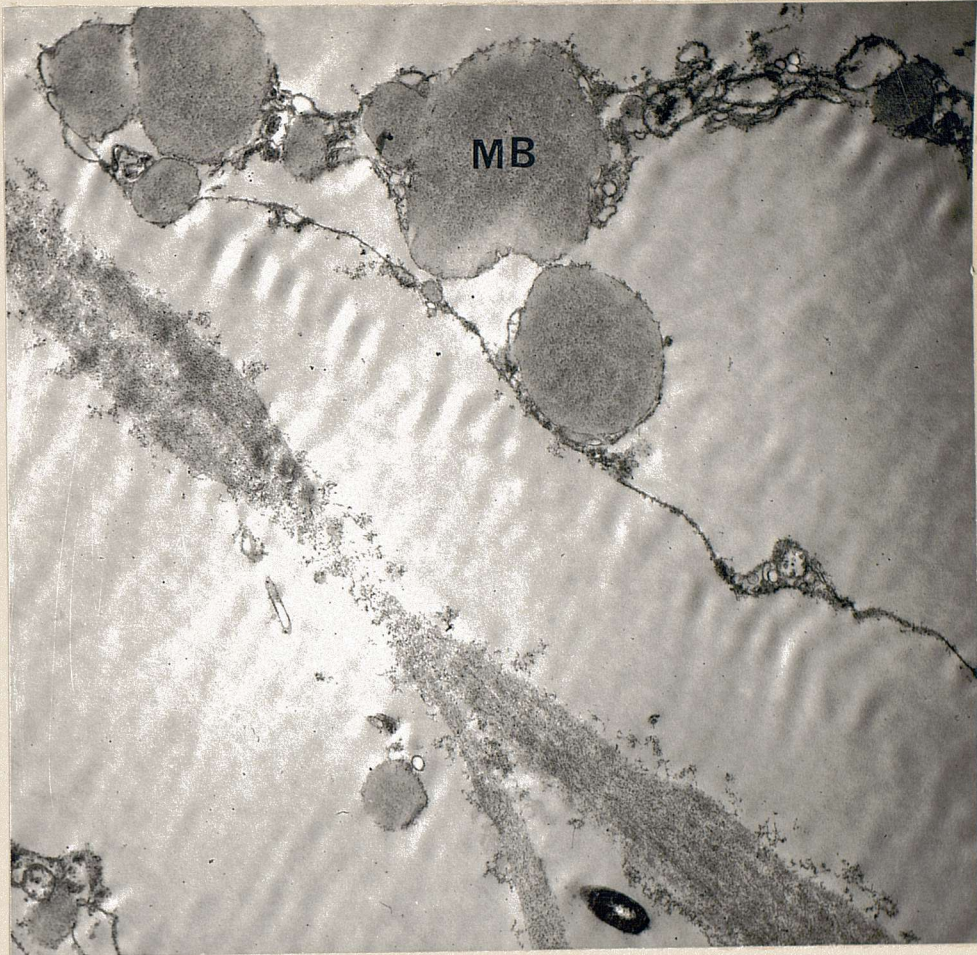


Plate 5.8 a.

($\times 10,000$)

MS

Late stage of infection at 25°C showing enlarged microbodies.

(osmic acid, lead citrate.)

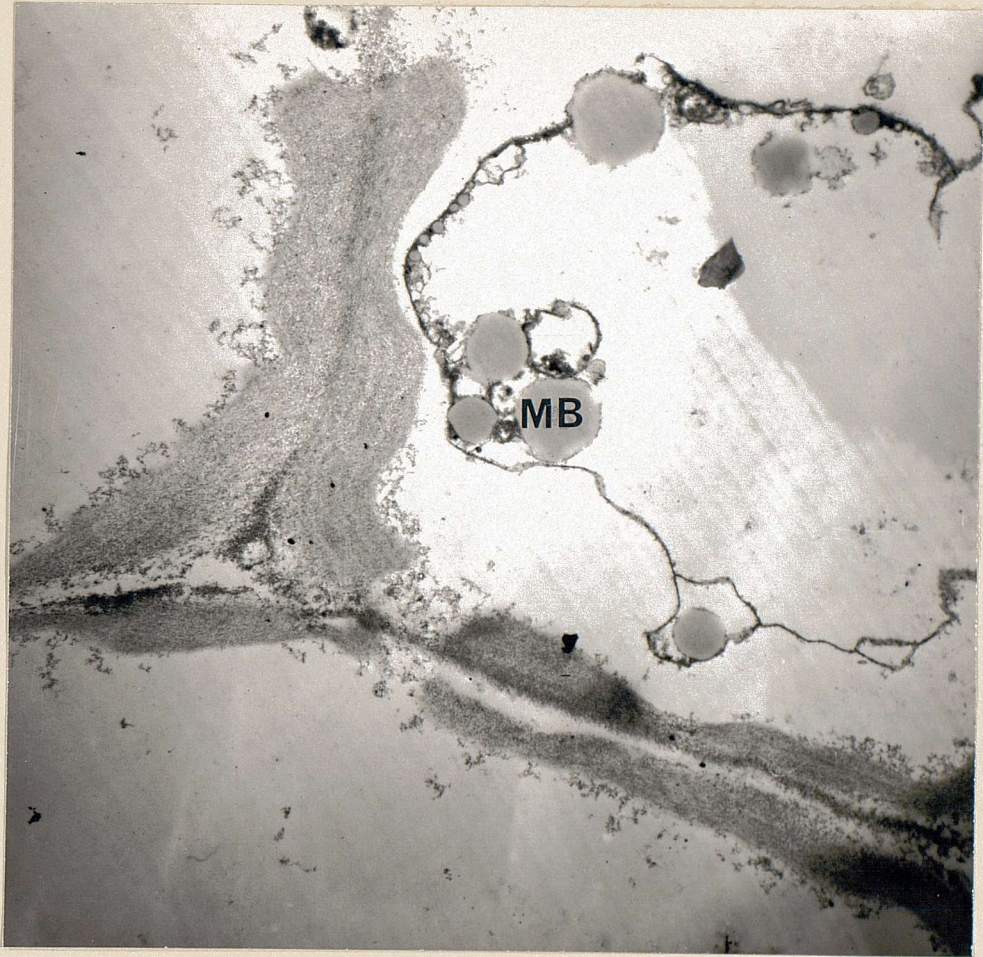


Plate 5.8 b.

(x 6,000, electron micrograph)

Macerated cells from infection kept at 25°C, showing the enlarged microbodies. MB

(osmic acid, lead citrate.)

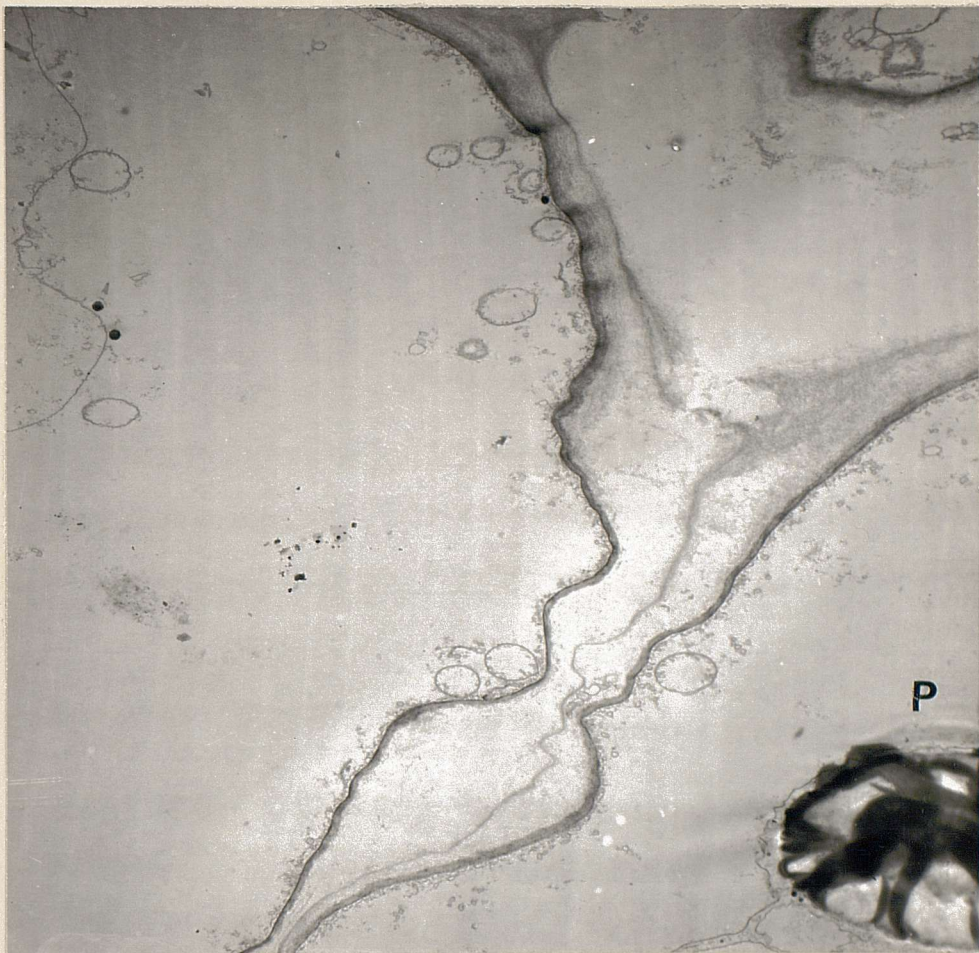


Plate 5.9 c.

(x 5,000, electron micrograph)

Macerated cells from infection kept at 5°C, note the better preserved cytoplasm, (e.g. the plastid containing the starch grain at ^{bottom right} top left), than those kept at 25°C.

(osmic acid, lead citrate.)

bacteria to enter the cell (plate 5.6).

3. Cytoplasmic damage.

At the terminal stages of cytoplasmic disruption, the cell is left with a more or less complete plasmalemma, although this is torn away from the cell wall where it may be attached to the plasmodesmata. This membrane does not appear to have suffered the same damage as seen in osmotic plasmolysis (Sitte 1966). The nuclear membrane is still intact, as are the membranes round the internal vacuoles, but the cytoplasm is reduced to membranes and what appear to be enlarged microbodies (plate 5.8 a & b). At temperatures below 25°C the microbodies do not become enlarged and there is less cytoplasmic damage. Plates 5.3 b and 5.8 c are of tissue maintained at 5°C and mitochondria, multifunctional plastids and endoplasmic reticulum survive next to severe maceration.

The range of cytoplasmic damage at any given point in a rot is very variable and any effect is localised to the extent of a few cells, but the severity of damage seen at higher temperatures is always greater than that at low temperatures. The state of the cytoplasm has an important bearing on the visible state of the rot to the naked eye. Different tissues equally macerated at the electron microscope level look and

smell quite different; slimy rots tend to have severe cytoplasmic damage.

Pitt (1969) has described the increase in diameter of lysosome-like particles in the potato storage parenchyma during infection by Erwinia. Using a histochemical test to localise acid phosphatase (Barka, 1960) very small points less than 0.5 μm in diameter gave a positive reaction in healthy tissue of King Edward used in this investigation, but positive reactions were given by bodies of over 5.0 μm diameter in diseased cells. The only bodies of **such** sizes apparent in healthy and rotted tissue respectively are the microbodies (Plates 6.7 b. & 5.8 a.).

If the microbodies seen in the electron micrographs possess the attributes of lysosomes (De Duve, 1959) they may be responsible for cytoplasmic damage. As these enlarged microbodies were not extensively developed at temperatures below 25°C, it is possible that their scarcity is responsible for the better preserved cytoplasm in low temperature rots. However it is possible that a 'cytase' produced by the host under the stimulus of infection may be responsible for both cytoplasmic damage and microbody development independently.

When stained with neutral red after 1% KOH (Strugger, 1935) to show cells in which the tonoplast was intact

and staining 'cherry red', none of the cells behind the bacterial infection front proved to have intact tonoplasts, nor did a layer of cells, 200 - 500 μm thick in front of the bacterial advance. From this it appears that substances diffusing from the bacteria or from the damaged cells cause the tonoplast membrane to 'leak', before the bacteria come into contact with the cells. Although leakage of the plasmamembrane cannot be detected in electron micrographs, the behaviour of the nuclear membrane is readily observable.

4. Nuclear damage.

Butler & Jones (1949) suggest that the nucleus of turnip root cells increases in diameter during infection by Erwinia, and it was decided to critically test this hypothesis in order to obtain an indication of the effect of soft rot on the cell membranes generally.

Experiment 5.2

To ascertain whether the nucleus increases in size with infection by Erwinia.

Materials & methods.

King Edward Grade 1 drybrushed tubers were surface sterilised with 0.1% mercuric chloride, and sterile slices taken and placed in an 18 h shake culture containing approximately 1.03×10^9 cells/ml of Erwinia. After incubation for up to 50 h at 25°C, samples were

removed and fixed in CRAF, embedded in 1947 ester wax, sectioned and stained in carbol fuchsin and fast green. The nuclear diameters of the first intact cells within 1,000 μm of the cut surface were measured with a micrometer eyepiece. In other tubers, sterile distilled water or nutrient broth replaced inoculum, and yet others remained untreated as controls.

Results.

Increase in nuclear diameter is characteristic of submergence in sterile distilled water, and nutrient broth as well as in Erwinia culture (Graph 5.1, 5.2, 5.3). Both of the former might possibly have been due to latent Bacillus infection as discussed in part 7. However, neither of the former treatments showed the rapid drop in nuclear size after 40 h seen in the Erwinia culture (graph 5.1).

Electron micrographs of nuclei at various stages of rot (plates 5.9 - 5.11 illustrate this sequence. When the diameter of the nucleus is at its greatest (plate 5.10), there is some budding off of fragments of the nuclear membrane (plate 5.11), followed by nuclear collapse.

Conclusions.

It appears that the soft rot affects the nuclear membrane, causing it to stretch and collapse. It seems

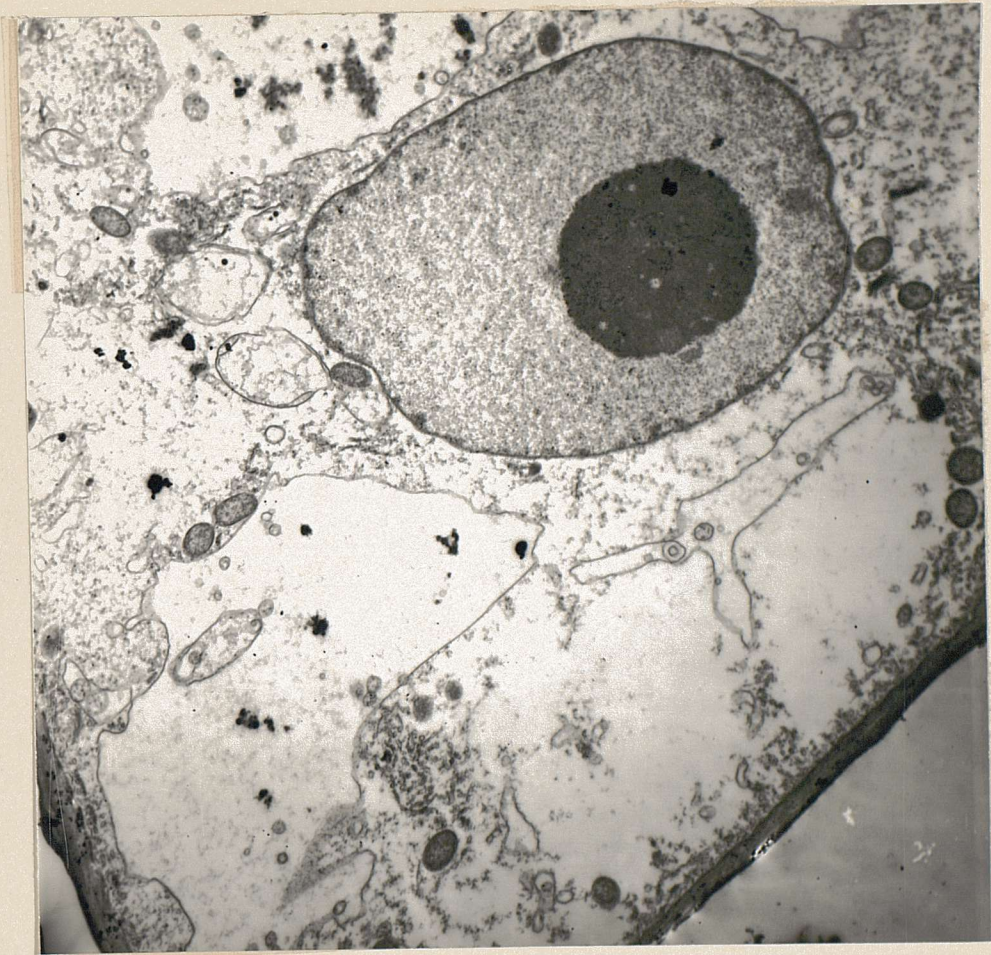


Plate 5.9

(x 6,000)

Nucleus and nucleolus from healthy tuber cell, showing the normal size and distribution of nuclear material.

(osmic acid, lead citrate.)



Plate 5.10

(x 10,000, electron micrograph)

Nucleus and nucleolus after 43 h exposure to soft rot,
note proportionally larger nuclear size, together with
some 'budding off' of the nuclear membrane (arrowed).

(osmic acid, lead citrate.)

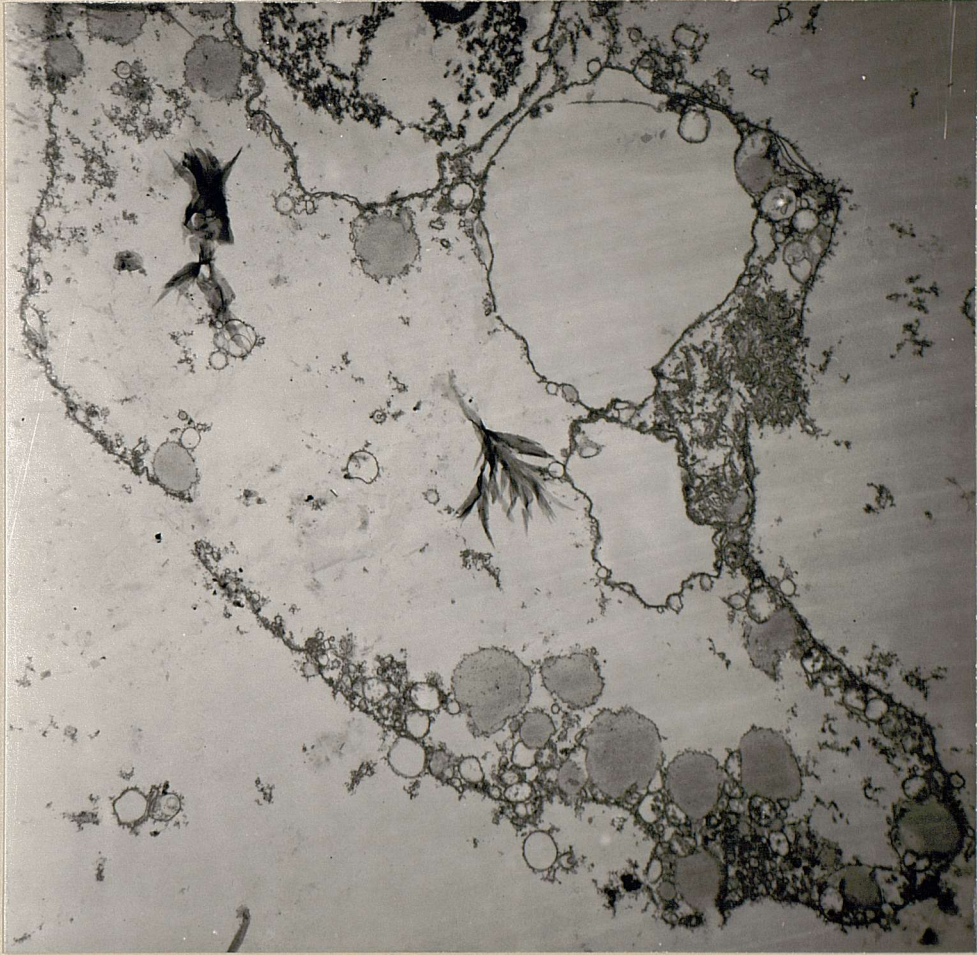


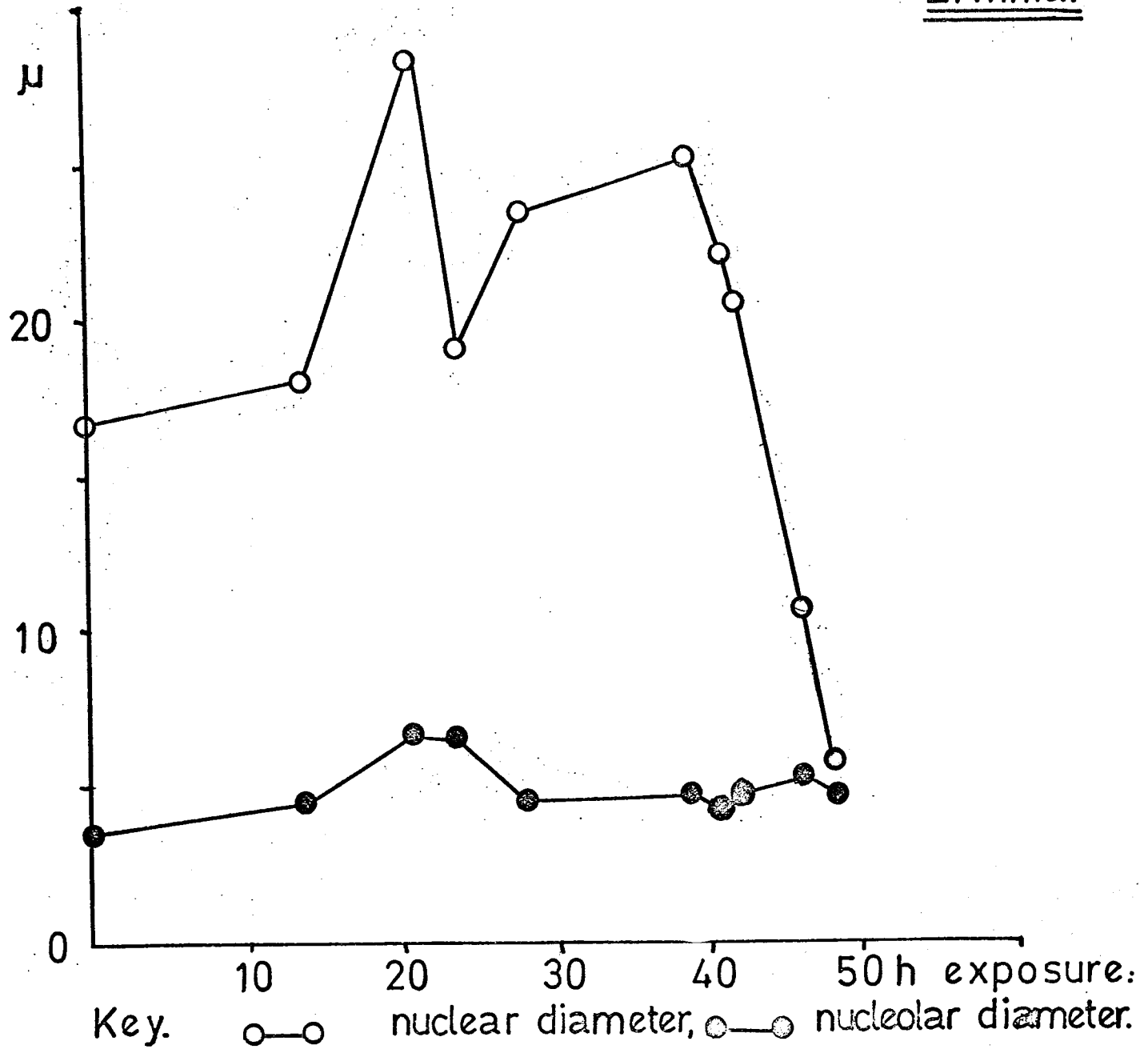
Plate 5.11

(x 6,000, electron micrograph)

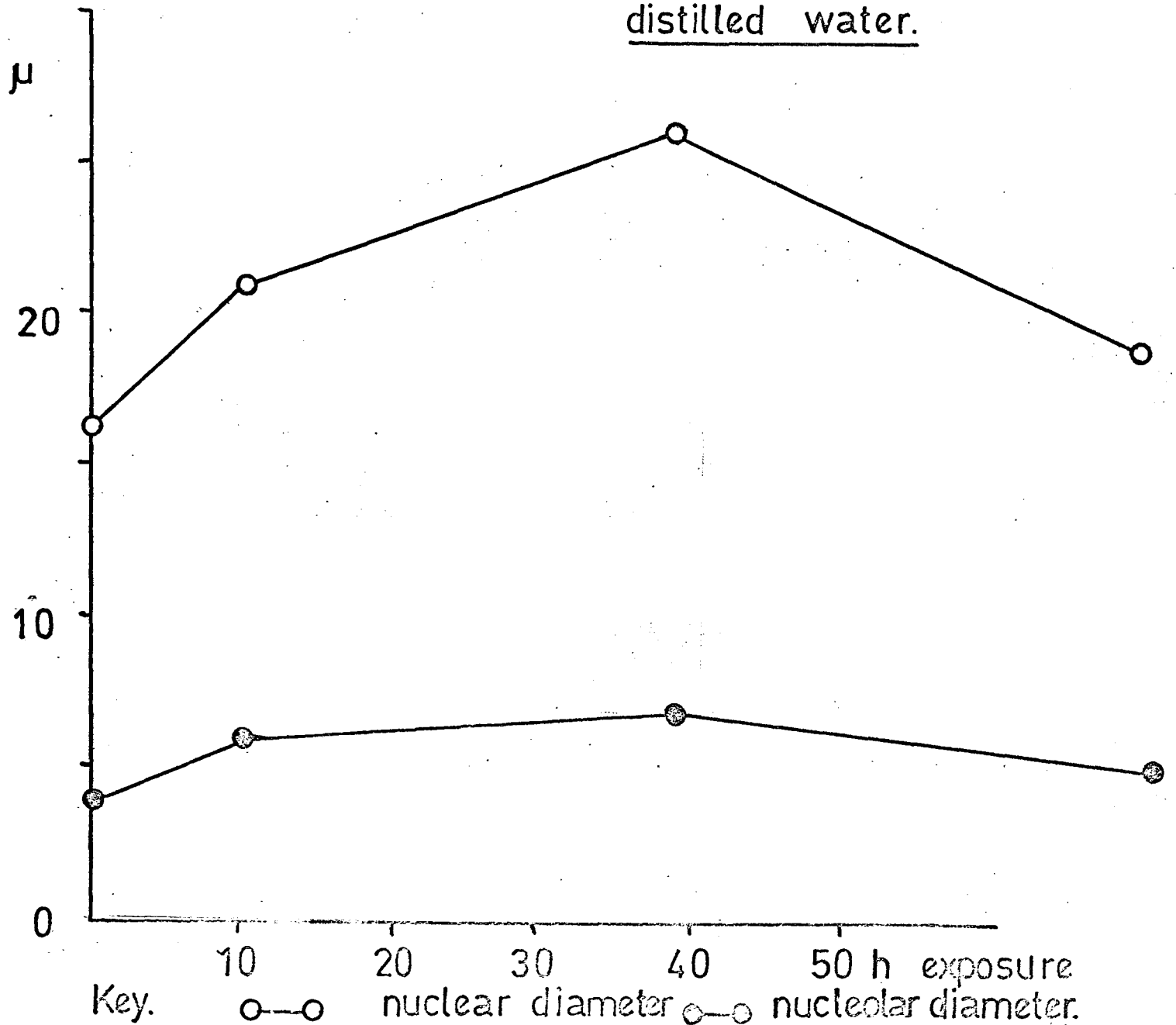
The remains of the nucleus and nucleolus in a cell after 36 days exposure to soft rot. Note the enlarged microbodies inside the plasmamembrane together with vacuoles and other membranes.

(osmic acid, lead citrate.)

Graph 5.1 Nuclear & nucleolar size/exposure time to Erwinia.

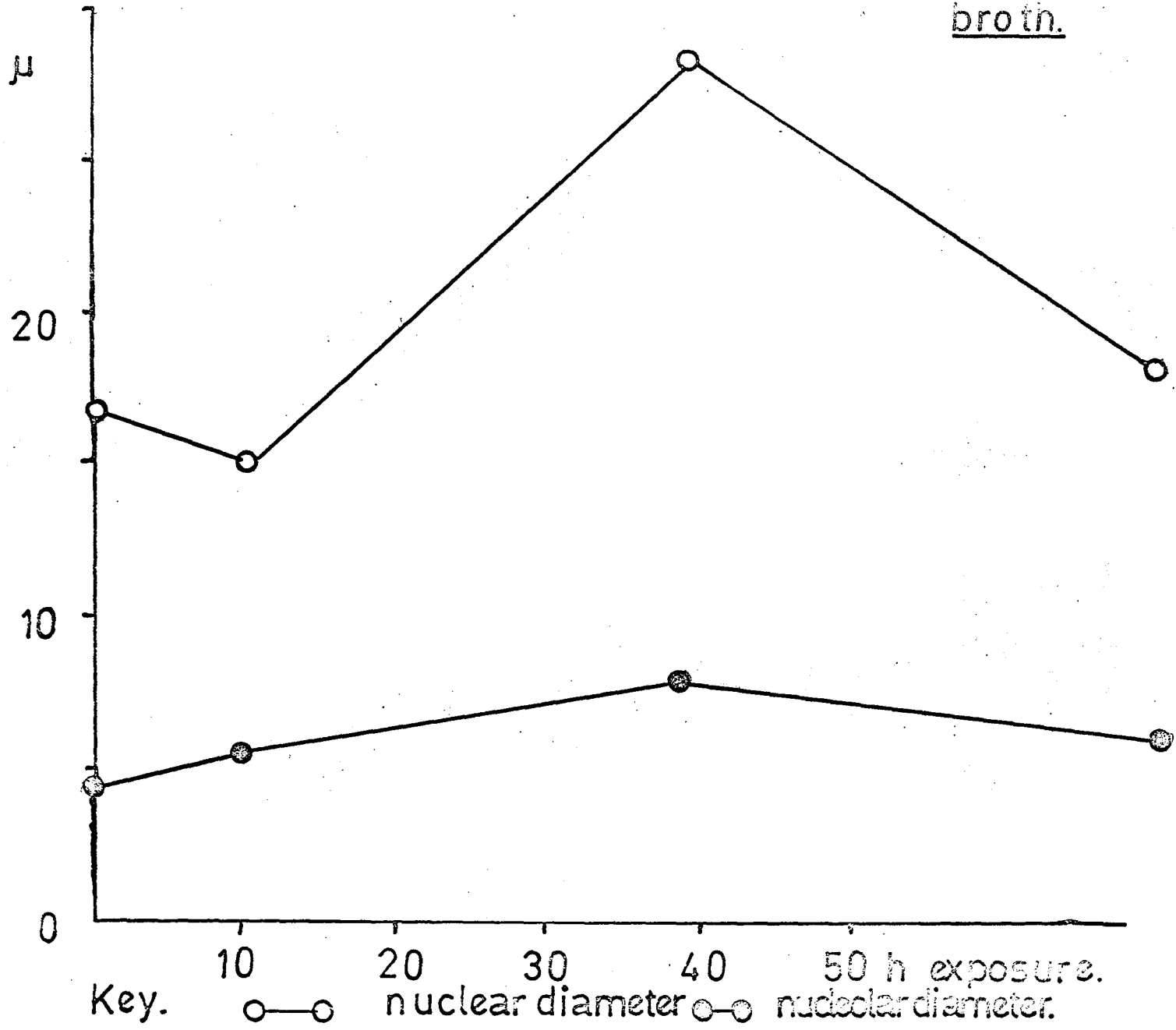


Graph 5.2 Nuclear & nucleolar size/time in sterile distilled water.



Graph 5.3 Nuclear & nucleolar size/exposure to nutrient

broth.



possible that this effect is not confined to the nucleus, (plate 5.10), but may extend to other membranes as the mitochondria (plate 5.3 b) and plastids may be swollen at early stages of cytoplasmic damage. It is difficult to compare the behaviour of the plasma membrane which appears closely adpressed to the cell wall in early infection but appears as if contracted in later stages (plate 5.11) due to cytoplasmic plasmolysis, but may be attached at the plasmodesmata (plate 5.7a. & b.) before this, c.f. the stages of plasmolysis (Sitte 1963).

Discussion.

The electron microscope give a unique picture of cell wall degradation, compared to the rather disappointing results of light microscopy. Using Albersheim's method it can be seen that pectin is irregularly distributed through the cell wall and this may account for the different nature of their reactions of the cell wall to soft rot. For example, the intercellular area which is rich in pectin forms the site of colony establishment, while the cell wall away from this splits in a ragged way and appears to swell after pectolysis and is held together until late stages of rot by the plasmodesmata. The latter do not appear to constitute a direct cytoplasmic connection between the neighbouring cells, although the cytoplasm may be attached there during the

later stages of soft rot, disruption of the plasmodemata does not therefore automatically involve cytoplasmic damage. Cytoplasmic damage appears further advanced in its terminal stages at the higher temperatures; here disorganisation occurs while the microbodies enlarge and the cell membranes expand and burst. At lower temperatures a 'cleaner' rot occurs, less slimey and odourless; its ultrastructure characterised by the absence of enlarged microbodies and well-preserved organelles. Whether Erwinia produces 'cytase' or not, the effect on the cytoplasm is profound at 25°C leading to the unpleasant complete breakdown of tissue seen in advanced rots.

PART 6. Host defence mechanisms.

Part 6.

Host defence mechanisms.

Introduction.

It is generally accepted that disease resistance may be due to chemicals or structures present before infection or to systems which develop after infection.

1. Preformed systems of resistance.

Structural resistance has been discussed in some detail in part 2. Resistance based on preformed substances is well known, and it is recognised that polyphenolic substances are ~~the~~ significant in this respect in certain diseases (Rubin & Artsikhovskaya, 1964). In the uninfected potato these substances are present in the 'eye' region around the buds, in and under the lenticel filling tissue, in and under the primary periderm (plate 6.1), and in the vascular parenchyma, but are not detectable elsewhere in large enough quantities for a positive Hoefpner-Vorsatz reaction. However the lenticel filling tissue, the primary and secondary periderm, and the vascular parenchyma are characterised by suberised cell walls as well as different anatomical structure from parenchyma making in situ assessment of the relative components of this preformed system difficult. Jobber (1969) has demonstrated in vitro effects on bacteria of chlorogenic acid. At

low concentration their growth rate was reduced, at higher ones cell death occurred. It is difficult to equate any in vitro phenomena with the situation in the potato where the chlorogenic and caffeic acid appears to be confined to the interior of the tonoplast. Even assuming a process which transports unoxidised or partially oxidised polyphenols to the plasmalemma, it appears unlikely that the concentration of chlorogenic acid present in the intercellular spaces is as high as in ^{side} the tonoplast. If the bacteria present in the intercellular spaces are exposed to small concentrations of chlorogenic acid they may be stimulated rather than inhibited (Jobber, 1969). Where polyphenols are present as preformed substances in ^{side} the tonoplasts of cells below the filling tissue they do not by themselves prevent infection through this channel. Whether preformed factors for resistance were present or not, this failed to stop infection developing after entry through any of the channels studied but this may have been due to the high concentration of inoculum used.

2. Systems developed after infection.

Of the systems which confer resistance after infection in plant diseases polyphenol oxidation and suberisation of the cell wall have been put forward as being active against soft rot. The role of plant

. polyphenols as a system which develops after infection has been considered by Lovrekovich, Lovrekovich & Stahmann (1967) and these workers suggest that an oxidised polyphenol zone may act as a barrier round an infection exposed to air. The development of suberin as a barrier to infection at the infection interface has been described by Rudd Jones (1948).

Both these systems were investigated by light and electron microscopy, and other techniques with the object of determining if either or both these systems were responsible for resistance. A variety of techniques was necessary, as in many situations both appeared to be active at the same locus.

Suberisation.

Introduction.

Infection interface suberisation has been described by Priestley & Woffenden (1923) and Rudd Jones (1948). Both found that the thickness of the suberin layer in each cell and the number of cells affected by suberin is highest at lower humidities and temperatures, Rudd Jones (1948) using bromothymol blue indicator; found that suberisation is accompanied by a change in pH from 5.6 to 6.3.; Herklots (1924) studying the effect of an artificially controlled pH on wound healing in the potato found that suberisation was promoted by shift to

alkaline condition, the pH at the interface environment therefore does not appear unsuitable for Erwinia. In his photographs of the suberin barrier in naturally infected tubers Rudd Jones (1948) shows a blackened cytoplasm possibly due to the presence of melanin granules.

The exact nature of the biochemistry of the processes of suberisation and polyphenol oxidation are not at present known but it does appear that both may be controlled by polyphenol oxidase (Politis 1948), and it has been also suggested that phlobaphenes, oxidation products of polyphenols may be laid down in the cell wall. If this is the case, distinguishing between the effectiveness of polyphenols and that of suberin as barrier substances may prove difficult.

Ultrastructure of the suberin barrier.

The nature of the suberin infection interface has not previously been described at an ultrastructural level. The mode of formation of the suberin barrier is evident at this magnification, as is that of imperfect suberisation which may be bye-passed as shown in part 2: both of these are undetectable by light microscopy of stained preparations, (plate 6.4).

The suberin laid down at the infection interface, although of variable thickness, and state of development resembles in its histochemistry under the light

microscope, the suberin barriers of the primary periderm, filling tissue and wound suberin. It also resembles these in its ultrastructure. The suberin is laid down extensively at the intercellular spaces from the edges inwards, so that the central hollow is encrusted with suberin; this deposition connects with two layers which are laid down in the cell wall (plate 6.5 a,b.) The two layers in the cell wall are characteristically absent at the plasmodesmata (plate 6.3a).

The cytoplasm in cells which have suberin in the cell wall is extensive and contains undamaged nuclei, numerous mitochondria, microbodies, and golgi bodies and associated small vacuoles, and plastid development is active (plates 6.5d, 6.6.). Also present in the cytoplasm are 'myelin' bodies similar to those found by Marinos (1967) in potato bud cells. These appear responsible for the production of large vacuoles, some of which contain melanin granules (plate 6.7.), some of these vacuoles appear contiguous with the plasmalemma (plate 6.8). Whether negative or positive pinocytosis takes place here was not established. The myelin bodies may contain starch, plasmoglobuli, phytoferritin, and always central tubules, and so have a similar collection of contents to those of the multifunctional plastids. Marinos (1967) has described the way in which the latter

produce small pockets between the outer membranes, which would if developed further resemble the configurations of membranes round the 'myelin' bodies, (plate 6.9). Plate 6.10 shows some of the outer membranes enclosing a vacuole which is apparently being budded off, the membrane thickness appears similar to that of the vacuoles present in the cytoplasm, one of which can be seen in the bottom left hand corner of the plate.

In this investigation suberin was often found in the cell walls of cells containing melanin in the tonoplast (plate 6.2.). In the artificially inoculated half tubers used in this investigation, ester wax sections (stained with ammonical gentian violet (Artschwager 1927), of melanin containing cells showed that after 40 hours suberin was present five cells thick at 0 and 58.3% R.H., three cells thick at 80.5% R.H. and absent at 100% R.H. where the relative humidity chambers were kept at 25°C. At lower temperatures suberin but not melanin may be present, for example after 92 hours at 5°C and 100% R.H., three to four cells were suberised but did not contain melanin.

At 25°C melanin granules are seen in those cells which are suberised as electron dense bodies in electron microscope sections fixed with 1% osmic acid (plate 6.3).

Polyphenol oxidase which has been alleged to mediate both

polyphenol oxidation and suberisation, has been studied in the potato tuber by Anderson (1969), who suggests that this enzyme is localised in a fraction intermediate in size between mitochondria and chloroplasts as well as in the latter. This organelle is said to be relatively dense which suggests inclusions are present; from a survey of possible candidates found in thin sections of potato cells, the multifunctional plastids appear a likely choice. Multifunctional plastids are abundantly present in both suberising cells and also in those cells in which melanin has been formed, e.g. young filling tissue, wound periderm, wound suberin, and vascular parenchyma.

The precise localisation of polyphenol oxidase in these organelles requires the application of enzyme etching and immunochemical staining.

The results of this activity in the suberising cells is the product of an impermeable coat around the cells in the cell wall and the intercellular spaces. The destruction of this coat by specific enzyme action is not established but it does appear possible that the coat can become detached from the cell wall by the dissolution of the region around the outside of the original intercellular space, and in the region between the cell walls (plate 2.14 & 2.15). Both these areas are

the ones richest in pectin as seen from the Albersheim's preparations. The relative stability of the periderm is due to the fact that the suberin here is present either side of a minute layer of pectin, and this tissue will resist the most persistent soft rot, together with some vascular tissue. Plate 6.11b shows periderm prepared by Albersheim's method. Pectolysis could therefore be an agent in the breakdown of the effectiveness of an incomplete suberin barrier, which evidently occurs in mature infections at high temperatures.

Polyphenol oxidation.

Introduction.

It is generally recognised that plant polyphenols and their oxidation products are of significance in a number of host parasite relationships (Wood, 1967). Lovrekovich, Lovrekovich & Stahmann (1967) reported the formation of a 'black infection barrier' composed of the oxidation products of phenols on the cut surface of potato tubers around an infection site. Experiments carried out in the course of the present investigation revealed a general picture comparable to that found by Lovrekovich and co-workers. Infection spread rapidly from the point of inoculation for the first 40 h; at high temperature the rate subsequently fell off and a black zone developed around the infected area (plates

6.12 a,b,). As time proceeded the black zone "moved" slowly outwards; the mechanism by which this occurred is not known, but the apparent movement could have been due to the masking of the pigment particles by the mass of bacterial cells in later stages of infection, as melanin may be found inside soft rotted cells.

Experiment 6.1.

To investigate the effect of differences in inoculum level and potato variety in the formation of a 'black infection barrier' and bacterial spread in a controlled environment.

Materials and Methods.

Grade 1 dry brushed Majestic tubers were washed in tap water, surface sterilised in 0.1% mercuric chloride, washed in sterile distilled water, dried and cut in two, and a central 7 mm diameter core removed with a cork borer leaving a well approximately 5 mm deep.

The central well was inoculated with 0.1 ml of a known concentration of bacterial suspension, which was spun down at 3,000 r.p.m. from approximately 24 h old cultures in nutrient broth.

The inoculated potato tubers were kept at 25°C and 100% R.H. and measurements of the diameters of the rots and the extent of the 'black infection barriers' were measured and samples taken for light and electron

microscopy.

Results.

Graph 6.1. shows a very similar state of affairs to that described by Lovrekovich et al (1967) in that variations in the level of the inoculum concentration caused great differences in the rates of bacterial spread and formation of the 'black infection barrier'.

Experiment 6.2.

To investigate the effect of temperature on bacterial spread and the formation of a 'black infection barrier' in a controlled environment.

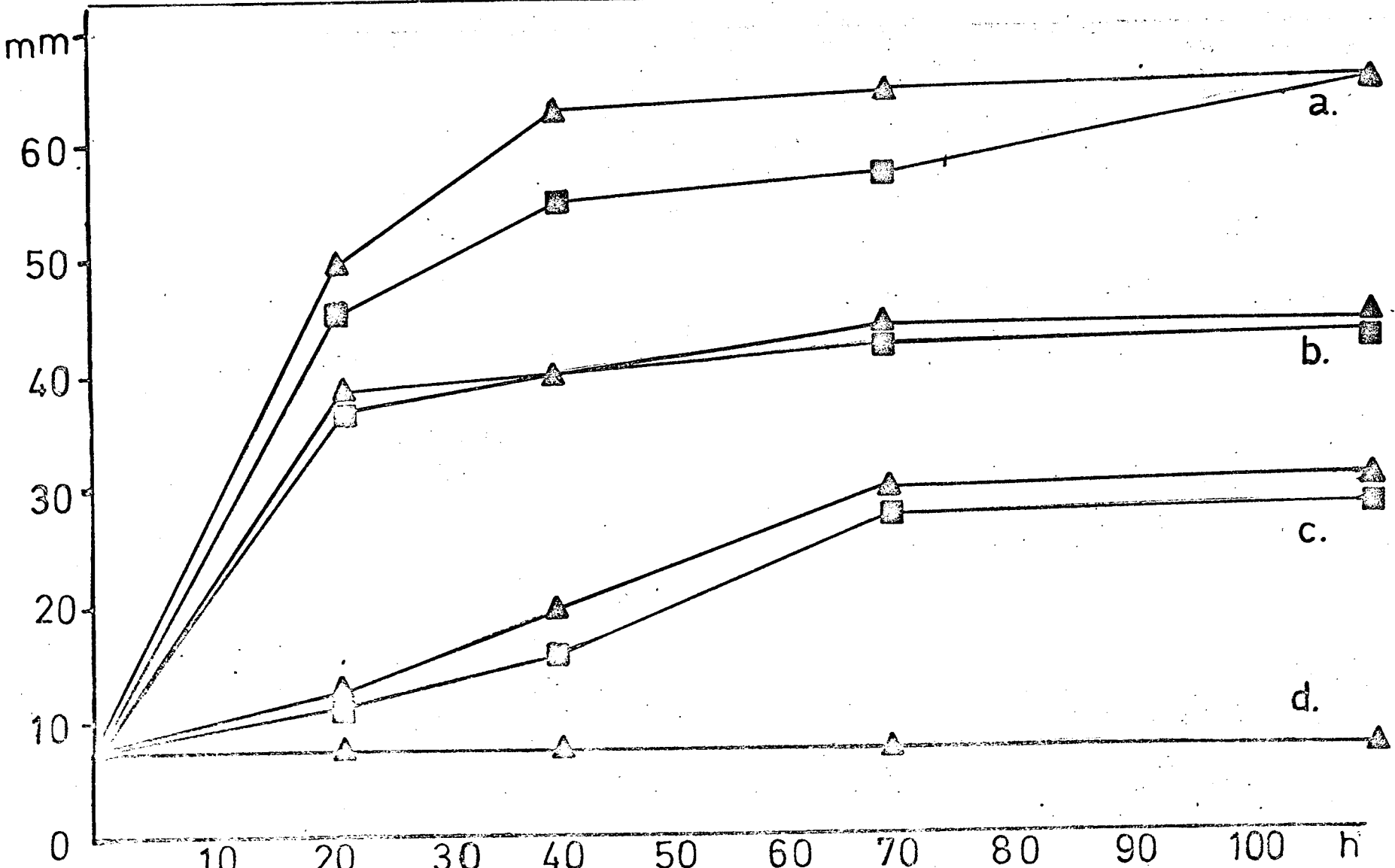
Materials and methods.

Tuber halves of Majestic and King Edwards were prepared as described in the previous experiment, inoculated with 0.1ml of approximately 4×10^{11} cells/ml. and kept at 100% R.H. and at 5', 10' and 25°C in relative humidity chambers. Measurements of the spread of infection were made, and the extent of the 'black infection barriers' where present was measured, and samples were taken for electron microscopy and light microscopy.

Results.

Graphs 6.2 & 6.3 show rapid initial spread at 25°C up to 40h with a tailing off afterwards, but the spread at 10°C and 5°C was much more gradual and was not

Graph 6.1 Effect of inoculum size. [Majestic, 100%RH, 25°C]

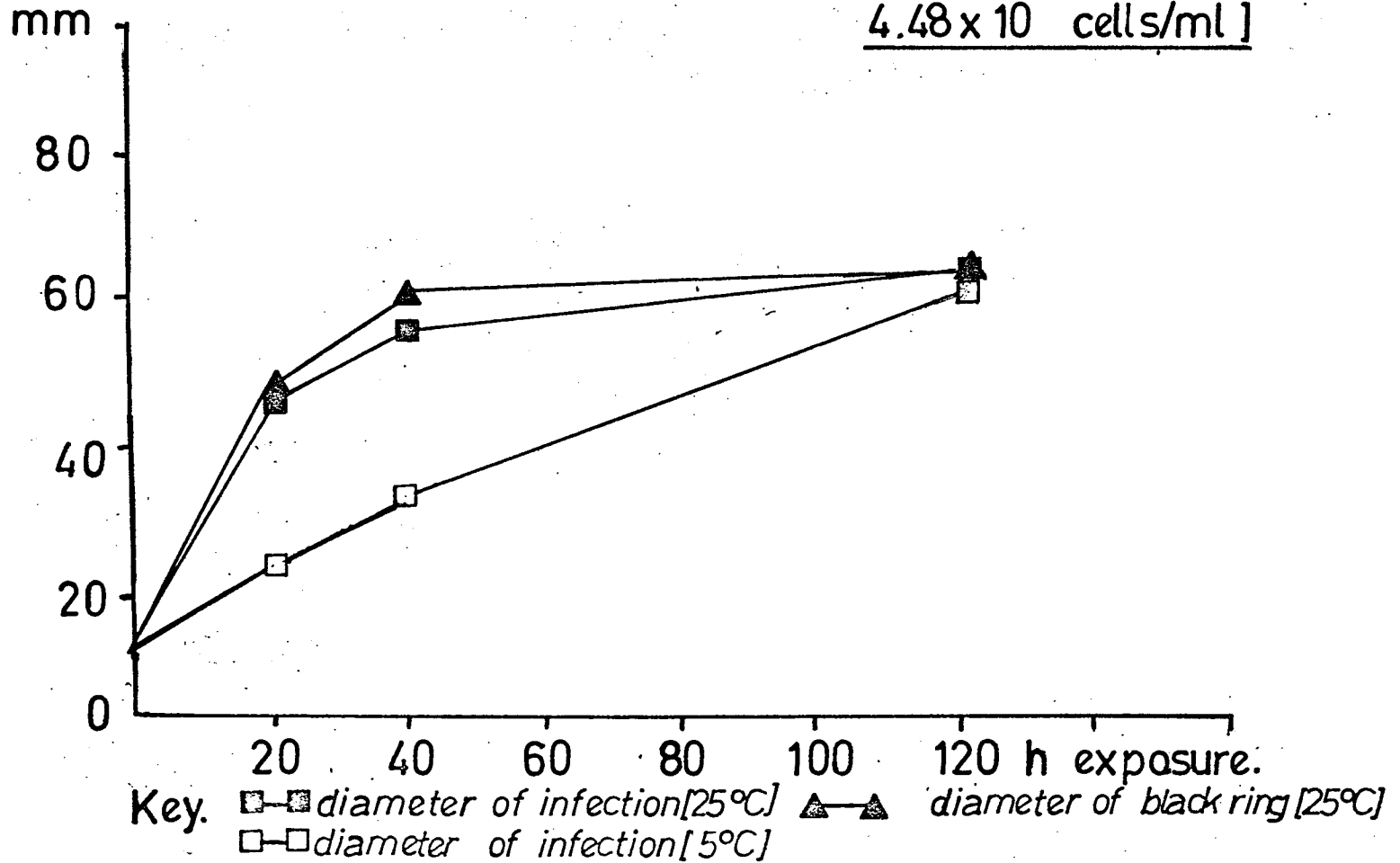


Key. \blacktriangle diameter of black ring. \blacksquare diameter of infection.

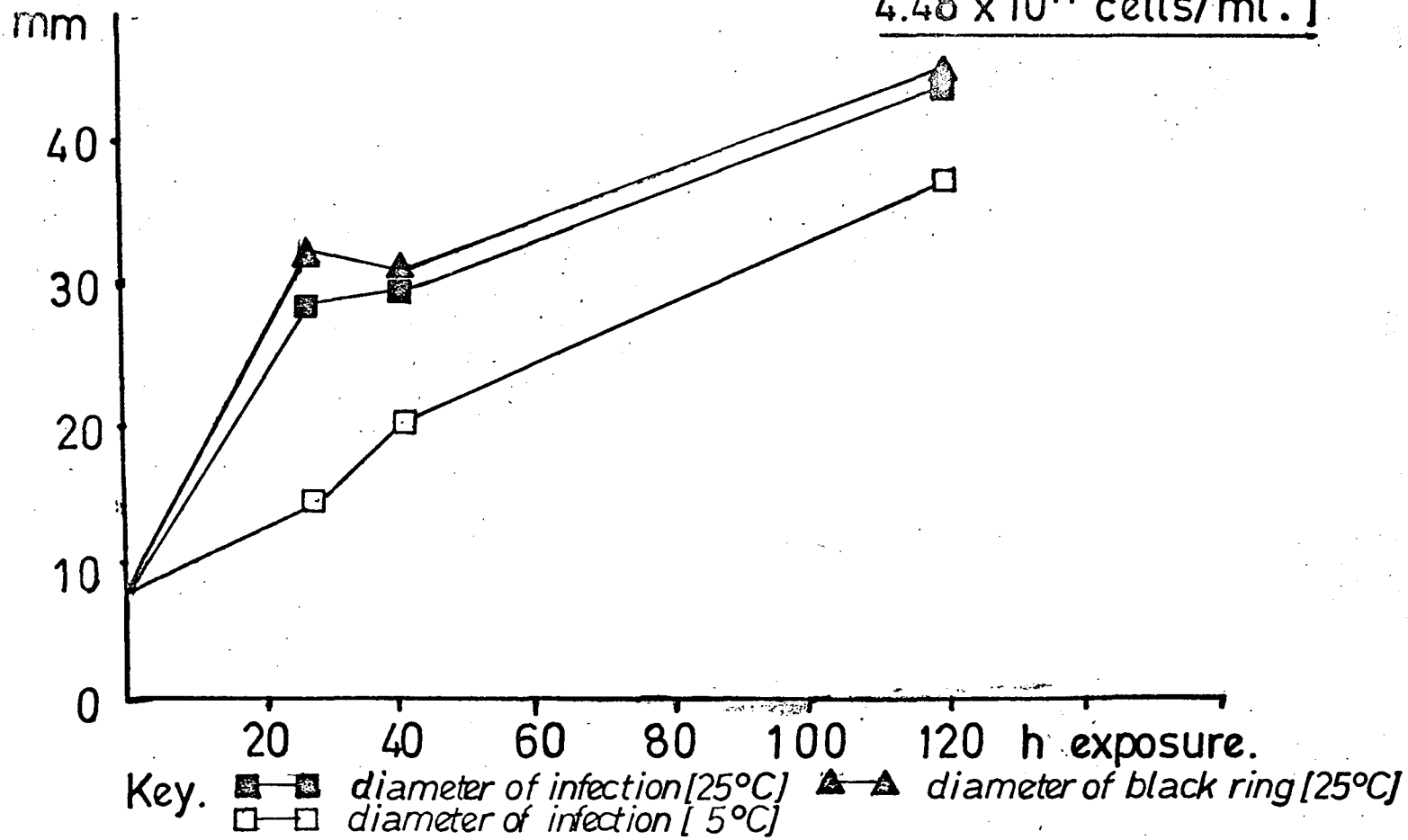
a. 4.50×10^{11} cells/ml c. 1.30×10^{10} cells/ml.
 b. 2.24×10^{11} cells/ml d. 1.20×10^9 cells/ml.

Graph 6.2 Effect of temperature. [Majestic, 100%RH

4.48 x 10 cells/ml]



Graph 6.3 Effect of temperature. [King Edward, 100%RH
4.48 x 10¹¹ cells/ml.]



accompanied by the presence of a black infection barrier. This effect of temperature resembled that described in part 3 for development of colonies perpendicular to an inoculated surface. There appeared to be a difference in the rates between the two varieties studied, and both appeared to require much higher inoculum levels than Red Nuget, used by the American workers, before infection was successful. In the present investigation, inoculum of 10^9 cells/ml. did not cause any more than a very local infection whereas in Red Nuget it caused an extensive rot.

Experiment 6.3.

To ascertain the effect of relative humidity of storage on the development of rot and infection barriers at 10°C.

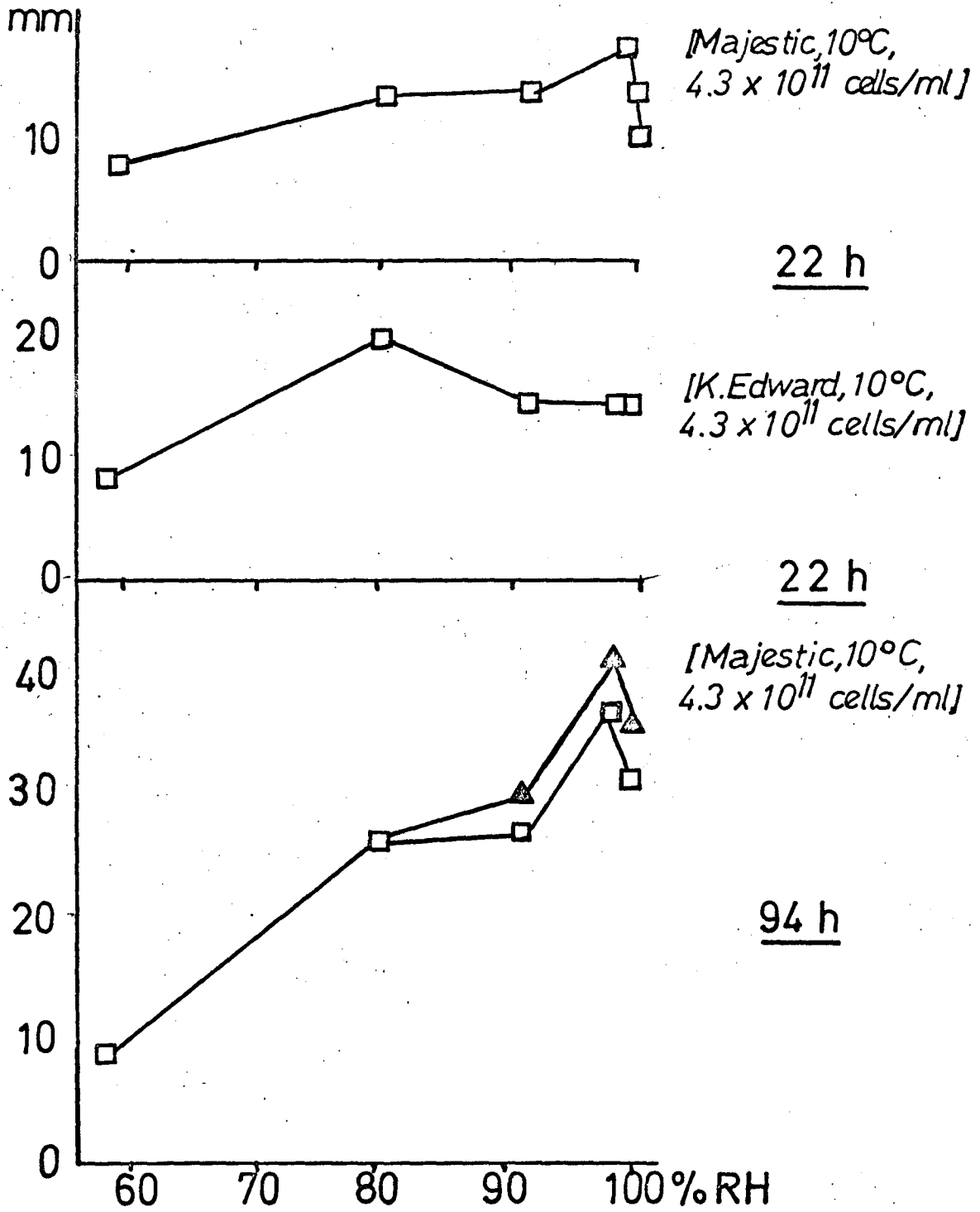
Materials and Methods.

Half tubers of King Edward and Majestic potatoes were prepared as described before, and inoculated with 0.1ml of approximately 4.3×10^{11} cells/ml of 23 h old inoculum. The inoculated tubers were kept at 58.3, 80.5, 93.9, 97.5 or 100% R.H. above diluted sulphuric acid in a closed vessel for 22, 67, 94 h and 40 days when they were measured and harvested for light and electron microscopy.

Results.

Graph 6.4 shows the results after 22 and 94 h; it :

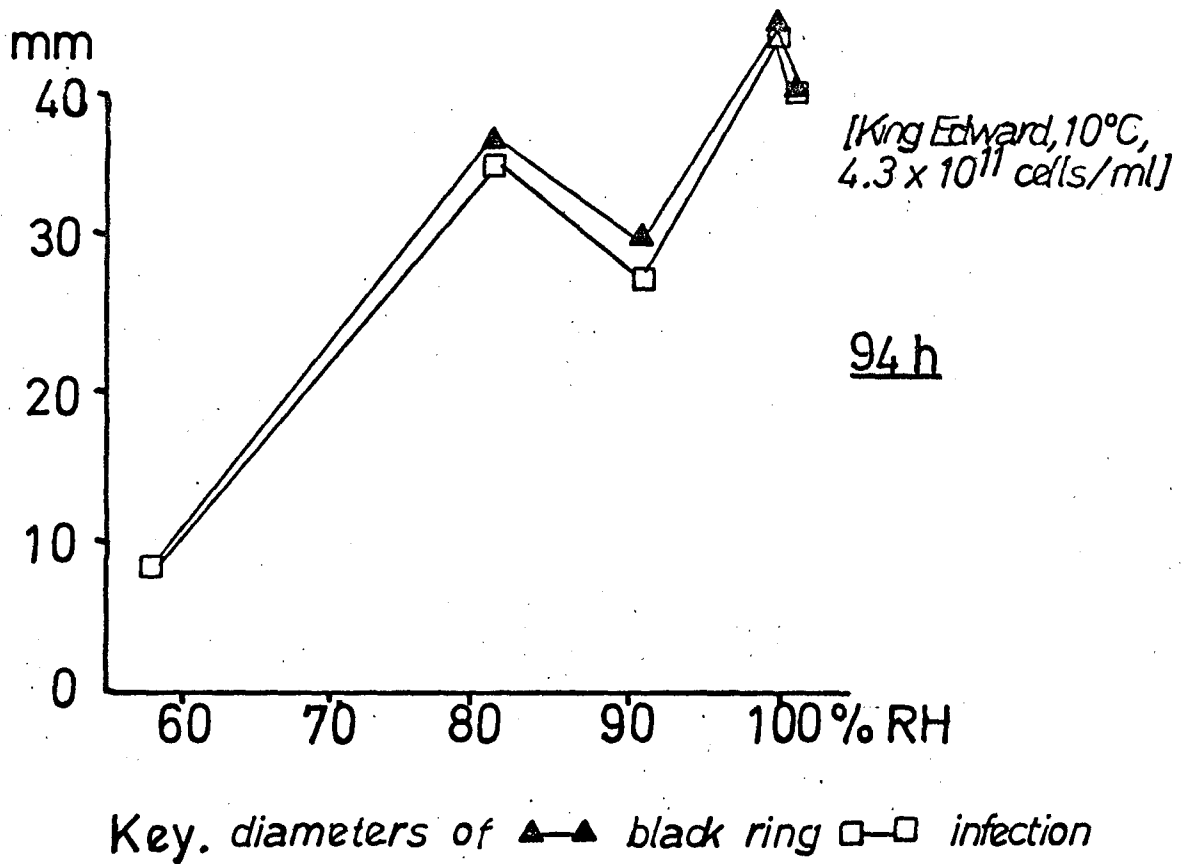
Graph 6.4 Effect of relative humidity. A.



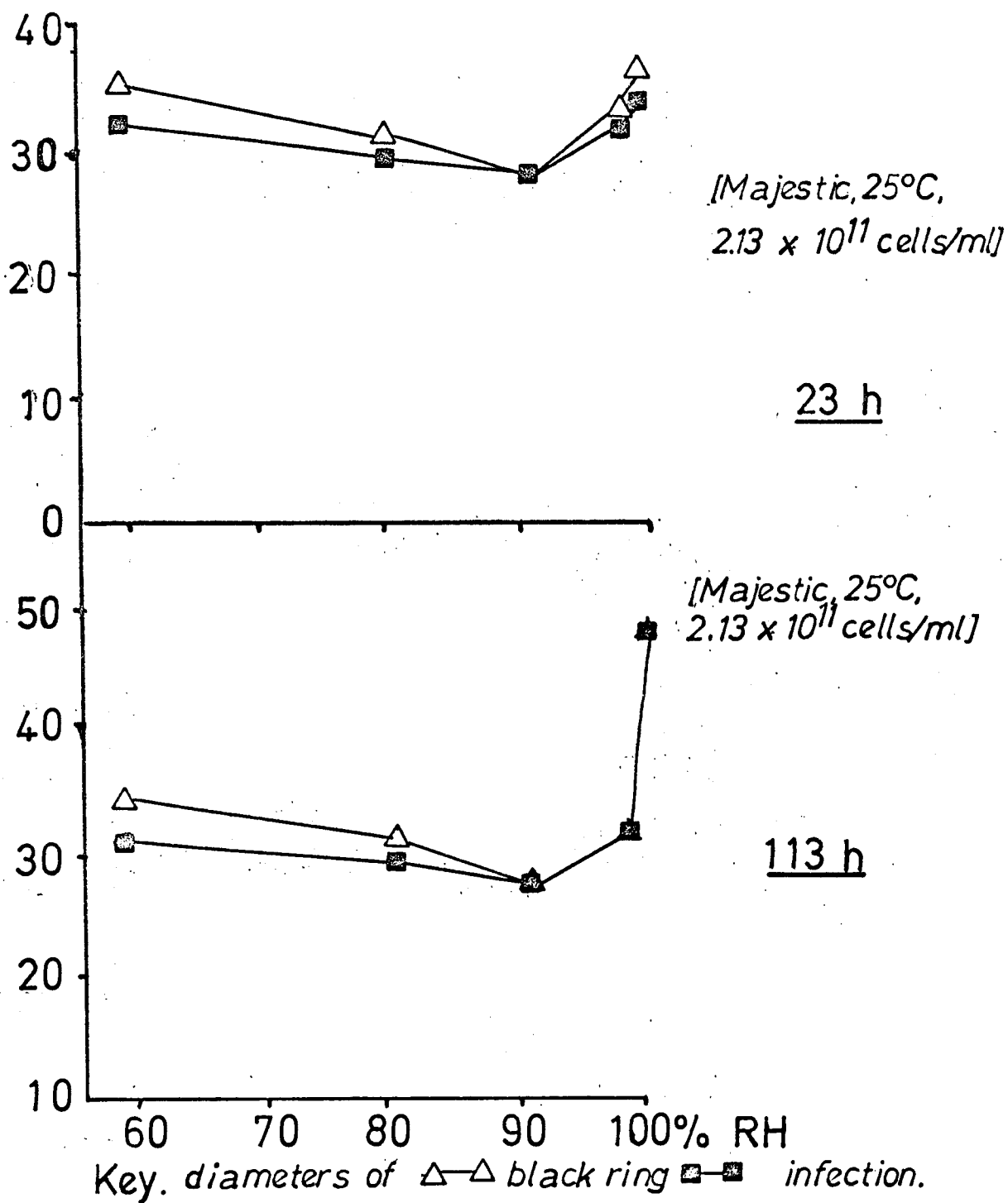
Key. diameters of ▲-▲ black ring, □-□ infection.

Graph 6.4 Effect of relative humidity.

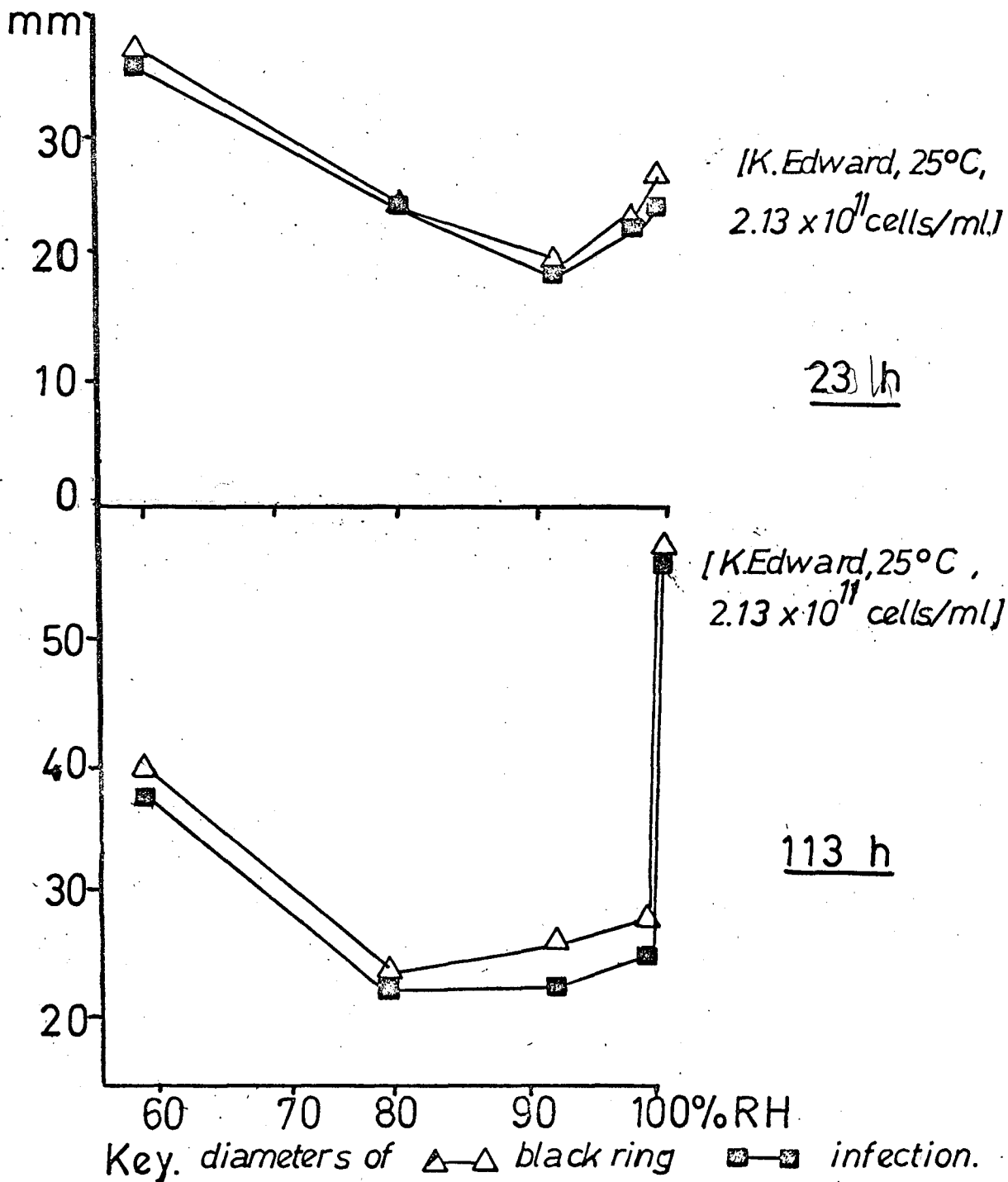
B.



Graph 6.5 Effect of relative humidity. A.



Graph 6.5 Effect of relative humidity B.



appears that in both varieties at 10°C, the rot and black ring development are most extensive at the higher relative humidities. This is confirmed at 40 days where only the potato halves at 93.9% R.H. and above were completely rotted.

Experiment 6.4.

To ascertain the effect of relative humidity of storage on the development of rot and of infection barriers at 25°C.

Materials and Methods.

Half tubers of King Edward and Majestic potatoes were inoculated as above with 0.1 ml of 2.13×10^{11} cells/ml of a 22h old culture, and kept under the same range of relative humidities at 25°C. Measurements were made after 23,44,68,113 and 137 h.

Results.

Graphs 6,5 a & b, show the measurements after 22 and 113 h. There is little extension of the rot except at 100% R.H.

Conclusions to both experiments.

It was concluded from both these experiments that the higher relative humidities favour the spread of the rot, although this is not the case initially at 25°C, perhaps due to favourable conditions for wound suberisation at the higher relative humidities.

Melanin Histochemistry.

The o-quinhydrone configuration of the melanin complex has been employed in the diagnostic ferrous iron technique for melanin (Lillie, 1957) and the less diagnostic Methanamine silver reaction (Gomori, 1952). Both of these tests used in this investigation confirmed the identity of the black granules found in the 'black infection barrier' cells, as melanin. Electron microscopy has shown that these melanin granules are present in the tonoplast of the cells.

Plate 6.13 shows melanin granules after treatment involving fixation in 1% osmic acid in 5% sucrose, here regular electron dense bodies are seen; the more complicated form which may also be found are shown in plate 6.14.

All these granules are present either in the central vacuole or in other ones which may be contiguous with it.

The blackened nature of the shoot tissues in the 'blackleg' syndrome of the disease is well known (Artschwager, 1920).

Reeve (1968; a & b) has described granules similar to those seen in this investigation on a light microscope level in the physiological disease of 'black heart' of potato tubers, where they are also found in the tonoplast.

It has been suggested by Jobber (1969), that melanin is inert with reference to bacterial activity.

Conclusions on the 'black infection barrier', its composition and properties.

When a comparable experiment to those of Dovrekovich was carried out on a freshly cut surface immersed in bacterial suspension, the progress of infection followed a similar pattern to that found by him being rapid at first, and then much slower, particularly at high temperatures. However, no black zone appeared. (See part 3.); which suggests that the black pigmentation does not constitute in itself the barrier but is merely a product whose appearance coincides with that of the true barrier under conditions of good aeration.

The position of the melanin in the tonoplast, away from the bacteria in the cell wall and the evidence of other workers (Jobber, 1969) that melanin is inert, leads one to believe that the true barrier to infection is not the black pigmentation but another factor occurring simultaneously. The evidence for suberisation as being an effective barrier when complete has been given. This appears to constitute a barrier to bacterial infection at the same locus as melanin.

Distribution of unoxidised polyphenol developed after infection.

Using the Hoefner Vorsatz test, a diffuse polyphenol rich zone was found outside the black zone described

above. It first appeared at 16h and formed a patchy ring by 23 h in tubers inoculated at 100% R.H. and 25°C. After 41 h this ring was close to the black ring owing to the spread of infection outwards; at 48 h it was adjacent to the black zone, a position it still maintained after 65 h. Neutral red staining showed that these cells still had intact tonoplasts as near as 1 cell diameter from the black zone.

This localised development of polyphenol was explained by light and electron microscopy, as being associated with dedifferentiating cells which appeared to have been retarded in development for ~~some~~ reason. The tissue outside the latter zone was differentiated into wound periderm in a normal way beneath the cover of wound suberin; in the case of the polyphenol rich cells suberin was present but suberisation was not complete, and wound periderm was not formed.

The factor which apparently inhibits the development of the wound periderm is not known but it evidently acts in advance of the bacteria and their pectolytic enzymes.

Infection interface polyphenol.

In the rots produced under less aerobic conditions (part 3), and also around the rots described above, a polyphenol rich layer a few cells deep was present outside the infected cells. This layer up to five cells

deep, was outside the cells which were suberised.

Polyphenols were found in dedifferentiating cells as well as those being suberised, for example beneath the lenticels, the primary periderm (plate 6.1), and in the vascular parenchyma. These cells are characterised under the electron microscope by the presence of electron dense bodies in their vacuoles. Whether these bodies are present in the living tissue or whether they are artifacts resulting from fixation and embedding remains uncertain, but in CRAF fixed material there appeared to be melanin present in these tissues judging by the techniques of Lillie (1957) and Gomori (1952). This suggests that melanin is present in small quantities in the layers rich in polyphenol.

Conclusion

As both suberisation and polyphenol production often occur in one and the same cell it is impossible to separate their individual roles as forming barriers at the infection front. In proliferated lenticels where polyphenol rich cells with unsuberised walls are present (plate 2.26a.) and such cells quite evidently do not act as a barrier to infection, this suggests that suberisation plays a larger part than phenolic compounds in the formation of barriers to infection.

Corollary Experiment.

It was decided to test the hypothesis that the change in spread of infection in aerobic and anaerobic conditions after 40 h at 25°C & 100%R.H. is due to purely physical action, rather than a specific host reaction as assumed in this chapter. The rate of penetration of Ferric chloride solution was investigated, in order to obtain a measure of the penetration of potato tissue by small particles.

Materials and Methods.

Potato tuber halves of King Edward were immersed in isotonic 5% sucrose solution containing 2.5% ferric chloride for up to 124 h and the distance stained recorded; storage was at 25°C & 100%R.H.

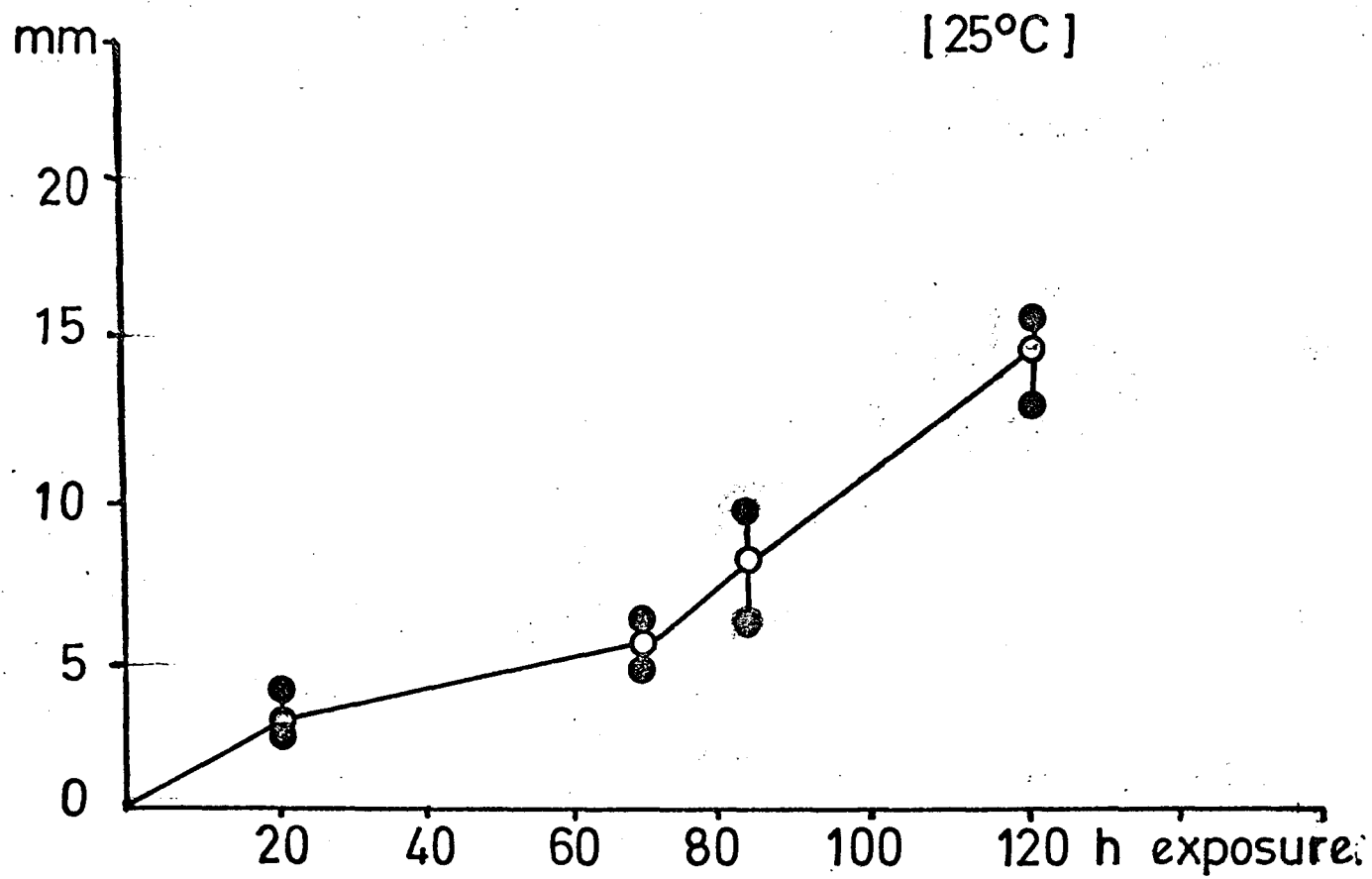
Results.

It can be seen from graph 6.6. that there was no indication of any difference in rate of spread through the tuber, before or after the first 2 days.

Conclusions.

It was concluded that the change in the rate of spread observed in bacterial infections after 40 h is due to some factor other than would act on a simple solution under similar conditions.

Graph 6.6 Penetration of 2.5% ferric chloride in 5% sucrose soln.



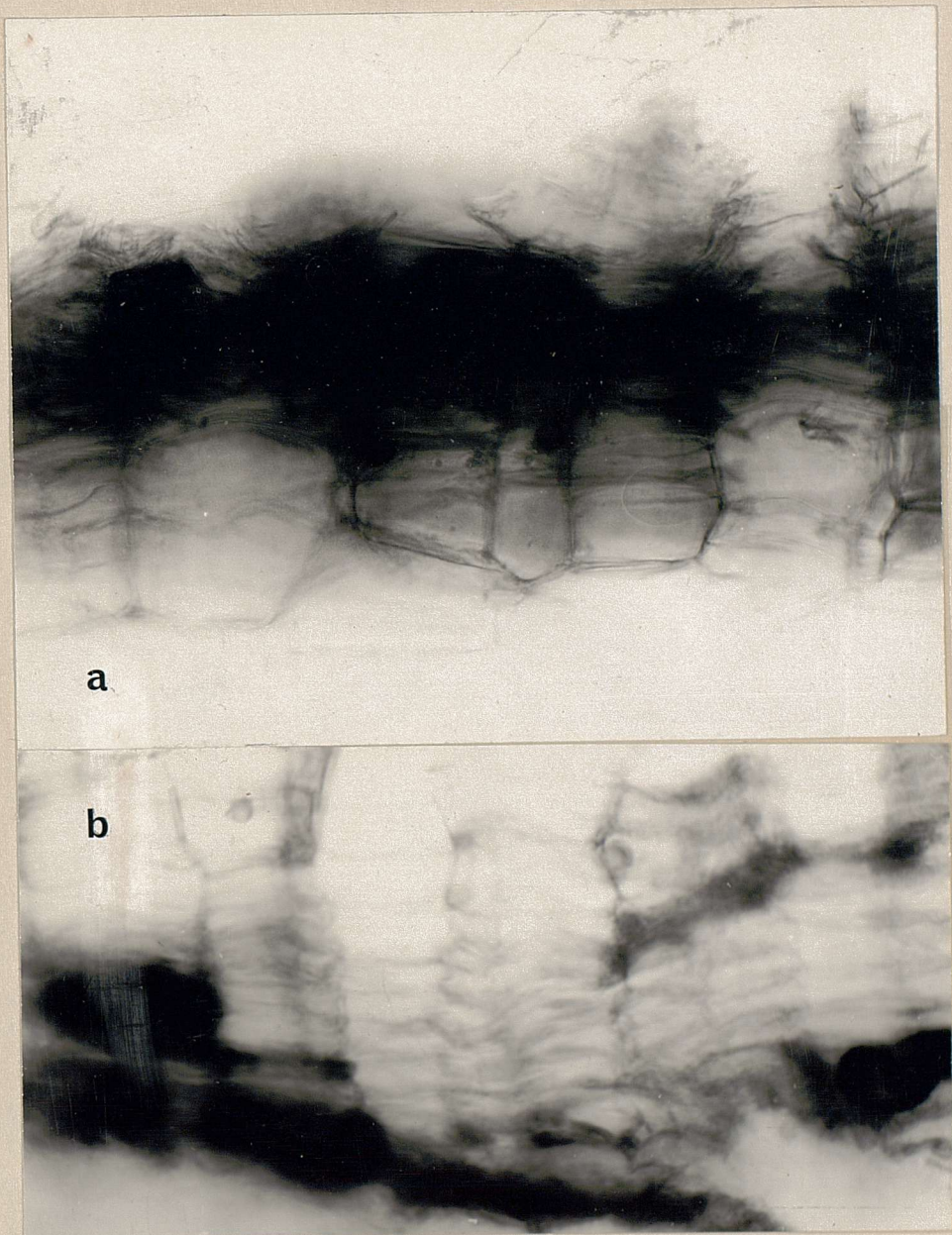


Plate 6.1 a. & b.

(x 200, light micrographs)

a. periderm, b. phellogen, stained to show the presence of polyphenolic substances, diffuse in the former, in tonoplasts of latter.

(Hoefpner-Vorsatz reagents.)

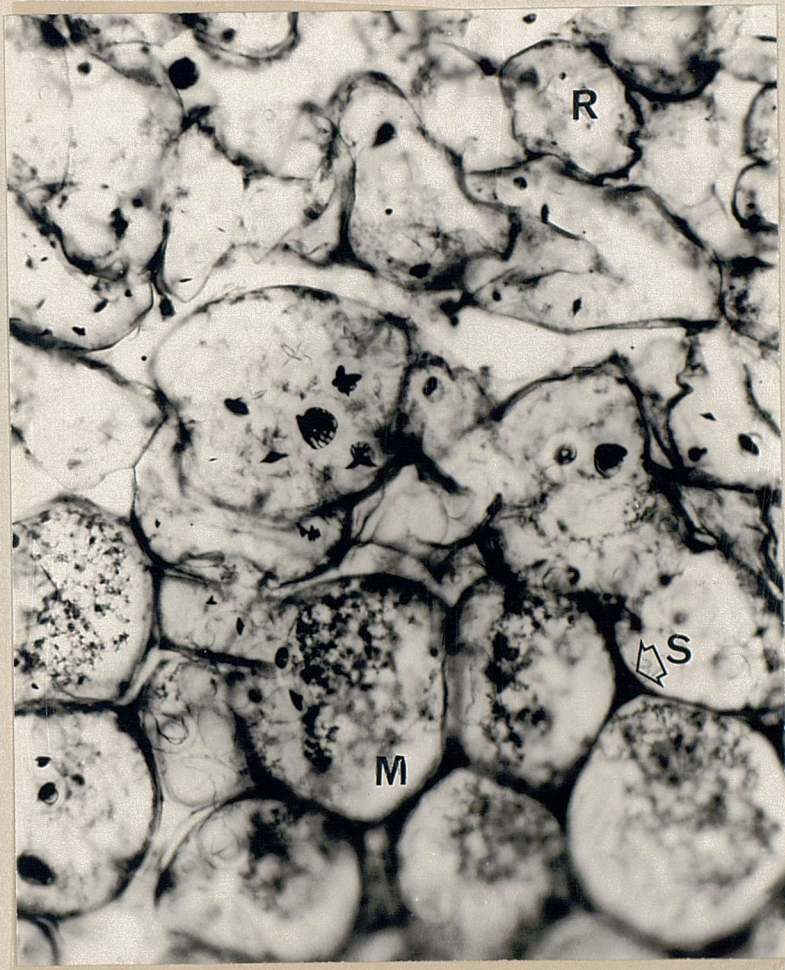
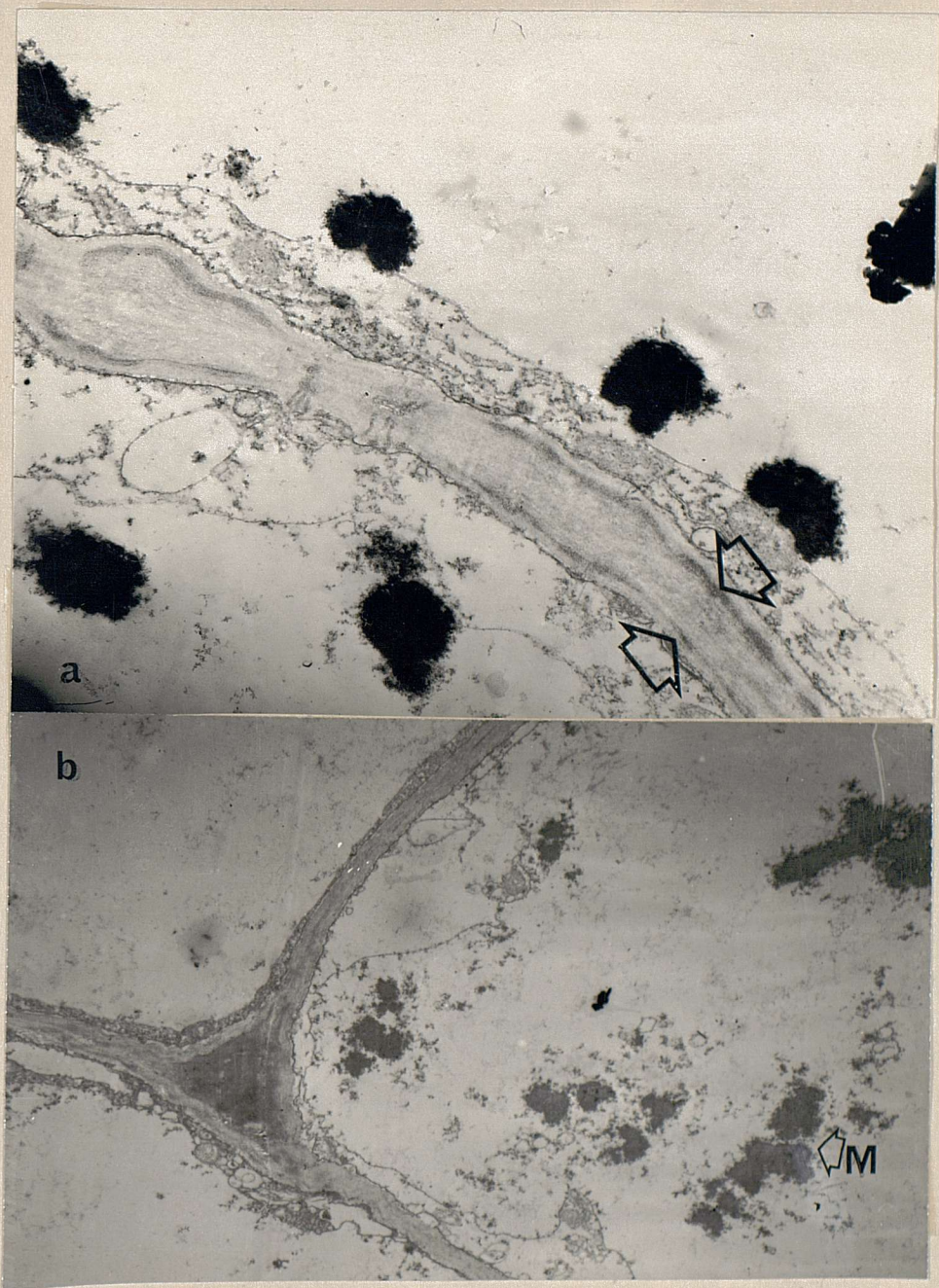


Plate 6.2

(x 160, light micrograph)

Infection interface under aerobic conditions, showing the melanin in the tonoplasts of cells comprising the 'black ring', these are also suberised M, melanin, S, suberin, R, soft rotted cells.

(osmic acid.)



Plates 6.3 a. (x15,000) b. (x 6,000) Electron micrographs.
Infection interface cells showing a. bands of suberin in cell wall, ^{carved} b. intercellular suberin plug. M, melanin granulos.
(osmic acid, lead citrate.)

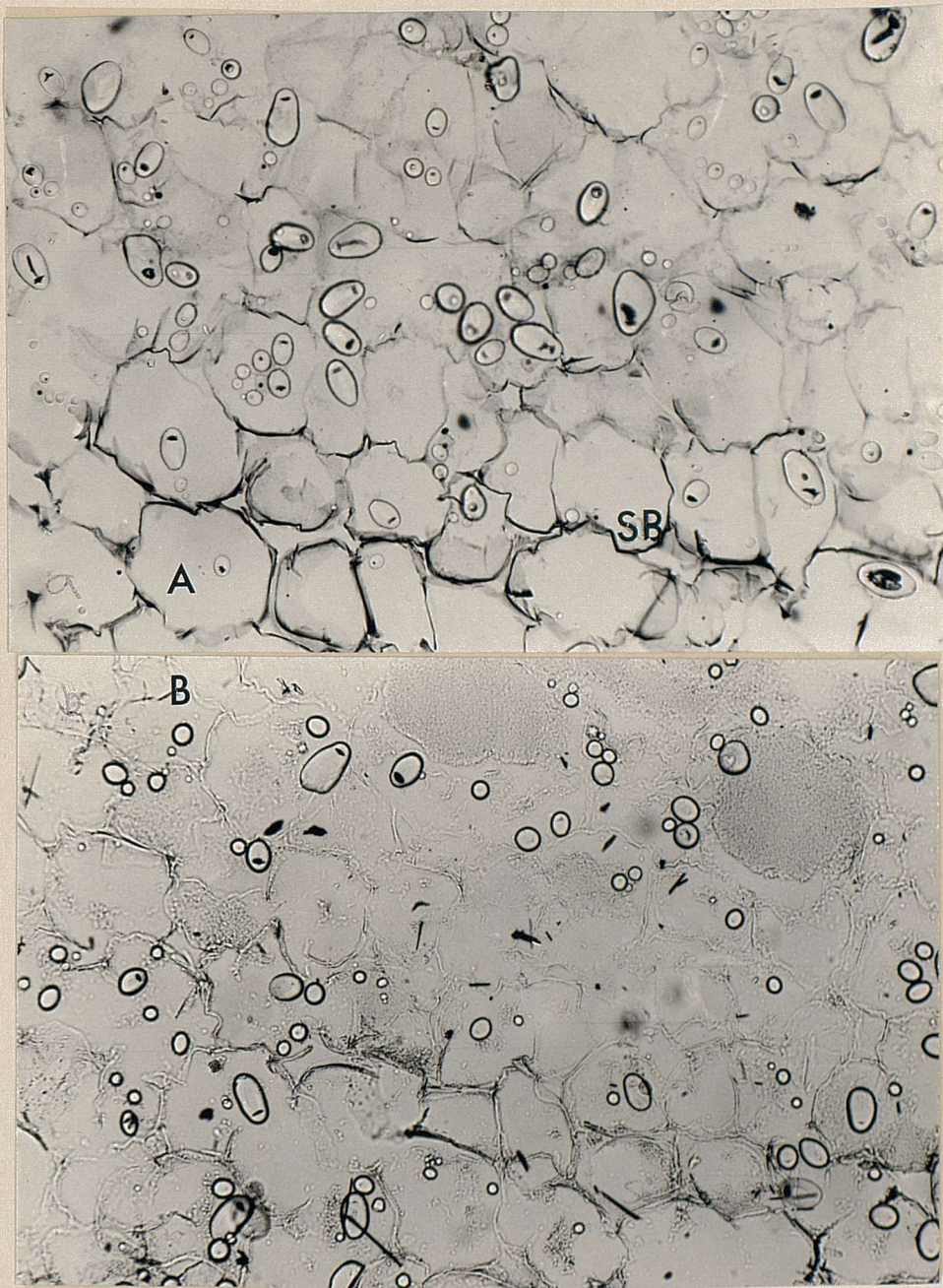


Plate 6.4 a. & b.

(x 160, light micrographs)

Sections through infection interface a. stained for suberin (ammoniacal gentian violet) b. bacterial colonies (carbol fuchsin, fast green); consecutive sections. *Sl*, suberin layer.

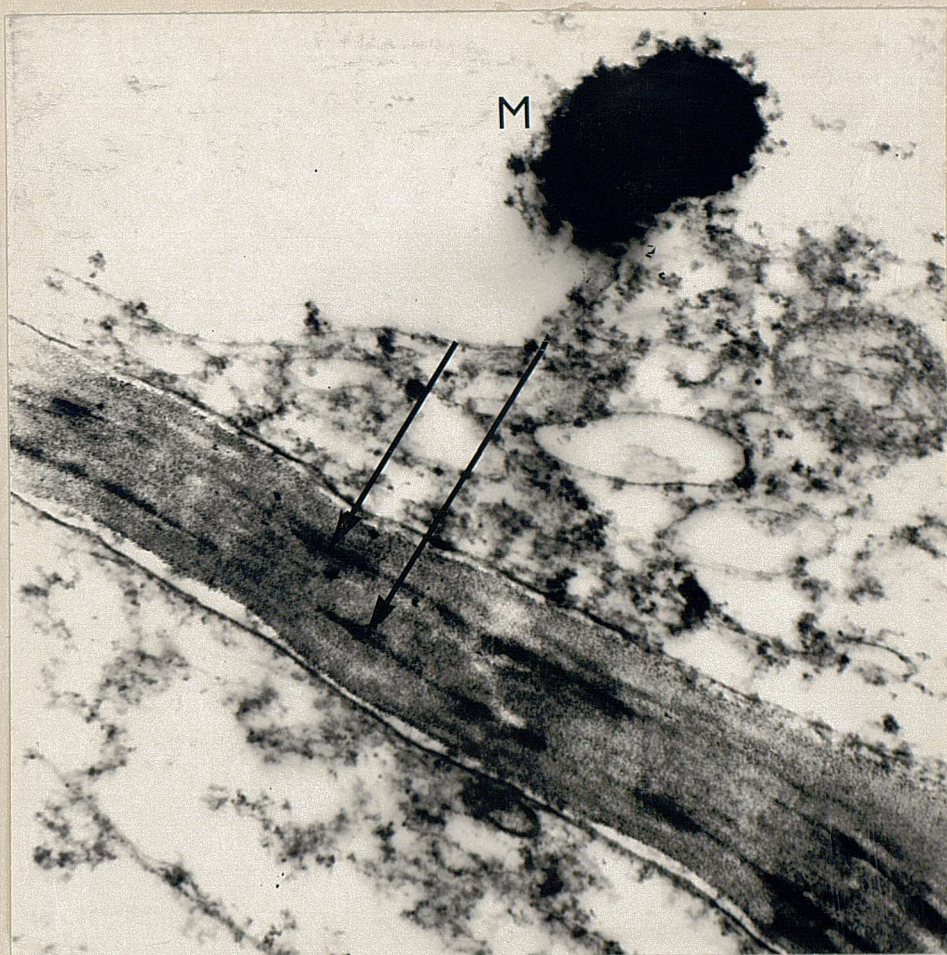


Plate 6.5 a.

(x 40,000)

Bands of suberin in the cell wall, arrowed, and melanin M, in the tonoplast.

(osmic acid, lead citrate.)

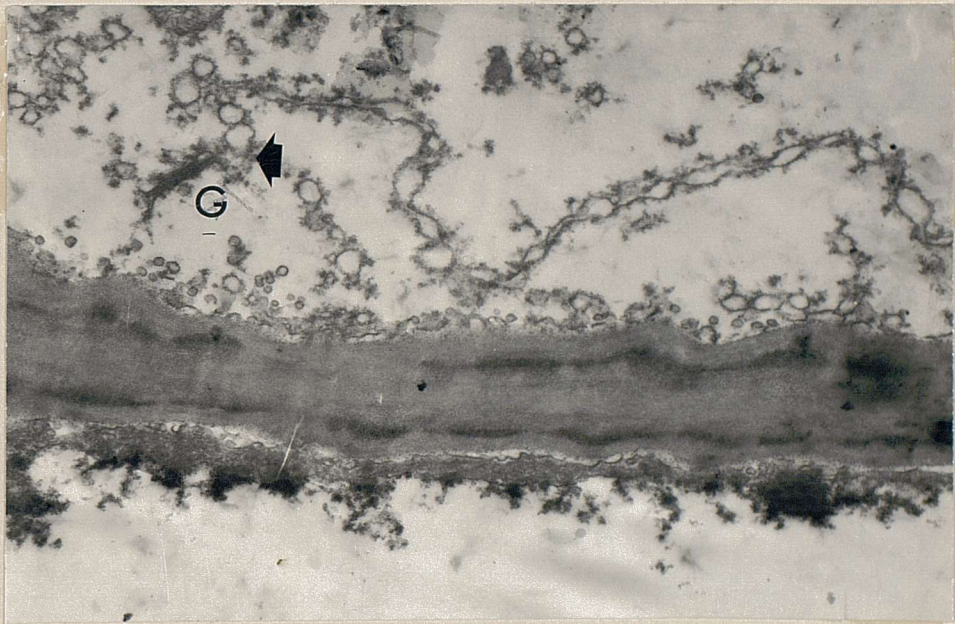


Plate 6.5 b. (x 12,500, electron micrograph)
Suberised infection interface cell wall, showing golgi
bodies and 'budded off' small vacuoles in neighbouring
cell cytoplasm, arrowed.

(osmic acid, lead citrate.)

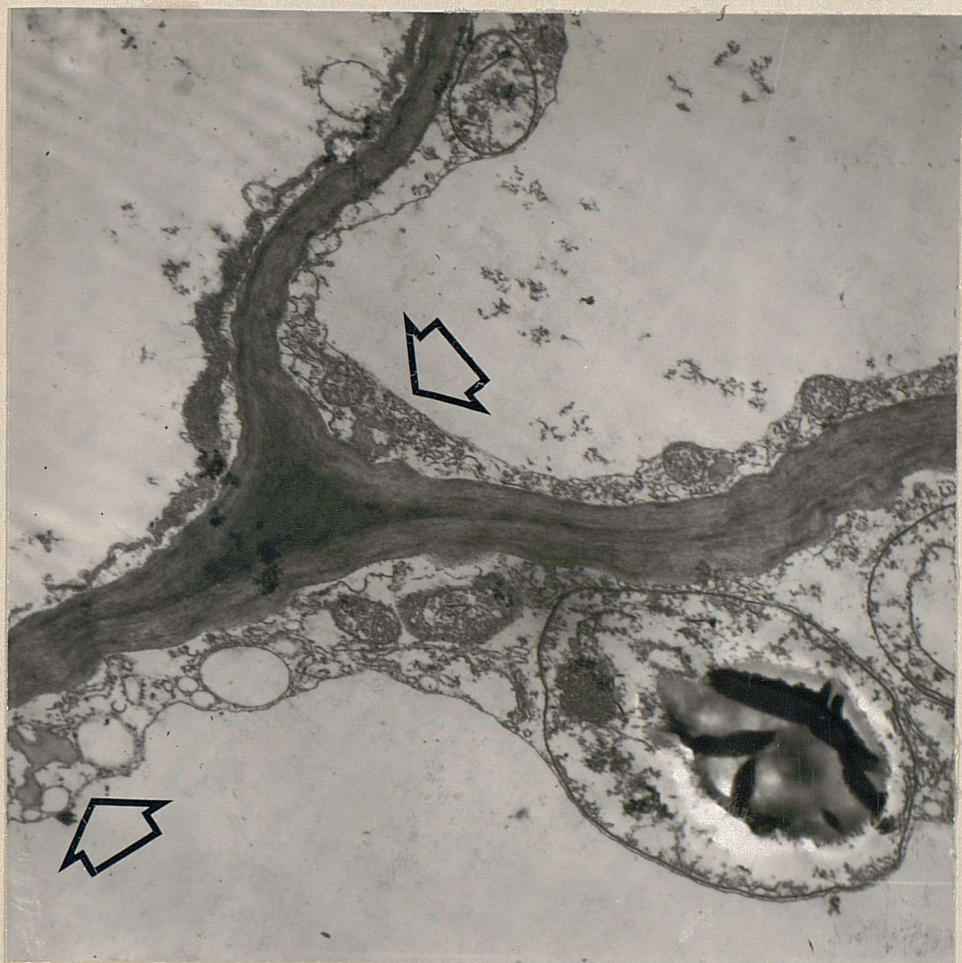


Plate 6.5 c.

(x 12,000, electron micrograph)

Infection interface cells showing intercellular suberin plug, note the microbodies arrowed, and the plastid containing starch grain, as well as other healthy cytoplasmic constituents, indicating an active metabolism.

(osmic acid, lead citrate.)

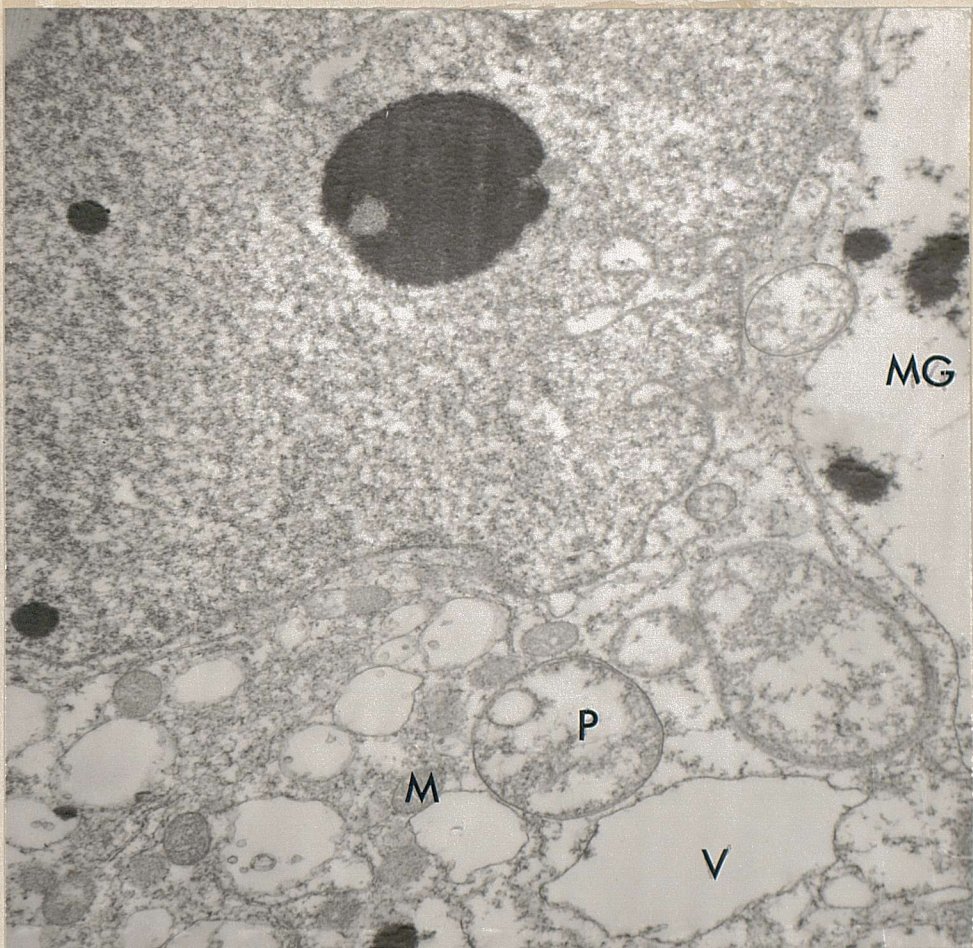


Plate 6.5 d.

(x 10,000)

Cytoplasm and nucleus of suberised infection interface cell,
note numerous small vacuoles ^V, mitochondria ^M and plastids, ^P
melanin granules in tonoplast. ^{MG}

(osmic acid, lead citrate.)

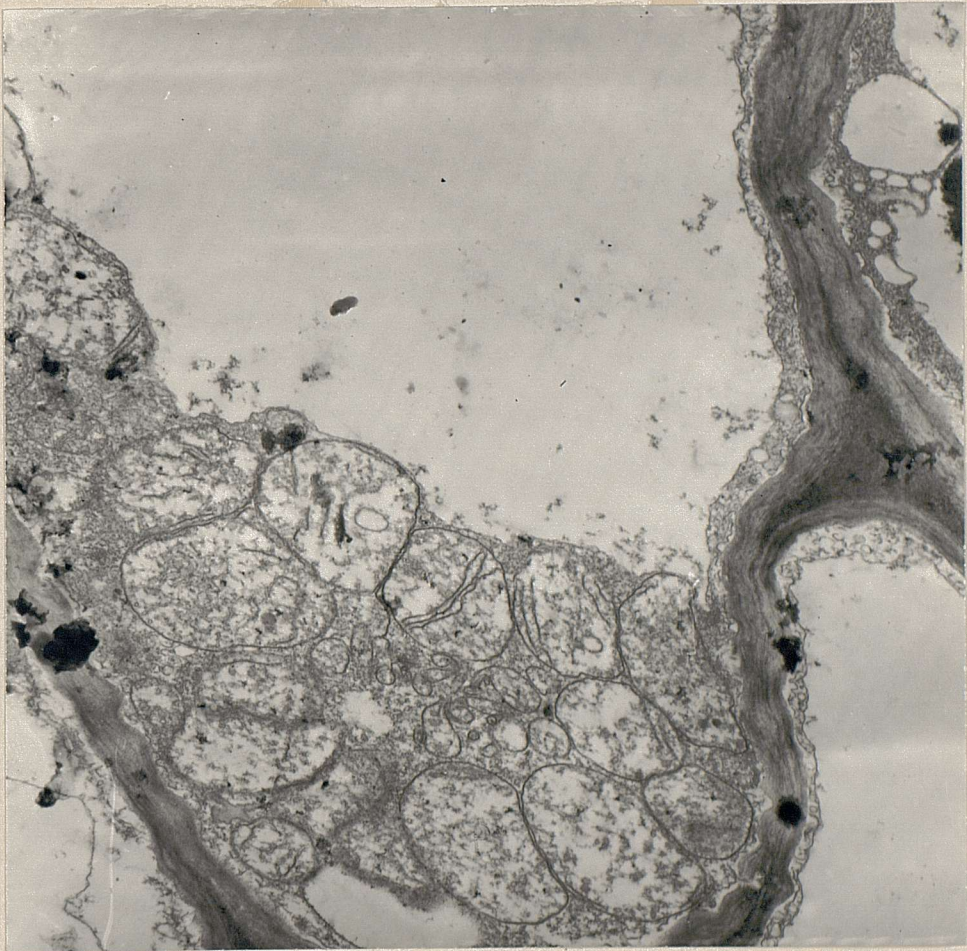


Plate 6.6

(x 5,000, electron micrograph)

The numerous plastids in the infection interface cells may be arranged in clusters typical of suberising cells. Intercellular plug of suberin present.

(osmic acid, lead citrate.)

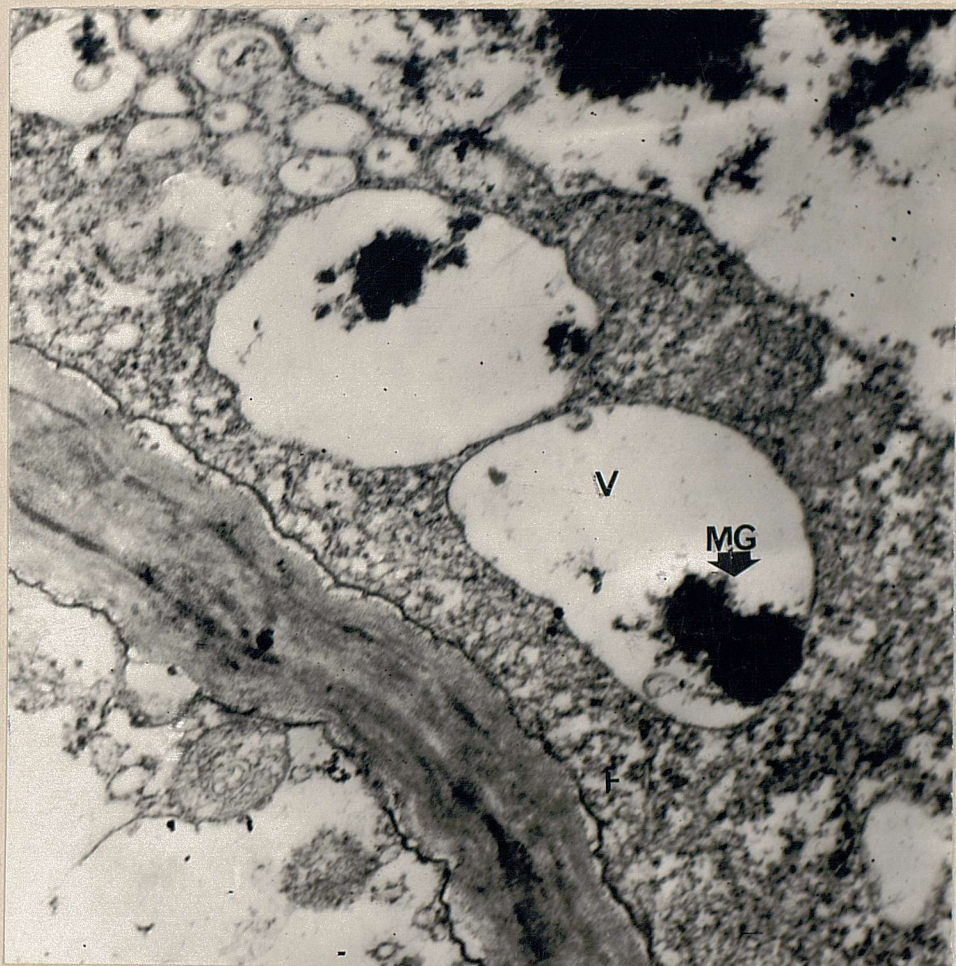


Plate 6.7 a.

(x 30,000, electron micrograph)

The vacuoles in the suberising infection interface cells
may contain melanin.^{MG}

(osmic acid, lead citrate.)



Plate 6.7 b.

(\times 12,000)

Suberising infection interface cell illustrating the presence of MY 'myelin' body; V, vacuole; L, microbody.

(osmic acid, lead citrate.)

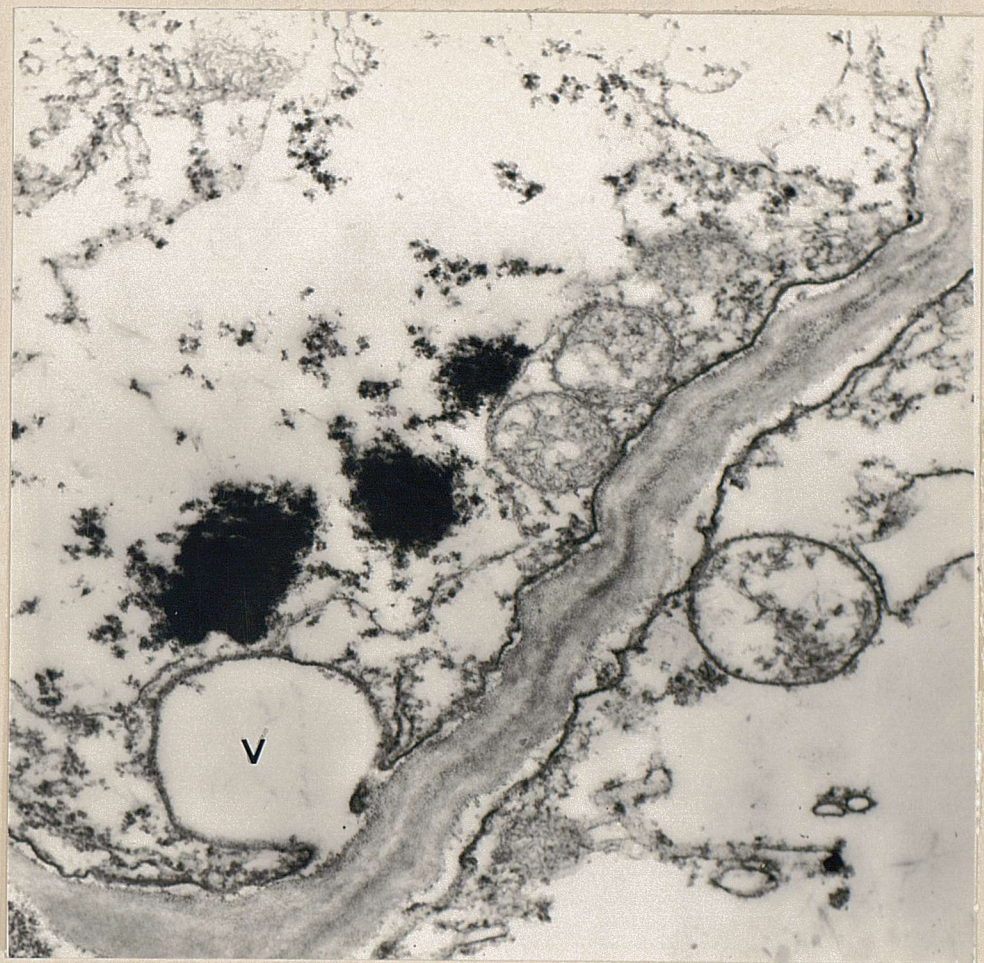


Plate 6.8

(x 30,000)

Infection interface cell showing small vacuole adjacent
to the plasmalemma.

(osmic acid, lead citrate.)



Plate 6.9

(x 30,000)

'Myelin' body from infection interface cell, showing
the characteristic structure.

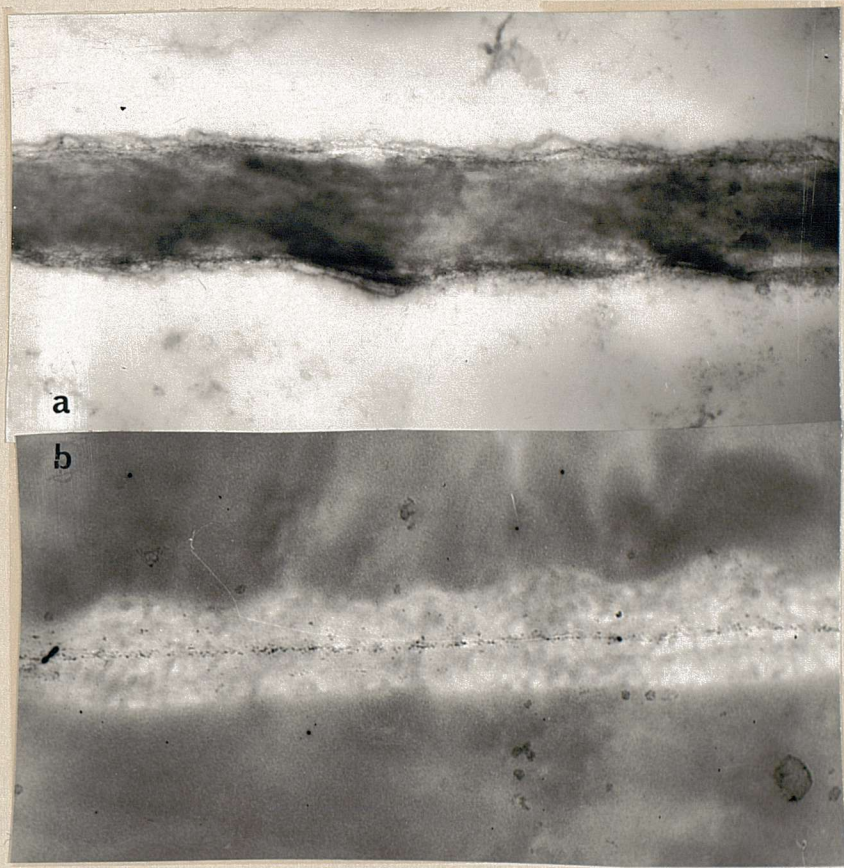
(osmic acid, lead citrate.)



Plate 6.10 *my* (x 20,000)

'Myelin' body which appears to be formed from a multi-functional plastid, apparently buds off vacuoles. From an infection interface cell.

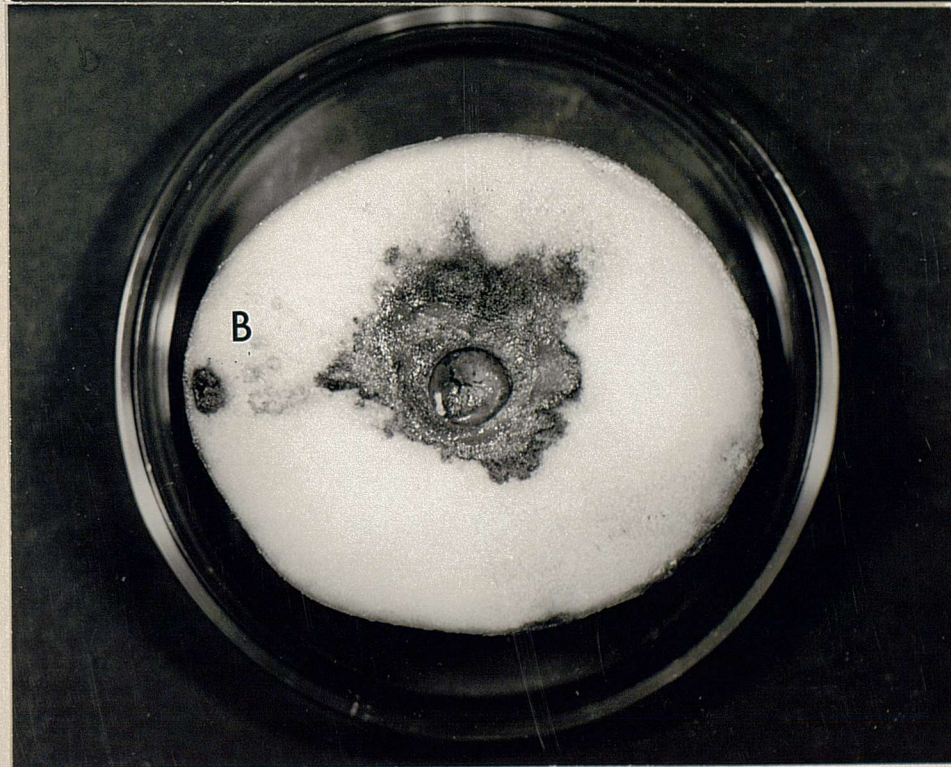
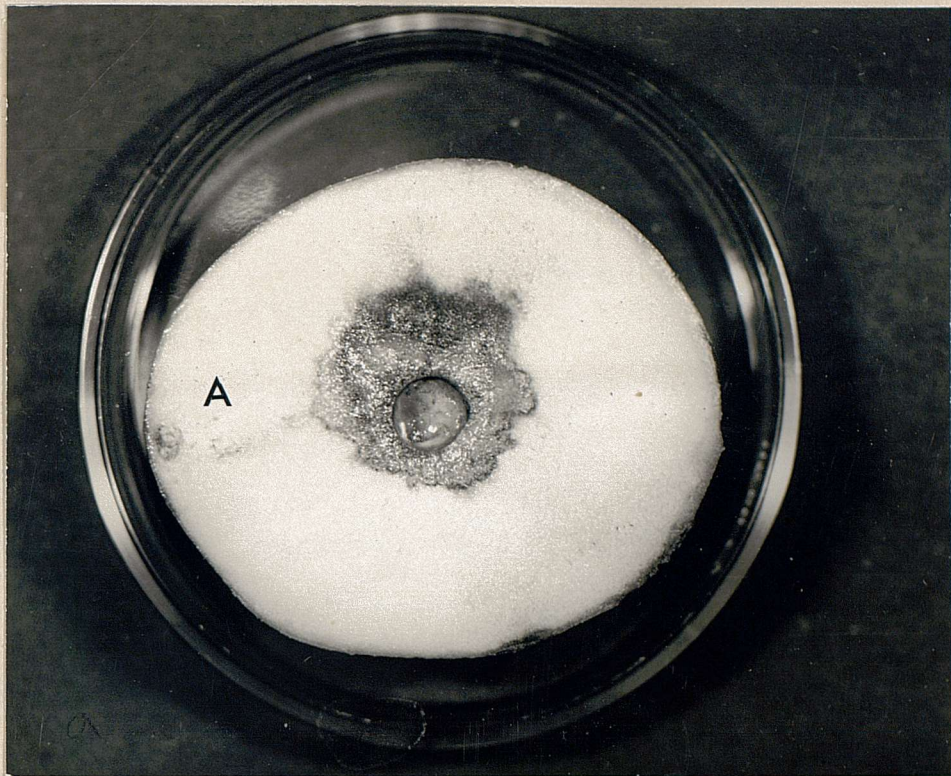
(osmic acid, lead citrate.)



Plates 6.11 a. (x 30,000) b. (x 40,000) Electron micrographs.

a. Section through periderm (osmic acid, lead citrate)

b. similar section stained by Albersheim's method.



Plates 6.12 a. & b. (x 2.5)

Bacterial rot spreading from central well in cut tuber surface, bounded by 'black band', after a. 70 h b. 92 h.



Plate 6.13

(x 15,000, electron micrograph)

Infection interface cell showing melanic granules in
the tonoplast, arrowed.

(osmic acid lead citrate.)

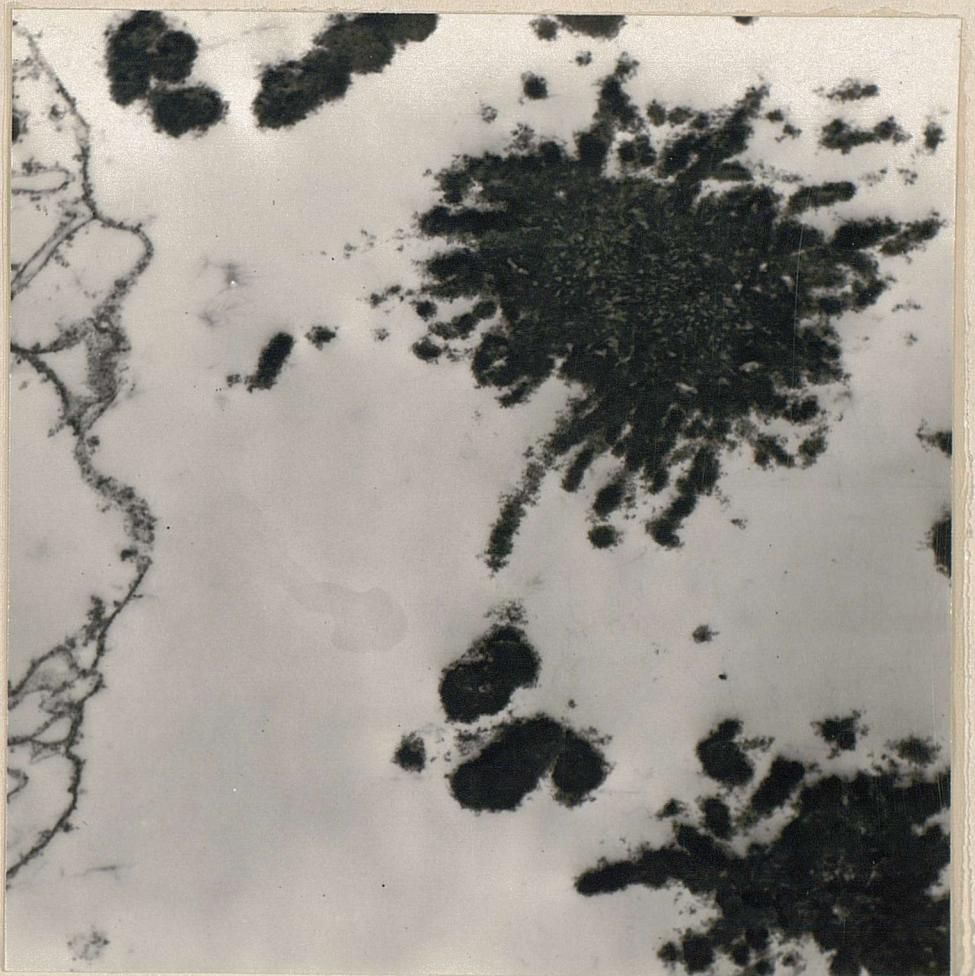


Plate 6.14 (x 20,000, electron micrograph)

Melanin granules of a more complicated structure than those in plate 6.13.

(osmic acid, lead citrate.)

PART 7. The secondary colonisation of soft rotted tissues
by Bacillus spp.

Part 7

The secondary colonisation of soft rotted tissue by
Bacillus spp.

Endospores resembling those of Bacillus megatherium described by Freer & Levinson (1969), were found in Kellenberger fixed, araldite embedded material under the electron microscope when sections of 36 day old completely macerated potato cells were examined. The cells had formed part of a tuber which had been inoculated initially by submersion in a pure 18 h suspension of Erwinia carotovora var. atroseptica under otherwise sterile conditions, (plates 7.1, 7.2). A pure culture of Bacillus megatherium (NCTC 10342) was examined using the same procedure; the spores present were similar to those in the macerated material (plate 7.3).

When a loop was taken and streaked on nutrient agar Bs. cereus var. mycoides and Bs. megatherium were isolated from the macerated cells together with Erwinia. This was found to be the case in all old established rots studied. These organisms were also found when sterile ^{slices} of potato tuber were plated out on nutrient agar at 25°C, and from an odourless rot which developed after a tuber was sprayed with sterile distilled water for 5 days. In the case of the latter, neutral red staining under the light microscope revealed that the cells were apparently living

although bacteria appeared to be present inside the cytoplasm, but as Smith (1905) remarks, fresh sections of rotted potato tuber tissue may give equivocal results due to their thickness.

Similar spores were present in many different mature rots, either alone or together with vegetative cells of Bacillus or Erwinia and in many different locations. Vegetative cells of Bacillus and spores at various stages of sporogenesis are frequent in the spaces between the cells before complete maceration (plate 7.4), but they may also be present inside the cells, often appearing near the starch grains which are by now loose from the cytoplasm (plate 7.5). They may also be found between the fibres that remain of the cell wall (plate 7.6), the cell wall is swollen and may be over 10 um thick compared to 1 um in healthy storage parenchyma, there may be some stretching too, as discussed in part 5; the environmental niche appears to be created by pectolysis. Endospores may be found in the viscous strands in late infection (plate 7.7); here the spores are embedded in a dense matrix of slime.

Hollis (1951) has suggested that Bacillus megatherium is present as a latent infection organism in the potato, but he did not think the same was true of Bs.cereus, although both have been successfully used in artificial

inoculations incubated at 37°C. Rudd Jones (1948) found inoculation using agar discs of Bacillus spp. difficult when applied to fresh wounds, and abnormal temperatures after inoculation, or elaborate pretreatment appear essential; injection with water, as well as other pretreatments used, freezing, steaming, or chloroform treatment, increase cell permeability and exosmosis of solutes into the intercellular spaces.

As a result of the possibility of confusion between Bacillus spp., presumably present as a latent infection and the Erwinia inoculated, methods of distinguishing between them were contemplated. Labelling the Erwinia seemed likely to be both ineffective and inefficient so criteria for distinguishing between the genera at the electron microscope level appeared essential. Glauert (1962) has put forward the characteristics of the surface layers, flagella and fimbriae, cell walls and type of division, granular elements of the cytoplasm, plasma membrane, the presence of intracytoplasmic membranous elements, and the nature of the nuclear material as being valid points of difference between bacterial genera. It was on this basis that the table was compiled, and the morphological differences are illustrated (plates 7.8-7.11). No Bacillus cells were seen during the investigation of healthy potato tissue

except in the filling tissue of the lenticels, but there remains the likelihood that they are dormant inside the tuber and can only colonise the tissues after they have been pretreated with water or Erwinia rotting.

Table of morphological differences.

Erwinia carotovora

Bacillus spp.

var. atroseptica.

- | | |
|---|--|
| 1. Cell division by simple 'pinching off'; the division furrow is not preceded by a cross wall or plate. This results in a more or less pointed end to the rod. | 1. A ring of material is synthesised and gradually grows inwards to form a complete cross wall. This results in a blunt ended rod. |
| 2. Does not produce chains of more than two cells. | 2. Chains of 2 - 4 cells commonly produced. |
| 3. Convoluted cell wall. | 3. Smooth cell wall. |
| 4. Granular cytoplasm, no glycogen present. | 4. Cytoplasm less granular, glycogen present (G). |
| 5. No spores recorded. | 5. Produces endogenous spores with characteristically structured walls. |

These points are illustrated in plates 7.8 - 7.11.

the structure of the spores are illustrated in plates 7.1 - 7.2.



Plate 7.1 (x 100,000) Transverse section.

Plate 7.2 (x 60,000) Longitudinal section.

Sections through endospores from 36 day soft rotted tissue.

(osmic acid, lead citrate.)



Plate 7.3 (X60,000)

Endospore of Bacillus megatherium from a pure culture,
longitudinal section.

(osmic acid, uranyl acetate.)



Plate 7.4

(x 6,000)

Vegetative cells and endospores resembling Bacillus spp. ^{Bs}
between the cells of a rotten tuber, also Erwinia ^E present.
(osmic acid, lead citrate.)



Plate 7.5

(x 6,000, electron micrograph)

Endospores near starch grain in macerated cell.

(osmic acid, lead citrate.)

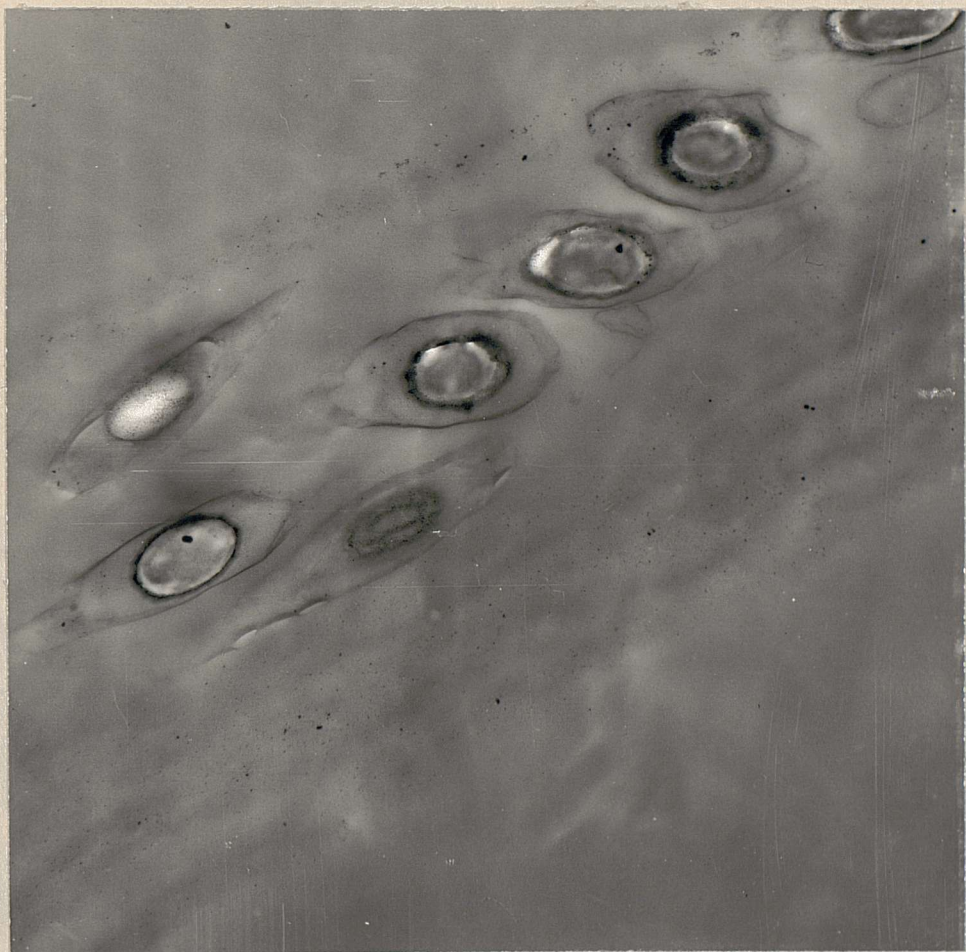


Plate 7.9

(x 20,000, electron micrograph)

Endospores in the cell wall of a macerated cell.

(osmic acid, lead citrate.)

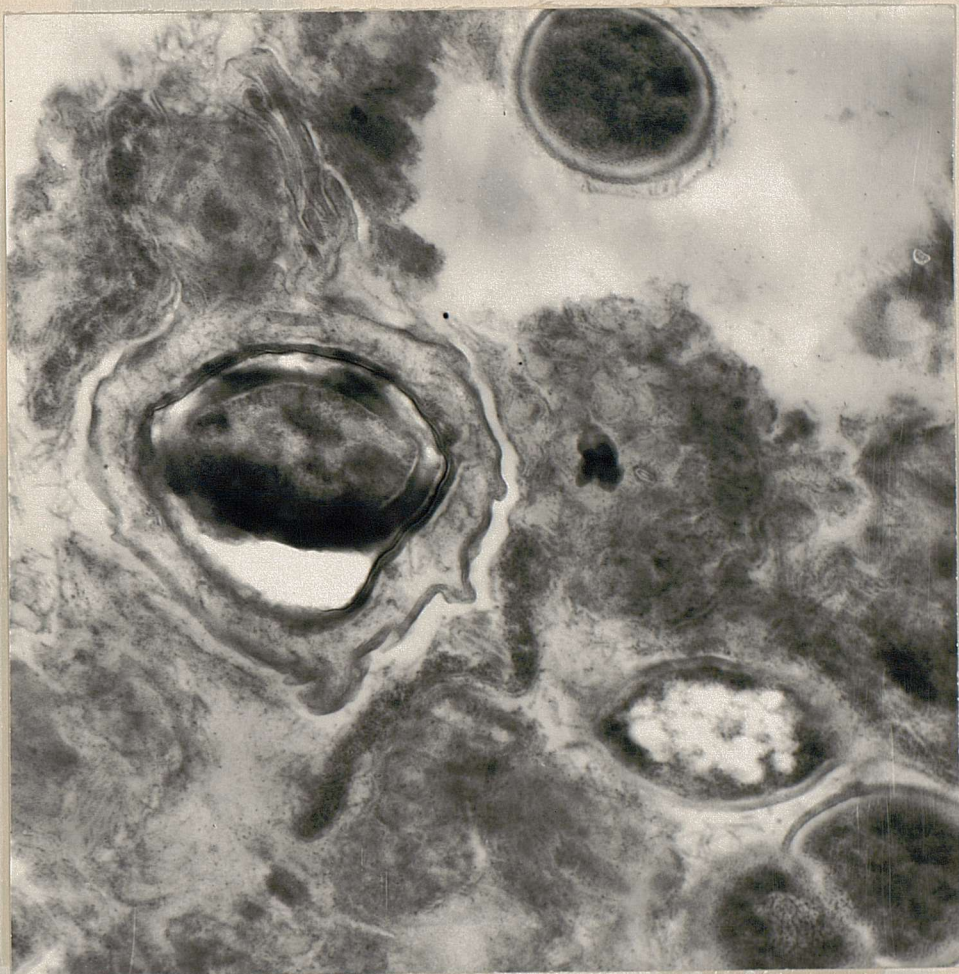


Plate 7.7

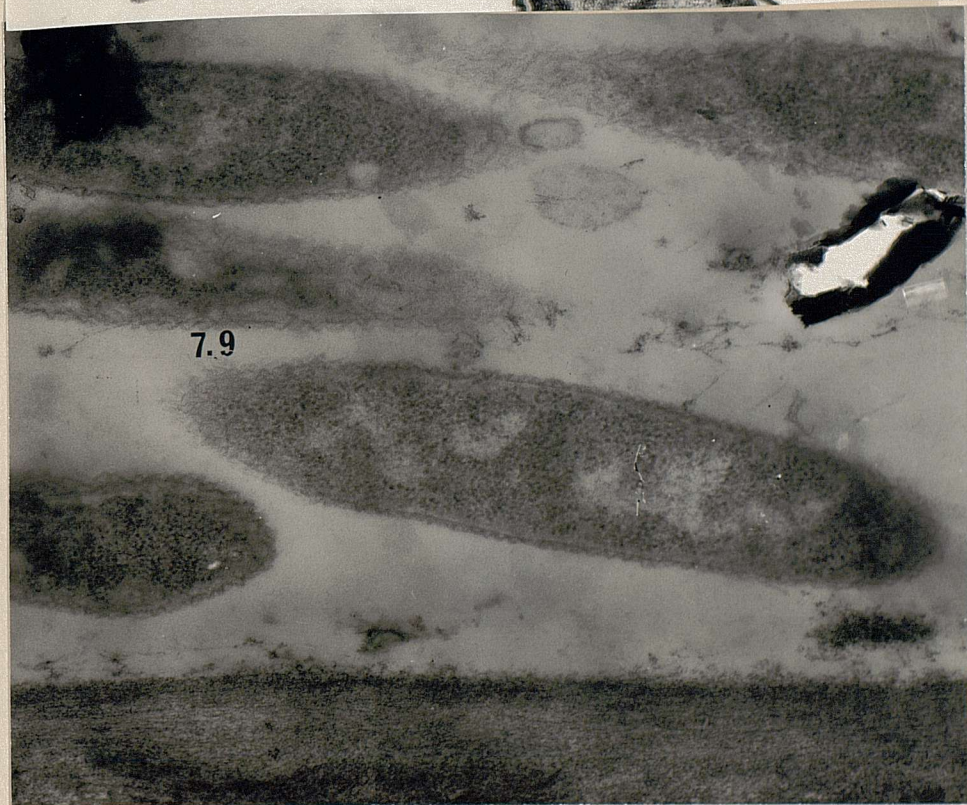
(x 60,000)

Endospores in slime from soft rotted tuber, together with
cytoplasmic remains.

(osmic acid, lead citrate.)



7.8

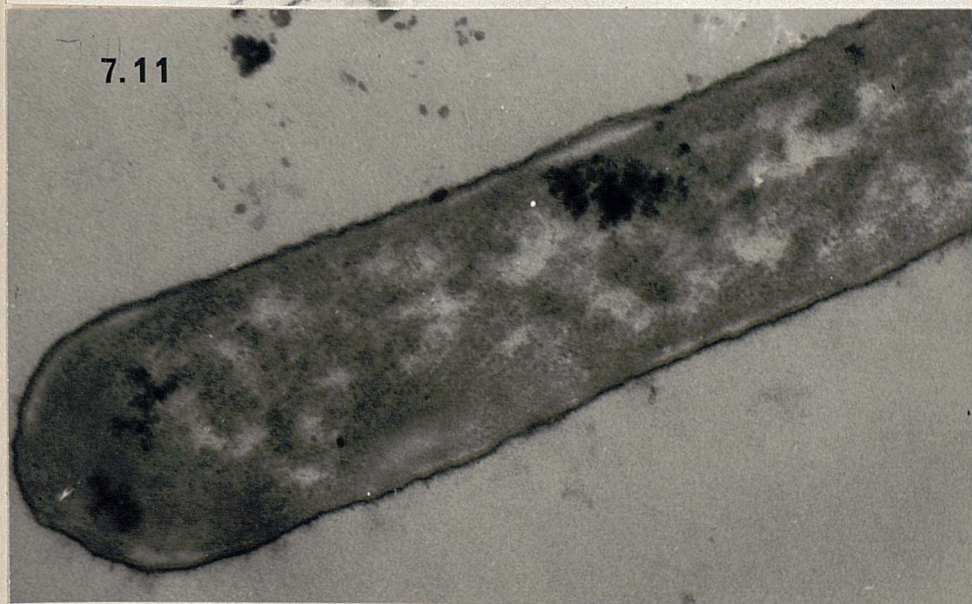


7.9

Plates 7.8 (x 40,000) 7.9 (x 40,000)

Erwinia carotovora var. atroseptica from pure culture;
from soft rotted tissue.

(osmic acid, uranyl acetate)



Plates 7.10 (x 40,000) 7.11 (x 40,000)

Bacillus megatherium from pure culture; Bacillus cell in rotten tuber. (osmic acid, uranyl acetate)

PART 8. Discussion and practical implications.

Part 8.

Discussion and practical implications.

Although the taxonomy, ecology and biochemistry of Erwinia carotovora var. atroseptica and the etiology of the disease it causes have been well studied, much is yet to be learned in these fields, and the ultrastructure of the pathogen and the details of its mode of infection have been largely neglected. Knowledge of the ultrastructural morphology of Erwinia is not only useful in the study of host parasite relationships, but also enables this primary pathogen to be distinguished from the Bacillus spp., which occur as secondary colonising organisms in otherwise unibacterial infections; it could also prove indispensable in ecological studies involving antagonistic organisms, or in comparative taxonomy.

(a) Entry.

In the potato tuber, the channels of infection open to the entry of bacteria appear to be lenticels in certain phases of development, and fresh wounds; the possibility of other channels such as the 'eyes', healed wounds or primary periderm being important in this respect seems remote. As fresh wounds have a limited life as open channels for infection, wound healing is a relevant process. The conditions under

which suberisation and wound periderm are best promoted are a combination of higher temperatures (20-30°C) and relative humidities (above 80% R.H.) with the absence of surface water. As the effects of the environment on healing beneath the surface are similar to those at the surface, and not those at the infection interface, this suggests that the peculiarities of the latter are due primarily to the vagaries of the bacterium.

The point of time at which the fresh wound ceases to be susceptible to soft rotting was determined by the artificial inoculation of healing wounds. In this way it was discovered that the absence of a layer of suberised cells allowed a bacterial rot to be initiated; the presence of suberin does not however, preclude infection at low relative humidity. When early stages of suberisation were studied by electron microscopy, the reason for the ineffectiveness of this barrier at low R.H. was revealed. At the ultrastructural level, suberised cell walls consisted of two bands of suberin in the cell wall with a suberin plug replacing the intercellular space. When any of these components was absent, pectolysis appeared to separate the remaining suberin from the other cell wall material, rupturing the barrier. Wound periderm which is formed after the suberin is laid down was seen to be similar in structure

to primary periderm, with no intercellular spaces, and relatively thin cell walls.

. From both light and electron microscopy it was found that the lenticel displays a wide variation in morphology at both the tissue and cell levels. At 58% R.H. a secondary periderm forms beneath the filling tissue, which becomes completely suberised and resistant to infection. Whereas when liquid water is present the unsuberised phelloderm protrudes between a ring of what is left of the filling tissue, and the meristematic cells and infection readily occurs. Between these extremes are found many intermediate types of lenticel with varying reactions to inoculation. Subsequent infection is greatest among those lenticels which were stored at the highest relative humidities prior to inoculation. Postinoculation variations in relative humidity have only temporary effects on the development of infection, although this proceeds at similar rates subsequently.

The role of the lenticel as a channel for infection is at present sadly neglected, and the lenticel could perhaps also act as point of entry for 'latent' infection organisms in the growing tuber. Routine infection via the lenticels on mother tuber disintegration, after rainfall has created the appropriate environmental

environmental conditions in the soil, as suggested by Perombelon appears possible.

(b) Invasion after entry.

The initial rate of colonisation of the tuber was investigated by measuring the development of colonies where the inoculum had entered through lenticels and fresh wounds. As well as the differences between the two channels, it was found that temperature determined the initial rate of colonisation. Particularly interesting is the slowing down of the rate at 25°C after 50 h., which Lovrekovich suggests is due to the development of a black oxidised polyphenol barrier. However this phenomenon was also seen in those less aerobic conditions where this 'black barrier' was not present as well as under conditions similar to those employed by Lovrekovich where melanin was present. This suggests that the black barrier, though often accompanying a check to the spread of rot does not cause the check, and further work is required to elucidate all the causal factors.

The ability of the host to take up inoculum appears to be an important factor influencing the incidence of rot, and further investigation of this under field conditions where the incidence of Erwinia in the soil may be low is necessary.

Once inside the tuber the bacteria primarily spread intercellularly through the parenchyma but vascular spread may occur, where the bacteria are confined to the vascular tissue by the suberised nature of the vascular parenchyma, which allows little lateral spread.

During the intercellular spread through the parenchyma, the Erwinia cell at first occurs in small compact zoned colonies but become generally dispersed as infection progresses and active spread gives way to the terminal stages of maceration. Each intercellular colony has a limited sphere of influence where cell wall and cytoplasmic damage occurs, but in general the rot is not homogeneous through the tissue until the final stages.

Cells rich in calcium oxalate monohydrate which occur beneath the lenticels and to a limited extent in the phelloderm of the primary periderm and the cortex cells of the tuber are among the very few cells to be colonised intracellularly, but the oxalate containing cells appear to stimulate bacterial colonisation.

Bacterial infection of potato tissue, as well as causing the complex pectolytic breakdown of parts of the cell wall resulting in maceration, also gives rise to cytoplasmic disorganisation. The latter involves the enlargement of the microbodies and the rupturing of the cell membranes. The effect of infection on the cytoplasm

is profound at higher temperatures leading to the complete disintegration of the cell contents in advanced rots. It is uncertain whether a specific cytase is produced, or if the parasite acts directly or through the cellulolytic agency of the microbodies.

From these studies it was found that there was often a considerable build up of infection of bacterial spread and tissue damage at an ultrastructural level without any visible evidence of soft rot near the tuber surface, for example in infection at low temperatures and at early stages of lenticel infection. The ultrastructure of these 'clean' infections reveals that little cytoplasmic damage had occurred compared with that in obvious rots, although the cell wall was severely damaged.

(c) Defence Mechanisms.

Host defence mechanisms in the potato are of two major types. The first involves systems which were in operation before inoculation, such as the polyphenolic substances found in the tonoplasts of cells in and under the primary and wound periderm, the 'eye' region, in and under the filling tissue of the lenticels, and around the vascular tissues as found in this investigation. Apart from the bud tissues of the 'eye' region these cells are also characterised by the presence of

suberin in the cell wall. Secondly there are the host defence systems developed after infection of which suberin is seen to be an effective barrier at the infection interface using electron microscope techniques. The other system proposed (Lovrekovich 1967), namely the oxidation of polyphenols to melanⁿin appears to have less effect but as it often occurs in the same cells as have suberised cell walls the position is difficult to evaluate. The typical cytoplasmic events accompanying these processes do not disprove the suggestion that the two processes may involve the same enzymic systems localised in the mitochondria and perhaps the plastids, and both events could be linked.

The fact that 'soft rot' in the field is rather more complicated than a straightforward infection of Erwinia is reinforced by the finding that Bacillus spp. always occur after otherwise sterile potato tuber tissue has been rotted by artificial inoculations of the former.

Since the application of electron microscopy has shown that ~~as~~ the bacteria are confined to the inter-cellular region, any toxin produced by the host will only be effective if present in that region. It appears that suberin can form a satisfactory infection barrier; the role played by phenolic compounds is much less

clear and requires further investigation since such substances are intracellular that there appears to be no evidence that this occurs intercellularly in healthy tissue or in tissues in the early stages of rot.

The combination of electron microscopy with pectolytic enzymology is an obvious development as is the use of ferritin-labelled antibody markers (Kay et al 1965) to illuminate the relationship between the substances involved in suberisation and polyphenol oxidation.

The former is quite feasible at present but the latter is dependant on a more detailed knowledge of the enzymology of the processes involved than is enjoyed at present.

Practical Implications.

From the evidence it appears that as well as protecting the tuber against wounding and providing healing conditions if this does occur, the tubers should also be treated in such a way as to promote the development of a resistant state in the lenticels. The latter must depend largely on the field conditions but may depend on the environment during transportation where this lasts several days as described in the U.S.A. by Smith and Ramsey (1947).

There is evidence that the state of dormancy of the tuber may be important, apparently once dormancy has

been broken the tuber is at its most susceptible (Webb 1969). All the tubers used in this investigation were stored prior to use at 5°C and 78% R.H, so they would have been in this susceptible state.

The implication of the experiments in part 2 is that it is possible for each load delivered to a potato store to differ from the others in some respect which affects susceptibility to soft-rot, the degree of spread from one homogeneous part of the store to another depending on the state of the neighbouring tubers. It should not be surprising that soft-rot should sometimes affect only isolated tubers which were presumably infected before conditions became unsuitable, sometimes cause localised rots where the pretreatment of part of the store predisposed them to rot, or sometimes spreading rots where the unfavourable pretreatment was not confined to one load or part of a load. Research aimed at following the progress of a load whose past history was well documented, through a well monitored store, should yield data which could be used to predict the so-called spontaneous rots. Knowledge of the amount of inoculum present and at what time it was in a state to cause infection would be useful as if no inoculum is present it is possible for even proliferated lenticels to be uninfected, leading to some workers imagining that some resistance factor :

associated with proliferation, as infected proliferated lenticels are indistinguishable to the naked eye from other lenticel rots.

As one of the potato handler's biggest problems has been his inability to predict which loads stored are likely to develop extreme soft rot symptoms before these are visible to the naked eye, it seemed important to devise a simple non-microscopic test for early stages of infection. Details of such a test based on triphenyl tetrazolium chloride can be seen in the appendix, in this way untrained personnel could screen each load minutes before unloading into storage bays classified on the soft rot risk, the greatest risk loads being utilised first, isolated from uninfected loads. The advantages of this system over others involving culturing or microscopy are speed and low cost in equipment.

The fact that 'soft rot' in the field is rather more complicated than a straightforward infection of Erwinia is reinforced by the finding that Bacillus spp. always occur after otherwise sterile potato tuber tissue has been rotted by artificial inoculations of the former. If screening for the early stages of Erwinia rot were carried out as described above, observations of any widespread 'spontaneous' rots, and the relation of 'latent' infection may be clarified, as well perhaps, the role of Bacillus spp.

Appendix.

A simple test to detect early stages of Erwinia rot.

The usefulness of a reliable test for the early stages of Erwinia to both growers and the potato industry has been stressed in part 8. Such a test, should for economic reasons be simple enough to be applied quickly by unskilled personnel. The following may be suitable for the basis of such a routine testing procedure.

The use of 2:3:5-triphenyl-tetrazolium chloride to detect the presence of dehydrogenase in infected tissue is well established (Bergmeyer, 1965). From this, T.T.C. has been applied to stain dehydrogenase bound to Erwinia cells for histochemical studies (Lovrekovich et al., 1967) and combined with agar as a diagnostic medium (Logan, 1963). The potential use of this compound to detect subvisual soft rot infection remains unexploited.

In part 2 of this investigation it was shown that a 1% aqueous solution of T.T.C. could be almost as effective in detecting Erwinia colonisation of the lenticels, as the examination of light microscope sections, providing a tangential sectioned surface was exposed, (plates 2.24, 2.25).

From parts 2 & 4, it appears that rots extend from the fresh wounds and lenticels largely through the storage parenchyma, therefore apart from some vascular infection, a complete 'peel' taken from a tuber would

give a fairly accurate indication if any rot is yet established in the tuber as a whole.

A representative sample of tubers taken from a batch before unloading has started, 'peeled', and the 'peel' from each of the tubers immersed for a few minutes in 1% aqueous T.T.C., should give a clear reaction where Erwinia is present even in low quantities. From the extent of reaction of the T.T.C., measured on the basis of the percentage cover of the inside surface of the cut peel, where potentially vulnerable storage parenchyma is exposed, the results from the sample tubers tested should indicate whether the batch could be classed as either a good or bad risk for soft rot damage. If the former was the case, the batch could be then unloaded into suitable bays or containers known to be free of the disease, while the bad risk tubers could either be stored in priority bays to be utilised quickly in such a way that the colonisation already established is removed, or in extreme cases, disposed of completely.

In this way, there is less pressure on the potato handler to provide accurate monitoring of the tubers before and after storage, or for very stringent control of storage conditions, although both of the latter are desirable.

It must be emphasised that these suggestions are made from laboratory evidence, and yet to be tested commercially.

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