

ULTRASTRUCTURE OF VACUOLES
IN ROOT TIPS

Thesis
Submitted for the Degree of
Doctor of Philosophy
in Botany

by
B.A. Fineran

University of Canterbury
July, 1969

OK
644
F495
1969

PREFACE

The aim of this project has been to gain experience in electron microscopy and to investigate selected problems of ultrastructure in root tips using techniques of thin sectioning and freeze-etching. The work was conducted while employed as a full time University Lecturer. Studies began in 1963 and were continued in 1964 during visits to the Physics and Engineering Laboratory (P.E.L.) D.S.I.R., Lower Hutt. Towards the end of 1965 practical work began at Canterbury following the acquisition of equipment and improved facilities. In 1968 a further visit was made to P.E.L. to use the recently installed freeze-etch equipment.

In the absence of experienced ultrastructural plant cytologists for guidance throughout most of the study, one of the principal tasks has been the selection of material and problems amenable for investigation. Exploratory work was carried out on the fine structure of meristem and differentiating root cap cells (Fineran, 1966 - included here as an appendix). From this study a more detailed investigation developed on the ultrastructure of vacuoles. The final results and discussions of this work on the vacuole and the necessary preliminary experiments on the preparation of root tips for freeze-etching form the basis of this dissertation. Each chapter represents a unified topic within the framework of the project. The literature relevant to each topic is reviewed in the chapter concerned.

An integral part of the project involved the establishment of an electron microscope laboratory in the Botany Department of this University.

I am grateful to Messrs W.S. Bertaud and D.M. Hall for basic instruction in electron microscopy; to Miss Suzanne Bullock for technical assistance during the later stages of the work; to Mr. R.W. Thomson for operating the freeze-etch machine; to Dr. D.G. Lloyd for assistance with the statistical analysis; to Professor Ann Wylie for literature translations and constructive criticism of the work and to my wife Judith for critical reading of the manuscript. The work was supported by equipment purchased from funds provided by the Golden Kiwi Scientific Research Distribution Committee (Grant No. 1 .A. 200/7917) and the University Grants committee (Grants Nos. 65/165, 67/212). Facilities for the study were made available through Professor Philipson of this Department and through Dr. M.C. Probine of the Physics and Engineering Laboratory, D.S.I.R., Lower Hutt.

CONTENTS

| | Page |
|---|------|
| 1. <u>INTRODUCTION</u> | 1 |
| 2. <u>MATERIALS AND METHODS: GENERAL</u> | 5 |
| 2. 1 Plant material | 5 |
| 2. 2 Chemicals and Solutions | 6 |
| 2. 3 Preparation of material for thin sectioning | 6 |
| 2.3. 1 Fixation | 6 |
| 2.3. 2 Dehydration and embedding | 7 |
| 2.3. 3 Ultramicrotomy | 7 |
| 2.3. 4 "Staining" | 7 |
| 2. 4 Preparation of materials for Freeze-etching | 8 |
| 2.4. 1 Pretreatment of specimens | 8 |
| 2.4. 2 Freeze-etching | 8 |
| 2. 5 Grids | 10 |
| 2. 6 Microscopes | 11 |
| 2. 7 Photographic Procedures | 11 |
| 2. 8 Range of material studied | 11 |
| 3. <u>EXPERIMENTS ON THE PRETREATMENT OF ROOT TIPS FOR FREEZE-ETCHING</u> | 13 |
| 3. 1 Introduction | 13 |
| 3. 2 Pretreatment of specimens | 15 |
| 3. 3 Observation and experiments | 17 |
| 3.3. 1 Attempts to initiate roots in glycerol | 17 |
| 3.3. 2 Attempts to initiate roots on stolons of <u>M. citrata</u> | 18 |
| 3.3. 3 Attempts to impregnate established roots by prolonged exposure to glycerol | 18 |
| 3.3. 4 Attempts to impregnate established roots by short exposure to glycerol | 20 |
| 3.3. 5 Attempts to pre-condition plants by watering with glycerol | 21 |
| 3.3. 6 Glycerination of excised roots | 21 |
| 3.3. 7 Glycerination and freeze-fixation in roots of germinated seeds. | 22 |
| 3.3. 8 Prefixation with glutaraldehyde prior to freeze-fixation | 23 |
| 3. 4 Discussion and Conclusions | 25 |
| 3. 5 Summary | 28 |

| | Page |
|--|------|
| 4. <u>AN EVALUATION OF THE FORM OF VACUOLES IN ULTRATHIN SECTIONS AND FREEZE-ETCH REPLICAS</u> | 30 |
| 4. 1 Introduction | 30 |
| 4. 2 Observations | 34 |
| 4.2. 1 Identification of vacuoles in thin section and freeze-etch replicas | 34 |
| 4.2. 2 Form of vacuoles in ultrathin sections | 35 |
| 4.2. 3 Form of vacuoles in freeze-etch replicas | 40 |
| 4. 3 Discussion | 41 |
| 4.3. 1 Evaluation of the shape of vacuoles in thin sections | 41 |
| 4.3. 2 Evaluation of the shape of vacuoles in frozen-etched root tips | 46 |
| 4.3. 3 Evaluation of irregular vacuoles from optical studies | 50 |
| 4. 4 Summary | 51 |
| 5. <u>EFFECT OF VARIOUS CONDITIONS OF FIXATION ON PRESERVATION OF VACUOLES FOR ELECTRON MICROSCOPY</u> | 53 |
| 5. 1 Introduction | 53 |
| 5. 2 Literature review | 53 |
| 5. 3 Materials and Methods | 56 |
| 5. 4 Results | 59 |
| 5.4. 1 Evacuation | 59 |
| 5.4. 2 Temperature | 59 |
| 5.4. 3 pH during KMnO_4 fixation | 59 |
| 5.4. 4 Fixative concentration | 61 |
| 5.4. 5 Duration of KMnO_4 fixation | 62 |
| 5.4. 6 Addition of indifferent compounds | 62 |
| 5.4. 7 Different concentrations of buffer | 62 |
| 5.4. 8 Different buffers at the same molarity and same pH in KMnO_4 fixation | 64 |
| 5.4. 9 Osmium tetroxide fixation | 64 |
| 5.4.10 Buffering at different stages during glutaraldehyde/osmium tetroxide fixation | 65 |
| 5.4.11 Glutaraldehyde/potassium permanganate fixation with buffering at different stages | 65 |

| | Page |
|--|------|
| 5. 5 Discussion | 66 |
| 5.5. 1 Importance of the various conditions of fixation examined | 67 |
| 5.5. 2 Effect of molarity | 68 |
| 5.5. 3 Effect of different ions | 68 |
| 5.5. 4 Relationship between molarity, osmolarity and tonicity | 69 |
| 5.5. 5 Potassium permanganate fixation | 70 |
| 5.5. 6 Effect of buffering in glutaraldehyde and osmium tetroxide fixation | 72 |
| 5. 6 Conclusions | 72 |
| 5. 7 Summary | 74 |
| 6. <u>ULTRASTRUCTURE OF THE TONOPLAST</u> | 75 |
| 6. 1 Introduction | 75 |
| 6. 2 Observations | 77 |
| 6.2. 1 The tonoplast in ultrathin sections | 77 |
| 6.2. 2 Appearance of the tonoplast in frozen- etched fresh root tips | 78 |
| 6.2. 3 Appearance of the tonoplast in prefixed root tips | 81 |
| 6.2. 4 Differentiation of the tonoplast | 83 |
| 6. 3 Discussion | 85 |
| 6.3. 1 Particles on the tonoplast in frozen fresh roots | 85 |
| 6.3. 2 Size of the particles | 86 |
| 6.3. 3 Distribution of particles on the two faces of the membrane | 86 |
| 6.3. 4 Grouping of particles on the tonoplast | 88 |
| 6.3. 5 Differentiation of the tonoplast with development of vacuolar inclusion bodies | 90 |
| 6.3. 6 Interpretation of exposed faces of the tonoplast in frozen-etched fresh root tips | 91 |
| 6.3. 7 Interpretation of the tonoplast in prefixed material | 94 |
| 6. 4 Summary | 97 |
| 7. <u>ULTRASTRUCTURAL OBSERVATIONS ON VACUOLAR INCLUSIONS</u> | 99 |
| 7. 1 Introduction | 99 |
| 7. 2 Observations | 101 |
| 7.2. 1 Occurrence | 101 |
| 7.2. 2 Types of inclusion bodies | 102 |

| | Page |
|---|------|
| 7.2. 3 Structure of inclusion bodies | 103 |
| 7.2. 4 Origin of inclusion bodies | 105 |
| 7.2. 5 Development of inclusion bodies after separation from the tonoplast | 107 |
| 7. 3 Discussion | 110 |
| 7. 4 Summary | 115 |
| 8. <u>SUMMARY AND GENERAL CONCLUSIONS</u> | 117 |
| 9. <u>LITERATURE CITED</u> | 120 |
| <u>APPENDIX I</u> | |
| <u>APPENDIX II</u> | |

ABBREVIATIONS ON PLATES

| | | | |
|----|-------------------------|-----|-------------------------|
| ER | endoplasmic reticulum | PL | plaques |
| GA | golgi apparatus | PDS | plasmodesmata |
| GR | groundplasm | PM | plasmalemma |
| GV | golgi vesicle | SG | starch grain |
| IB | vacuolar inclusion body | SP | spherosome |
| L | lipid body | T | tonoplast |
| M | mitochondrion | To | outer face of tonoplast |
| N | nucleus | Ti | inner face of tonoplast |
| NE | nuclear envelope | V | vacuole |
| NI | nucleolus | W | cell wall |
| P | plastid | | |

I. INTRODUCTION

The development of the electron microscope (see Hall, 1966) and associated techniques (see Pease, 1964; Kay, 1965; Sjöstrand, 1967) has led to the growth of a whole new field of biological research. Prior to 1930 cytological inquiry was limited to resolutions mainly above 1,000 Å. Today, with modern electron microscopes, it is possible to resolve particles to within a few Ångströms (see Wischnitzer, 1962, 1967). In early years difficulties in sectioning limited the application of electron microscopy to cytology but since the late 1940's, with the development of ultramicrotomes (see Porter, 1964; Sorvall, 1965; Sjöstrand 1967), progress has been rapid.

Because material prepared for thin sectioning is treated chemically, preparations are subject to artifacts. The artifact problem is diminishing as more observations are confirmed using different techniques, but the fact that dead cells are studied remains a source of criticism. A technique which largely circumvents chemical treatment of cells for electron microscopy is freeze-etching (Steere, 1957; Moor et al., 1961; Moor, 1966b; Koehler, 1968). In freeze-etching, living cells are very rapidly frozen, then fractured, etched and a replica of the exposed surface studied. The technique provides an independent approach for assessing results from thin sectioning; it also provides new information in the form of three dimensional images and surface views of cell structures.

The application of electron microscopy to plant cytology developed late compared with its application to animal cytology (Buvat, 1963a); this was due partly to difficulties of specimen preparation (Pease, 1964) and to the small number of workers in the field. The first important papers describing fine structure of cells in seed plants appeared in the late 1950s (e.g. Buvat, 1958; Lance, 1958; Sitte, 1958; Perner, 1958; Schnepf, 1959; Whaley et al., 1959a & b, 1960a & b; Porter & Machado, 1960; Hohl, 1960. For other references see "The International Bibliography of Electron Microscopy" vols. 1 & 2 and Sitte, 1961). During this period studies were confined mainly to meristematic cells (e.g. Sitte, 1957; Buvat, 1958; Whaley et al., 1960a; Hohl, 1960); difficulties in preparing highly vacuolated cells hindered progress with other tissues. Even in 1963 information on the ultrastructure of differentiated tissue in higher plants was scant (Buvat, 1963a; Esau, 1963). Since then, however, research papers have appeared at an increasing rate (see texts by Côté, 1965; Frey-Wyssling & Muhlethaler, 1965; Clowes & Juniper, 1968; Pridham, 1968).

An organelle characteristic of plant cells is the vacuole. The word 'vacuole' was first applied to contractile vacuoles of protozoa by Spallanzani, in 1776, but it was not until about the mid-nineteenth century that the name became applied to the more stationary structures of plant cells (see Zirkle, 1937). Vacuoles have been regarded as cell inclusions (e.g. Holman & Robbins, 1954) but modern research has clearly shown that they are cytoplasmic in nature (e.g. Frey-Wyssling & Muhlethaler, 1965; Clowes & Juniper, 1968). The vacuole is a single

membrane bounded organelle which usually contains a large amount of water. During cell differentiation, the vacuoles enlarge, fuse and eventually occupy a large proportion of the cell. Unlike animal cells which grow by increasing the mass of protoplasm, plant cells expand with great economy of protoplasm production by filling much of the cell with vacuolar material. In addition to water, vacuoles contain various dissolved and colloidal substances, and solid matter in the form of crystals and portions of cytoplasm. The nature of the contents is, however, variable among vacuoles of a cell and in one vacuole at different stages of differentiation. Vacuoles perform a variety of functions. They are important in the support of primary tissues by means of the turgor pressure developed within them. Vacuoles are also sites for storage, synthesis and deposition of waste products. Current studies indicate that vacuoles often contain enzymes and may function as lysosomes (e.g. Matile & Moor, 1968).

Since the 1880's a vast literature has accumulated on the physiology and cytology of vacuoles (reviewed by Bailey, 1930; Zirkle, 1932, 1937; Guilliermond, 1941; Drawert, 1955a & b; Kramer, 1955; Pisek, 1955; Dangeard, 1956; Voeller, 1964; Dainty, 1968). During the past decade observations have been extended to the ultrastructural level. However, compared with other organelles of the cell (see Frey-Wyssling and Mühlethaler, 1965; Clowes & Juniper, 1968) comparatively little work has been devoted specifically to the fine structure of vacuoles. Studies have been made in an attempt to explain the origin of vacuoles (e.g. Buvat, 1958; Poux, 1962; Marinos, 1963a; Barton 1965; Bowes, 1965b; Ueda, 1966).

Observations have been made on the tonoplast in thin section (e.g. Grun, 1963). Recently there has been a growing interest in the cytoplasmic inclusions found within vacuoles (e.g. Gifford & Stewart, 1968; Buvat & Coulomb, 1968).

However, few systematic investigations have been made (1) to evaluate the shape of vacuoles in electron microscope preparations, (2) to determine the effect of various conditions of fixation on the preservation of vacuoles (3), to examine the organization of the tonoplast in freeze-etch replicas and (4) to survey the various types of cytoplasmic inclusions found within vacuoles of root tips. In this project these aspects of the vacuole have been studied using thin sectioning and freeze-etch techniques. Root tips from five species were used. Before freeze-etching could be applied to problems of vacuole ultrastructure methods of preparing root tips for freeze-etching had to be developed. This preliminary work on freeze-etching is presented first.

2. MATERIAL AND METHODS: GENERAL

Technical details not previously published (Fineran, 1966 - see Appendix I) are outlined below. Schedules applying to particular experiments are described in the relevant chapters. General information was obtained from texts by Mercer and Birbeck (1961), Kay (1961, 1965), Pease (1960, 1964), Sjöstrand (1967), Sorvall (1965), Vogel (1946), Hall (1966) and Wischnitzer (1962).

2.1 Plant Material

Root tips were taken from Lonicera nitida, Mentha citrata, Allium cepa (onion), Zea mays (corn), Triticum vulgare (wheat) and Avena sativa (oat). Species selected were those found to be most suitable for study at the time. Allium, Zea, Triticum and Avena were introduced after difficulty was experienced in handling the other species for freeze-etching. Lonicera and Mentha were derived from clonal stocks and roots obtained adventitiously. Cuttings of Lonicera were struck in soil in pots and roots took several weeks to develop. Cuttings of Mentha were placed in $\frac{1}{2}$ strength Hoagland's nutrient solution and roots were initiated within about a week. Whenever possible plants were transferred from the greenhouse or laboratory to a controlled environment (provided by a growth cabinet) prior to experiments.

Roots of Zea, Triticum and Avena were obtained from seeds germinated in an incubator in the dark for 12 to 24 hours at 25°C. The Allium roots were produced adventitiously on bulbs grown on water. All seeds and bulbs were purchased locally.

2.2 Chemicals and Solutions

Analar and laboratory grade chemicals were used wherever possible. Glass distilled water was employed in preparing aqueous solutions.

2.3 Preparation of Material for Thin Sectioning

2.3 1 Fixation

Root tips 1-2 mm long were selected for most studies. Occasionally median longitudinal slices 1 mm thick were used; otherwise specimens comprised excised whole root tips. Specimens were cut in tap water prior to KMnO_4 fixation and in the fixative for other schedules.

Potassium permanganate (KMnO_4) (Luft, 1956; Mollenhauer, 1959), osmium tetroxide (OsO_4) (Palade, 1952) and glutaraldehyde (abbreviated GA) (Sabatini *et al.*, 1963) were the principal fixatives employed, singly and in combination. Three main or "standard" schedules were followed:

1. Standard aqueous KMnO_4 : 2% solution (0.126M; pH ca 8.5) with fixation for 2 hours at room temperature (16-20°C).
2. Standard buffered KMnO_4 : 2% solution made up in 0.140 M veronal acetate-HCl buffer pH(s) 6.5-7.5) with fixation for 2 hours at room temperature.
3. Standard GA/ OsO_4 : 6% GA (Biological grade) in 0.066 M Sorensen's phosphate buffer (pH 6.8) for 4 hours, followed by washing in buffer (3 changes during 3 hours), then post fixed in 1% OsO_4 in buffer for 12 hours followed by buffer washes again; all stages

were conducted at 4°C.

pHs were checked using a radiometer (Copenhagen). During the first 30 minutes of fixation specimens were evacuated at 64 cm mercury (provided by a water vacuum pump) to remove air from intercellular spaces and to assist penetration of fixative.

2.3 2 Dehydration and Embedding

Among various methods tested (Davis, 1959; Mollenhauer, 1959; 1964; Juniper, 1962; Mohr & Cocking, 1968) the procedure using Araldite (Glauert & Glauert, 1958; Luft, 1961) outlined by Merrillees et al. (1963; Merrillees pers. comm. to W.S. Bertaud, 1962) proved to be the most suitable after some modification (Fineran, 1966). Specimens were embedded in gelatin and BEEM capsules.

2.3 3 Ultramicrotomy

After 1964 sections were cut on an LKB Ultratome II using glass and diamond knives.* Glass knives were prepared on an LKB glass breaker. Most of the illustrations presented are based on sections cut with diamond knives.

Sections were picked up from the trough using Sjöstrand Type one-hole grids and after staining transferred to standard mesh grids (Method adapted after Galey & Nilsson, 1966).

2.3 4 "Staining"

Specimens were block stained during dehydration in saturated uranyl acetate in 70% acetone overnight (method

*Diamond knives obtained from Friedrich Dehmer, 8202 Bad Ailbing, Germany.

modified after Jensen, 1964 and Wooding & Northcote, 1965). Sections were post-stained by floating the one-holed grids on drops of stain on a wax block. The drops of stain were placed around a hollow containing pellets of NaOH (to absorb CO₂) and the whole covered with the lid of a petri dish pressed into the wax. Stains were centrifuged for 10 minutes before use. Millonig's (1961a) lead tartrate and Reynolds' (1963) lead citrate were the principal stains employed. The micrographs presented are based on sections stained with lead tartrate. A staining time of 10 minutes was used for KMnO₄-fixed tissue and upwards of 2 hours for GA/OsO₄ preserved material.

2.4 Preparation of Material for Freeze-Etching

2.4 1. Pretreatment of Specimens

Two procedures were adopted: (a) Roots were grown in glycerol at different concentrations and for various periods prior to freeze-fixation. (b) Specimens were prefixed in GA in glycerol followed by freeze-fixation. The experiments involved in preparation of root tips for freeze-etching are described in chapter 3.

2.4 2. Freeze-Etching

Root tips 1-2 mm. long were excised in glycerol of the same concentration used for pretreatment. Large roots were further cut to remove the median 1 mm. thick longitudinal slice using an apparatus similar to that figured by Branton and Moor (1964).

Specimens were placed on 3 mm copper discs and orientated under a stereoscopic microscope. The copper disc was then plunged into liquid Freon - 12 for a few seconds and quickly transferred to liquid nitrogen for storage. Freeze-etching was carried out on a Balzers apparatus similar to that described elsewhere (Moor, et al., 1961, 1963; Moor, 1964; Hall, 1967). Root tips were fractured, etched for 1 minute at $\overset{-}{\underset{\wedge}{100^{\circ}\text{C}}}$, shadowed with platinum/carbon (Moor, 1959) at an angle of 30 degrees and then carbon was applied from a vertical position. Silver was occasionally evaporated on to this replica to give added strength during subsequent handlings. The yield of specimens with good shadowing was low but towards the end of the work some improvement was achieved by increasing the length of platinum wire from 5 to 8 cm with the carbon fixed at 5 mm. After etching and shadowing, replicas were floated on to distilled water containing a drop of detergent.

Three methods were used to digest adhering plant material from the replica:

1. Water supporting the replica was gradually replaced over $\frac{1}{2}$ hour by concentrated nitric acid and left for 1 hour. The concentrated acid was then progressively replaced by fuming nitric acid and the replica left in this for 45-60 minutes. Sometimes the replica was placed in a water bath at 76°C during this period. On cooling, the fuming acid was cautiously diluted with water until all trace of acid ^{was} removed. This method (modified after Hall, 1967) gave mainly clean

replicas within a few hours but care was required in handling specimens.

2. The replica was transferred from distilled water to 75% sulphuric acid (4 hours). The acid was then diluted to about 10% and the replica placed in 5% chromic 5% nitric acid overnight. Next morning the replica was brought down to water and thoroughly washed in several changes before being gradually transferred to bleach (a commercial product similar to "Janola" containing a concentrated solution of sodium hypochlorite and NaOH). Replicas remain in the bleach for $\frac{1}{2}$ hour before being returned to water. The method produced reasonably intact replicas though specimens were sometimes contaminated.
3. Replicas were prepared following the general schedule outlined by Branton and Moor (1964) using bleach for 2 hours and 70% H_2SO_4 for 4 hours. Variable results were obtained using this schedule.

Solutions were changed using a fine pipette. Where possible replicas were transferred between solutions using a small loop of platinum wire.

2.5 Grids

Copper grids were used throughout the study. Sections were mounted on 100 mesh grids covered with a collodion film (see Kay, 1965 p.62) reinforced with carbon. Other types of grids were used occasionally for special purposes. An Hitachi Vacuum evaporator (Type HUS-3B) was used for preparation of carbon films.

Freeze-etch replicas were mounted on bare 400 mesh grids and occasionally on 200 mesh grids with a support film of collodion.

2.6 Microscopes

Sections and replicas were examined using Philips (EM 100B & EM 200) and Hitachi (HS-7 & HU11-B) electron microscopes. Thick - thin sections were observed under phase contrast microscopy with a Reichert Zetopan.

2.7 Photographic Procedures

35 mm Kodak fine grain positive film, developed in D16 for 4 minutes, was used in the Philips electron microscopes. Ilford plates N 60, N 50 & N 40, developed in ID2, were employed with the Hitachi instruments. Occasionally for special purposes Kodak Electron Image plates were used.

Electron micrographs were printed on Ilford papers. The freeze-etch illustrations presented are printed so that the shadow is white with the direction of shadowing indicated by an arrow near the bottom right corner. For the dissertation the original plates were photocopied and printed on Agfa-Gevaert projection duplex paper. Catalogue numbers of negatives used in preparation of the plates are given in Appendix II. The numbering of the illustrations continues on from that of the preliminary work (Appendix I) and starts here at 15.

2.8 Range of Material Studied

Besides direct observations under the electron microscope, results are based on examination of over 2500 electron micrographs. Approximately 300 different fixations were prepared and about 1500 specimens sectioned. A total of 57 freeze-etchings were made and 130 grids prepared. One to four root tips, depending on size, were

frozen-etched each time.

Qualitative and quantitative sampling of cell structures was made from micrographs taken at fixed magnifications and directly from the screen of the Philips EM 100B electron microscope.

3. EXPERIMENTS ON THE PRETREATMENT OF ROOT TIPS FOR FREEZE-ETCHING

3.1 Introduction

The technique of freeze-etching is finding increasing use in botanical research (see Koehler, 1968). The method, based in principle on the work of Hall (1950), was first applied to biological objects by Steere (1957) and improved by Moor and associates (Moor et al., 1961, 1963; Moor, 1964, 1965, 1966a & b). While several studies have been devoted to bacteria (e.g. Giesbrecht & Drews, 1966; Moor, 1966d; Remsen, 1966; Remsen & Lundren, 1966; Remsen et al., 1967a, 1968; Nanninga, 1968), fungi (e.g. Moor & Mühlethaler, 1963; Moor, 1964, 1967; Matile et al., 1965; Hess et al., 1966, 1968; Branton & Southworth, 1967; Matile & Wiemken, 1967; Remsen et al., 1967b; Sassen et al., 1967; Hess, 1968; Hess & Stocks, 1968), algae (Ettlinger et al., 1965; Herrmann & Staehelin 1965; Jost & Zehnder, 1966; Staehelin, 1966, 1968a & b, Leak & Burke, 1967; Leak, 1968a), structure of plastids (Frey-Wyssling & Schwegler, 1965; Mühlethaler et al., 1965; Bamberger & Park, 1966; Branton & Park, 1967; Dilley et al., 1967; Park & Branton, 1967; Guerin-Dumartain, 1968) and other aspects of plant ultrastructure (e.g. Eggman, 1966; Moor, 1966c; Hall, 1967; Johnson, 1968) comparatively little work has been done on root tip tissue.

The first successful attempt to freeze-etch root tips was made by Branton and Moor (1964) and Moor (1966b). In preliminary experiments Branton and Moor found that root tips were more difficult to freeze than yeast cells (Moor & Mühlethaler, 1963) but they were finally successful in freeze-etching roots of Allium cepa which had been initiated and

grown in 20% glycerol for 5 days. Other illustrations of freeze-etched root tips have appeared (Moor, 1965; Branton, 1966; Northcote, 1967; Frey-Wyssling, 1966, 1967) but no detailed study had been published at the start of the present work. Since then, other papers have appeared (Matile, 1968; Matile & Moor, 1968; Northcote & Lewis, 1968). The difficulty of pretreating root tips for freeze-etching, in fact anything except unicells and fungi, appears to be one reason for the paucity of investigations on root tips and other organs of higher plants. Also, because fracturing is random, there is the problem of working out the location of cells in replicas of a multicellular tissue.

Before freeze-etching can be successfully carried out freeze-fixation must be perfected (Moor, 1966b). For this, biological material must be rapidly and uniformly frozen throughout and fulfill two physical requirements; it should (i) be freezeable without production of ice crystals and (ii) be etchable. As most plant tissue contains above 80% water, freeze-fixation without freezing damage is not possible unless either extremely high freezing speeds are used or an anti-freeze agent can be introduced to increase the frost hardiness of the specimen. The use of an anti-freeze agent is usually preferred. This process involves the gradual impregnation of a nontoxic hydrophilic substance into the cytoplasm and vacuole in such a way that the metabolism of the cell is not destroyed. Preferably, the tissue should become adjusted to the presence of the anti-freeze agent and continue growth so that the freeze-etch replica ultimately prepared represents the condition in a viable cell. So far, glycerol has proved to be the most successful anti-freeze agent for plants (Moor, 1964, 1966b). If the tissue need

not be viable at the time of freeze-etching chemical pre-fixation (Moor, 1966b) may be employed with subsequent or simultaneous glycerol impregnation.

This chapter reports results of experiments carried out while attempting to prepare root tips for freeze-etching. The work was exploratory and not intended as a systematic study of the pretreatment of root tips. A variety of plant material and approaches were tested before a suitable method was found of preparing living roots for this process. The investigations also included the preparation of root tips for freeze-etching using combined chemical and physical fixation.

3.2 Pretreatment of specimens

Ten main groups of experiments were carried out in an endeavour to impregnate roots with glycerol prior to freeze-fixation. Controls were established under similar conditions but without glycerol. Plants were grown at room temperature.

- (1) Unrooted shoots (Table 1) and
- (2) rooted plants (Table 2) of Lonicera nitida, Mentha citrata and Allium cepa were placed in 5% - 20% glycerol in water (V/V) or half strength Hoaglands (V/V) for two months or more. In one set of experiments the plants remained in 5%, 10%, 15% and 20% solutions throughout this period.
- (3) Other series of plants (Table 3) were first placed in 5% glycerol and over a period of weeks brought up to 20% glycerol at 5% stages.
- (4) Stolons of Mentha were layered into 5%, 10%, 15% and 20% glycerol in water for 3 weeks. Light was excluded from the immersed portion of the stolon.
- (5) Lonicera and Mentha grown in soil in pots were pre-conditioned to

- glycerol by watering with a 5% solution every second day for 1 to 4 weeks. One set of plants was returned to normal watering after a week's treatment with glycerol.
- (6) Rooted cuttings of Lonicera and Mentha were placed in 15% and 20% glycerol overnight and for up to 3 days (Table 4).
 - (7) Excised roots of Mentha, 5 cm long, were floated on to 20% glycerol for 6 hours.
 - (8) Seeds of Zea mays, Triticum vulgare and Avena sativa were left to germinate on filter paper covered with 20% glycerol for several weeks (Table 1).
 - (9) Seedlings of Zea, Triticum and Avena with roots each 1 cm long were allowed to grow with the base of the seed and root covered by 20% glycerol.
 - (10) Roots of Zea, Triticum and Avena seedlings germinated in water were placed on filter paper impregnated with glycerol and arranged to allow aeration of the roots (Table 5). This was achieved by placing seedlings on top of narrow tubes of filter paper (inserted inside glass tubing for support) with the base of the tube standing in glycerol. Specimens were left to incubate for 5 and 7 days in contact with 20% glycerol. In other experiments the roots were exposed to 20% glycerol for 3 days then to 25% for 3 days. At the beginning of the experiments Zea roots were 0.5 - 1.0 cm long, those of Triticum 2 - 5 mm long. Most roots of Avena had not emerged from the coleorhiza.
 - (11) Root tips of Lonicera, Mentha, Avena and Triticum were prefixed prior (Moor, 1966b; Matile, 1968) for 1½ to 3 hours to freezing in 3% and 6% GA in 0.025 M phosphate buffer, containing 0.001 M CaCl₂,

made up in 30% glycerol (Table 6). 30% glycerol was used to ensure good freezing.

3. 3 Observations and Experiments

3.3 1 Attempts to initiate roots in glycerol

If living roots are to be successfully frozen-etched they must absorb sufficient anti-freeze agent to prevent the formation of ice crystals. At the same time growth must be maintained though perhaps at reduced levels.

Concentrations of glycerol between 5% and 20% were applied to cuttings of Lonicera and Mentha but all attempts to initiate new roots failed (Table 1). This approach was unsuccessful irrespective of whether plants were placed immediately in concentrations of 15% and 20% or brought up over a period of weeks to 20% glycerol at 5% stages. Seeds of Zea, Triticum and Avena placed in 20% glycerol failed to germinate (Table 1) and could not be induced to germinate when subsequently transferred to water. These results demonstrate the difficulty of initiating roots in glycerol for freeze-etch studies.

In a later series of experiments, limited success was obtained initiating roots of Mentha and Allium in 15% glycerol using previously rooted plants placed in glycerol for several weeks (Table 3). At this concentration insufficient glycerol had been absorbed to prevent extensive freeze damage (Fig. 15). (See also illustrations by Moor, 1966b). Although 15% glycerol is therefore too low a concentration for root tips,

fungal contaminants growing on the material had absorbed enough anti-freeze agent to give reasonable freeze fixation. (Fig. 16).

3.3 2 Attempts to initiate roots on stolons of *Mentha*

The results of the above experiments suggested that exposing the whole plant to glycerol was too severe for the organism to adjust its metabolism. If only part of the plant were exposed to glycerol, leaving the remainder to grow as normal, perhaps a better response might be expected.

Stolons of *Mentha*, from plants growing in pots, were therefore layered into glycerol of different concentrations. After several weeks no roots had been produced from the stolons. The distal portion died but the rest of the plant was unaffected by glycerol. This result shows that even a part of a plant may be intolerant to glycerol.

3.3 3 Attempts to impregnate established roots by prolonged exposure to glycerol

Although Branton and Moor (1964) and Moor (1964, 1966b) found water-grown root tips of *Allium cepa* to be useless for freeze-etch studies, it was not stated whether this applied to other species.

Rooted plants of *Allium*, *Lonicera* and *Mentha* placed in 5%, 10%, 15% and 20% glycerol were all adversely affected (Table 2). The symptoms became more marked with increasing concentration and duration of exposure. Plants brought up gradually to 20% glycerol at 5% stages over a period of weeks (Table 3) were less severely affected than those placed directly

in solutions above 10% concentration. In all instances roots lost turgidity and most did not recover; only a few showed signs of re-growth on transferring to glycerol. After several weeks, shoots of Mentha had died above the level of the solution but those of Lonicera remained alive apart from severe wilting at the tips. Of the rooted cuttings, Lonicera indicated better tolerance towards glycerol than did Mentha. No difference in response was observed between plants grown in glycerol/water or glycerol/half strength Hoagland's nutrient solution. Added nutrients thus offer no advantage in promoting growth of roots in glycerol. The use of half strength Hoagland's nutrient solution was discontinued in subsequent experiments.

~~Frozen-etched~~ ^{Roots} of Lonicera, Mentha and Allium placed in 15% and 20% glycerol for several weeks showed extensive freeze damage ^{on freeze-etching} (cf. Fig. 19). By contrast fungal contaminants - which often heavily infested the solutions - exhibited good freeze-fixation (Figs. 16, 44). The frozen-etched root tips showed little cellular detail, but it was not known whether this had been destroyed by prolonged treatment with glycerol or whether the structures had not been revealed during fracturing. To determine this, root tips from these experiments were prepared for ultramicrotomy. The sections indicated that while some cells showed fair preservation (Fig. 17), despite the reaction of KMnO_4 with glycerol (see Pease, 1967), most were disorganised, often with disintegrated cell walls (Fig. 18). These results indicate that prolonged exposure to glycerol is deleterious and explains the paucity

of cytoplasmic detail in the frozen-etched material.

3.3 4 Attempts to impregnate established roots by short exposure to glycerol

As prolonged exposure to glycerol proved harmful, subsequent experiments were carried out using shorter periods of exposure (Table 4). It seemed possible that sufficient glycerol might be absorbed before the tissue began to degenerate.

Rooted plants of Lonicera and Mentha placed in 15% and 20% glycerol overnight, and for up to 3 days, lost turgidity. Most roots of Mentha exhibited poor freeze-fixation with extensive development of ice (Fig. 19), although a few cells contained peripheral cytoplasm with some preservation (Fig. 20). Roots of Lonicera showed fair preservation in places (Fig. 21) but results were inconsistent. Despite freezing damage, the tissues, in contrast to those exposed to glycerol for long periods, revealed organelles. The plasmalemma, tonoplast and membranes of other organelles were occasionally seen in face view. The membranes often showed a slightly dimpled appearance suggestive of shrinkage of the membrane over underlying large crystals of ice or of plasmolysis caused by the glycerol (cf. Fig. 24).

The experiments show that roots of Lonicera and Mentha are incapable of giving reliable freeze-fixation on short exposure to glycerol. However, fungi show reasonable preservation (Fig. 22). From this it may be concluded that fungi are better adapted to grow in glycerol; a 15% solution overnight will give good preservation.

3.3 5 Attempts to pre-condition plants by watering with glycerol

The poor response of rooted plants to glycerol indicated that the transition from normal growth to that in glycerol was too abrupt. If the change were gradual perhaps an improvement might result.

To test this, established plants of Lonicera and Mentha growing in soil in pots were pre-conditioned by watering with 5% glycerol. Within a few days the plants developed the usual symptoms of exposure to glycerol, Mentha being affected more than Lonicera. Plants returned to normal watering after a week's treatment with glycerol failed to recover.

3.3 6 Glycerination of excised roots

Impregnation of excised plant material for freeze-etching has been carried out using concentrations of glycerol from 5 to 40% for 2 hours and up to 3 days (Moor, 1966b; Bamberger & Park, 1966; Branton et al., 1967; Hall, 1967).

Excised roots of Mentha placed in 20% glycerol for 6 hours showed extensive freezing damage (Figs. 23 & 24) similar to that in intact roots exposed to 20% glycerol overnight (Fig. 19). This suggests that the level of glycerol capable of being absorbed by Mentha roots is quickly attained. Organelles with a high concentration of water exhibited coarse reticulate patterns of ice; groundplasm showed smaller crystals while organelles with a low aqueous content displayed only slight effects of freezing (Fig. 23). Fracturing in such badly fixed root tips is often better than in well frozen material. Also, specimens are more deeply etched.

While this schedule gave mainly poor results, smaller pieces of tissue, longer soaking periods, higher concentrations of glycerol or gradual infiltration (cf Richter, 1968a & b) might prove to be more suitable.

3.3 7 Glycerination and freeze-fixation in roots of germinated seeds

Preliminary trials were carried out using seeds germinated overnight in water then placed on filter paper covered with 20% glycerol. Roots touching the impregnated paper, but not immersed in the solution, remained healthy while those covered by glycerol softened and eventually darkened - possibly due to insufficient oxygen.

Using the method outlined (p. 16), which allowed adequate aeration, many roots showed slight growth and remained turgid on exposure to glycerol (Table 5). Zea and Triticum roots grew a few mm. Although Avena roots did not emerge from the coleorhiza, the coleorhiza grew slightly. The worst symptoms of exposure to glycerol were shown by Zea.

Good freeze-fixation and cellular detail was obtained from Avena and Triticum grown in 20% glycerol for 7 days (Figs. 25 & 26). Results in Zea were variable; a few roots showed reasonable preservation (Fig. 27) but most were only fair to poor (Fig. 28). In other experiments using Avena and Triticum grown in 20% glycerol for 5 days, freeze-fixation was poor compared with roots exposed for 7 days. Avena showed better preservation than Triticum but many specimens still exhibited effects of freezing. Only a few roots of Triticum showed good

freeze-fixation (Fig. 29), otherwise preservation was fair to poor.

To determine whether higher concentrations of glycerol would improve preservation, seedlings of Zea, Triticum and Avena were exposed to 20% glycerol for 3 days followed by 25% glycerol for 3 days. Apart from occasional specimens (Fig. 30), Avena and Triticum displayed mainly good freeze-fixation (Fig. 31) similar to roots grown in 20% for 7 days. Zea showed improvement over earlier experiments (Fig. 32). Nevertheless, results with Zea were variable, some specimens showing effects of freezing (Fig. 34).

Although freeze-fixation was generally good in Avena and Triticum variation occurred between specimens of a given trial. Poor preservation was occasionally present towards the periphery of a specimen and at the cut end of the root. A higher concentration of water in peripheral cells than in those near the meristem may explain this variation. In most experiments a few cells also showed plasmolysis, either as small blebs between the cell wall and plasmalemma, or as large contracted areas (Fig. 33). Occasionally one cell would be affected in this way although adjoining cells exhibited good preservation (Fig. 33). Different physiological conditions and different abilities to absorb glycerol among cells probably accounts for some of this variation in freeze-fixation.

3.3 8 Prefixation with glutaraldehyde during glycerination prior to Freeze-fixation

To overcome the difficulty of growing plants in glycerol some workers have employed chemical fixation prior to freeze-

fixation (Moor, 1966b; Park & Branton, 1967; Bamberger & Park, 1966; Matile, 1968, Matile & Moor, 1968).

Glutaraldehyde is usually favoured as it is known to effect cross-linkage of protein with minimal disturbance to cell structure (Sabatini et al., 1963). The above workers prefixed material in GA and then soaked in glycerol. This schedule was modified in the present experiments by making up the GA in 30% glycerol to allow simultaneous prefixation and glycerination.

In all experiments (Table 6) using Lonicera, Mentha, Avena, Triticum and Zea pretreated with GA/glycerol good freezing was obtained throughout (Figs. 35-44). The groundplasm and contents of organelles showed a finely granular appearance becoming slightly coarser in large vacuoles, but seldom did the granularity approach that frequently seen in well preserved vacuoles of frozen living cells. The indistinct radiate pattern of freezing sometimes noted in well frozen living roots (Fig. 31) is often present in prefixed material interspersed with non-etched areas (Fig. 39). The use of 30% glycerol in prefixed preparations, compared with 20% glycerol, probably accounts for the small degree of etching.

The quality of freeze-fixation in the different species is similar (Figs. 35, 36, 38). No difference is noted between specimens frozen as whole root tips and those prepared as median longitudinal slices 1 mm thick. Equally good preservation was obtained by using 3% and 6% GA and by fixing for $1\frac{1}{2}$ to 3 hours. Within the limits of these trials, it appears that

TABLE 1
ATTEMPTS TO INITIATE ROOTS IN SOLUTIONS OF GLYCEROL

| Trial | Species | Number of cuttings, bulbs and seeds used at each concentration of glycerol | Trials using glycerol in water % conc. | | | | Trials using glycerol in $\frac{1}{2}$ Hoaglands % conc. | | | | Duration of trials in weeks | Reaction of plants to growth in glycerol | Growth of controls | |
|-------|-------------------------|--|---|----|----|----|---|----|----|----|-----------------------------|--|--|------------------------------------|
| | | | 5 | 10 | 15 | 20 | 5 | 10 | 15 | 20 | | | | |
| 1 | <u>Lonicera nitida</u> | 6-8 | x | x | x | x | x | x | x | x | x | ca. 12 | No roots produced. shoot tips wilted within a few days, defoliation began after ca. a week. Deterioration faster in higher concentration. Most plants dead by end of experiment. | Roots produced after ca. 3 weeks. |
| 2 | <u>Mentha citrata</u> | 6-8 | x | x | x | x | x | x | x | x | x | ca. 8 | As above. Plants wilted and died before those of <u>Lonicera</u> . | Roots produced within a week. |
| 3 | <u>Zea mays</u> | 12-20 | x | x | x | x | | | | | | 3 | Seeds failed to germinate. | Seeds germinated within 12-24 hrs. |
| 4 | <u>Triticum vulgare</u> | 24-30 | x | x | x | x | | | | | | 3 | As above. | As above. |
| 5 | <u>Avena sativa</u> | 24-30 | x | x | x | x | | | | | | 3 | As above. | As above. |

TABLE 2

REACTION OF ROOTED PLANTS TO GROWTH IN GLYCEROL OF DIFFERENT CONCENTRATION

| Trial | Species | Plants per trial | Concentrations of glycerol tested | | | | | | | | Duration of trials (weeks) | Reaction of plants to glycerol treatment |
|-------|-------------------|------------------|-----------------------------------|-----|-----|-----|--------------------------------------|-----|-----|-----|----------------------------|---|
| | | | Glycerol/water | | | | Glycerol/ $\frac{1}{2}$ Hoaglands | | | | | |
| | | | 5% | 10% | 15% | 20% | 5% | 10% | 15% | 20% | | |
| 1 | <u>A. cepa</u> | 2 - 4 | x | x | x | x | | | | | 6 | Little evidence of re-growth of roots at any concentration. Most roots lost turgidity. All bulbs failed to sprout further on transfer to water. |
| 2 | <u>L. nitida</u> | 6 - 8 | x | x | x | x | x | x | x | x | 4 - 8 | Shoot tips wilted and in higher concentration eventually died. Some defoliation occurred at all levels becoming greater with increased concentration. Little sign of regrowth of roots and most lost turgidity. |
| 3 | <u>M. citrata</u> | 6 - 8 | x | x | x | x | x | x | x | x | 4 - 8 | All shoots died above level of solutions. Little sign of regrowth of roots; most showed loss of turgidity. Effects of glycerol became evident earlier than in <u>Lonicera</u> . |

TABLE 3

REACTION OF ROOTED PLANTS ON GRADUAL EXPOSURE TO GLYCEROL OF INCREASING CONCENTRATION FROM 5-20%

| Trial | Species | Plants per trial | Reaction of shoots on exposure to glycerol | Condition of roots after exposure to glycerol | Freeze-etch | | |
|-------|-------------------|------------------|---|---|---|----------------------|---|
| | | | | | Material freeze-fixed | Number freeze-etched | Observations freeze-fixation |
| 1 | <u>A. cepa</u> | 5 | Bulbs ceased sprouting on transfer to glycerol | Loss of turgidity in most but some showed gradual and partial recovery. A few mm of regrowth occurred in some after 4 weeks in 15%. Some new roots were also produced by that stage. No sign of growth on transfer to 20% glycerol. | Root tips initiated in 15% glycerol | 1 | Walls and some organelles visible; little structural detail preserved. Gross freezing damage in vacuole and groundplasm (Fig. 15) |
| | | | | | Established roots showing slight regrowth in 15% before transferring to 20% glycerol. | 1 | As above. |
| 2 | <u>L. nitida</u> | 6 | Most tips wilted and some died. Remainder of shoot persisted with various amounts of defoliation. | Most lost turgidity and did not fully recover. Many became soft and brownish in appearance. No new roots initiated. Little regrowth of existing roots. | Roots from 15% glycerol with slight regrowth. | 1 | Occasional outline of cells seen. No organelles identified. |
| 3 | <u>M. citrata</u> | 8 | Wilted within a few days. After 3 weeks in glycerol all were dead above level of solution. Beneath the glycerol stems remained living & on some a few new axillary shoots 1-2cm long developed. | Most lost turgidity and became brownish in appearance. A few showed slight recovery but little regrowth. A small number of roots 5-10mm developed on the new axillary shoots and occasionally elsewhere on the stem in 15% glycerol. No change on transfer to 20% glycerol. | Roots showing slight regrowth in 15% glycerol | 4 | Little tissue fractured. Some freeze damage. Bacterial and fungal contaminants showed good freeze-fixation. (Fig. 16) |
| | | | | | Shoot tips developed in 15% glycerol | 1 | Fractured shoot tissue not found. Microbial contaminants showed fair freeze-fixation but considerable freezing in surrounding media (cf. Fig. 22) |

TABLE 4
 ROOTED PLANTS PLACED IN GLYCEROL FOR SHORT DURATIONS PRIOR TO FREEZE-FIXATION

| Trial | Species | Glycerol treatment | Reaction to treatment | Freeze-etch | |
|-------|-------------------|--------------------|--|----------------------|---|
| | | | | Number Freeze-etched | Observations freeze-fixation |
| 1 | <u>M. citrata</u> | 15% for 3 days | Plants wilted & most roots lost turgidity. Many roots darkened in growing zone behind tip. | 1 | Cell walls visible in cross fracture. Extensive effects of freezing throughout. Peripheral cytoplasm poorly preserved in some cells (Fig.20). Microbial contaminants with reasonable fixation |
| 2 | <u>M. citrata</u> | 20% overnight | Plants wilted and roots lost some turgidity. Several roots darkened in growing zone. | 3 | Good cross fracture of cells and tissue but extensive freezing damage throughout (Fig.19). Plasmalemma & other membranes show fair preservation. Reasonable preservation in microbial contaminants. |
| 3 | <u>L. nitida</u> | 20% overnight | Shoots showed a little wilting. Roots lost some turgidity. | 1 | Fair preservation in places (Fig.21). Overall fixation better than in <u>Mentha</u> . |

TABLE 5

FREEZE-FIXATION OF SEEDLING ROOTS EXPOSED TO GLYCEROL

Roots in contact with 20% glycerol for 7 days

| Trial | * Species | Effect on exposure to glycerol | Number freeze-etched | Observations freeze-fixation |
|-------|-------------------|---|----------------------|--|
| 1 | <u>Z. mays</u> | Some roots grew a few mm. The radicle of several seeds had emerged before treatment but showed slight growth thereafter. Roots remained turgid except for some which darkened and softened. | 3 | Results variable. Some roots displayed fair preservation of gross structure but with freezing damage (Fig. 28). Others showed a few cells with reasonable fixation. One group of root tips showed good preservation throughout (Fig. 27) |
| 2 | <u>A. sativa</u> | Roots did not emerge from coleorhiza, which showed slight growth in some specimens. Roots remained turgid. | 3 | Very good preservation throughout (Fig. 25) except for one tip which showed freezing damage in peripheral cells. Mostly fine textured appearance in ground-plasm and vacuoles. |
| 3 | <u>T. vulgare</u> | Roots had emerged from coleorhiza prior to treatment but displayed only slight growth afterwards. Most roots remained turgid. | 3 | Most roots showed good fixation (Fig. 26). A few displayed a small amount of freezing in places. |

Roots in contact with 20% glycerol for 3 days followed by 25% for 3 days

| Trial | Species | Effect on exposure to glycerol | Number freeze-etched | Observations freeze-fixation |
|-------|-------------------|--|----------------------|--|
| 4 | <u>Z. mays</u> | Shoots grew 1/4"-1" and developed adventitious roots at base. The primary roots grew 1-3 mm. Some roots in contact with glycerol darkened and became soft, others remained turgid. | 4 | Some batches showed fairly good preservation (Fig. 32) while others displayed various effects of freezing (Fig. 34). Results better than in trial 1. |
| 5 | <u>T. vulgare</u> | Only slight growth of root detected. Roots remained turgid. | 2 | Good preservation throughout. Some effect of freezing towards periphery of a few specimens. |
| 6 | <u>A. sativa</u> | Roots remained turgid but did not emerge from coleorhiza. The coleorhiza showed a slight amount of growth. | 5 | Very good preservation in most cells and root tips (Fig. 31). A few showed slight freezing damage in peripheral cells (Fig. 30). |

Roots in contact with 20% glycerol for 5 days

| | | | | |
|---|-------------------|---------------------|---|---|
| 7 | <u>T. vulgare</u> | Similar to Trial 2. | 2 | A few root tips showed good preservation (Fig. 29), otherwise fair to poor. |
| 8 | <u>A. sativa</u> | Similar to Trial 3. | 2 | Reasonably good to fair preservation. Poor in peripheral cells of some root tips. |

* 20 - 30 seedlings were used per trial.

TABLE 6
 PREFIXATION WITH GLUTARALDEHYDE PRIOR TO FREEZE FIXATION

| Trial | Species | Source of root tips | Portion of root tip used | % Conc. glutaraldehyde | Duration of fixation (hours) | Number freeze-etched |
|-------|-------------------------|------------------------------------|---|------------------------|------------------------------|----------------------|
| 1 | <u>Mentha citrata</u> | Cutting in $\frac{1}{2}$ Hoaglands | Whole tips | 6 | 3 | 1 |
| 2 | <u>M. citrata</u> | as above | as above | 6 | 2 | 1 |
| 3 | <u>Lonicera nitida</u> | Plants potted in soil | Whole tips and median longitudinal slices | 6 | 3 | 2 |
| 4 | <u>L. nitida</u> | as above | Whole tips | 6 | 1 $\frac{1}{2}$ | 2 |
| 5 | <u>L. nitida</u> | as above | as above | 6 | 2 | 1 |
| 6 | <u>Zea mays</u> | Germinated seeds | Medial longitudinal slices | 6 | 1 $\frac{1}{2}$ | 1 |
| 7 | <u>Z. mays</u> | as above | as above | 6 | 2 | 5 |
| 8 | <u>Z. mays</u> | as above | 1mm ³ of promeristem | 6 | 2 | 4 |
| 9 | <u>Z. mays</u> | as above | as above | 3 | 2 | 1 |
| 10 | <u>Avena sativa</u> | Germinated seeds | Whole tips and median longitudinal slices | 6 | 2 | 1 |
| 11 | <u>Triticum vulgare</u> | Germinated seeds | Whole tips | 6 | 2 | 1 |

conditions necessary for prefixation are not critical.

While preservation of cellular structure in prefixed root tips of Avena, Triticum and Zea is comparable to that of well frozen living roots (of Figs. 36 & 26) - indicating the validity of prefixation (Park & Branton, 1967; Branton & Park, 1967; Matile & Moor, 1968) - differences are nevertheless noted. Some organelles failed to show the smooth outlines of those in frozen living roots (Figs. 38, 40, 42). Nuclei occasionally displayed envelopes with a wavy outline in cross fracture (Fig. 41) or a deeply indented surface in face view; although this condition is often seen in sectioned material it is uncommon in interphase nuclei of well preserved frozen-etched living cells. The endoplasmic reticulum is sometimes angular in form, while vacuoles (Fig. 40), and plastids (Fig. 41) occasionally show small surface indentations or irregular extensions (Fig. 42). The appearance of these structures suggests that disturbances have occurred during pretreatment, probably as a result of shrinkage. However, not all components are affected suggesting that organelles react differently to fixation depending on the structure and its physiological activity at the time of killing.

3.4 Discussion and Conclusions

These experiments show that root growth of Avena sativa, Lonicera nitida, Mentha citrata, Triticum vulgare and Zea mays is inhibited by glycerol. The effects of exposure are mainly irreversible and confirm previous observations (Moor, 1964, 1966b). Moor found that roots grown in the normal way and then transferred to glycerol would not absorb

sufficient anti-freeze agent, irrespective of whether complete roots were used or sections. Even when concentrations of glycerol were gradually increased, an irreversible plasmolysis occurred beyond a certain level. Recent studies by Richter (1968a & b), however, have shown that the epidermis of Campanula and certain other plants will absorb glycerol without irreversible effects provided the glycerination is done very gradually.

The response to growth in glycerol of plants examined in the present study seems to depend on: the presence of established roots, the area exposed for transpiration, reserve material, and the condition of the plant. Rooted plants placed in glycerol reacted less severely than unrooted material, indicating that ^{the} presence of roots reduces the effects of the anti-freeze agent. Presumably some water is absorbed from the glycerol solution which minimises the loss caused by transpiration. As Mentha is a plant of wet habitats, this may explain its low resistance to water loss on exposure to glycerol; this is in contrast to Lonicera. Bulbs and seeds offer advantages over cuttings in that foliar transpiration is absent. The reserve material of bulbs and seeds may also help sustain growth after plants are placed in glycerol. The state of growth of the plant is probably also important if material is to be grown in glycerol.

For successful freeze-etching, organs should be initiated in glycerol so that their growth becomes adjusted to the presence of the anti-freeze agent (Moor, 1964, 1966b; Branton & Moor, 1964). However, the present experiments show that the radicles of seeds germinated in water can become adjusted to glycerol if transferred at an early stage, provided the roots are adequately aerated. Northcote and Lewis (1968)

have independently obtained good results with pea roots using similar methods. The potentialities of very gradual infiltration with glycerol (e.g. Richter, 1968a & b) have not been fully explored in the present work.

Not all roots of seedlings appear to be equally suitable for growth in glycerol. The large primary root of Zea, in contrast to the small ones of Avena and Triticum, absorbs glycerol with difficulty. Roots within the coleorhiza become better impregnated with glycerol than those at later stages of development.

Twenty percent glycerol gives good freeze fixation of viable root tips, confirming other studies (Branton & Moor, 1964; Northcote & Lewis, 1968). However, for some roots, e.g. Zea, concentrations up to 25% may be necessary. In addition, the time required to absorb glycerol apparently varies in different roots and among organisms. Whereas established roots absorb glycerol within a few hours, a longer period is required if they are to become fully adjusted to glycerol and resume growth. This is shown by Avena and Triticum which require about 7 days incubation in the anti-freeze agent to give good freeze-fixation. Compared with root tips, fungi and bacteria readily metabolize glycerol within a few hours and from solutions of low concentration. Therefore, in their pretreatment for freeze-etching, these organisms present few difficulties compared with tissues of higher plants (e.g. Moor & Mühlethaler, 1963; Hess, 1968).

Chemical fixation prior to freeze fixation gives reliable freezing of root tips. It offers an alternative approach for plants difficult to grow in glycerol. The uniform freezing of prefixed material is

apparently due to (a) use of a high-concentration of glycerol (30%), and (b) easy penetration of glycerol as a result of the lowered permeability conferred by chemical fixation. Although prefixation gives results comparable to those in frozen viable material (Park & Branton, 1967; Matile, 1968; Matile & Moor, 1968; Nanninga, 1968) the possibility of artifacts caused by combined chemical/physical fixation must nevertheless be considered. The present root tips show slight alteration, apparently caused through shrinkage, in the morphology of some organelles. This is probably due to dehydration by glycerol on death of the cell before the structures have become fully stabilized by GA. To avoid shrinkage glycerination should probably follow prefixation (Park & Branton, 1967; Matile, 1968; Matile & Moor, 1968). Although simultaneous prefixation and glycerination sometimes causes slight damage, the degree of disturbance is small comparable to that seen in so called "well preserved" sectioned material.

Summary

Results of preliminary experiments on pretreatment of root tips of Lonicera, Mentha, Allium, Zea, Avena and Triticum for freeze-etching are outlined. Attempts to initiate roots in solutions of glycerol were mainly unsuccessful. Established roots of Lonicera, Mentha and Allium transferred to glycerol of different concentrations and for various durations failed to give satisfactory freeze fixation. Excised roots transferred to glycerol also gave poor results.

Successful freeze-fixation of viable roots was obtained using seedlings of Avena, Triticum and Zea. The seeds were germinated 12 to 24 hours in contact with water, then placed on top of tubes of filter

paper impregnated with glycerol; this allowed aeration of the roots. Most roots grew slightly on exposure to glycerol and remained turgid. Avena and Triticum gave good freeze-fixation after (a) 7 days in contact with 20% glycerol, and (b) in 20% glycerol for 3 days followed by 25% glycerol for 3 days. Good preservation was also obtained with Zea but results were more variable than in Avena and Triticum.

Root tips prefixed in 3% and 6% phosphate buffered GA in 30% glycerol gave consistent freeze-fixation in all species. Results were in general comparable to those obtained from frozen living roots.

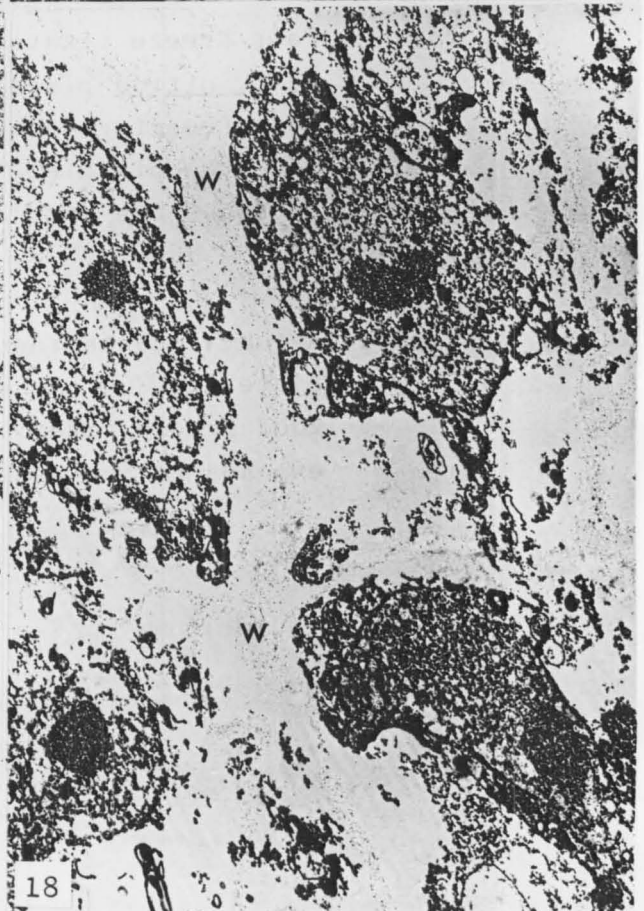
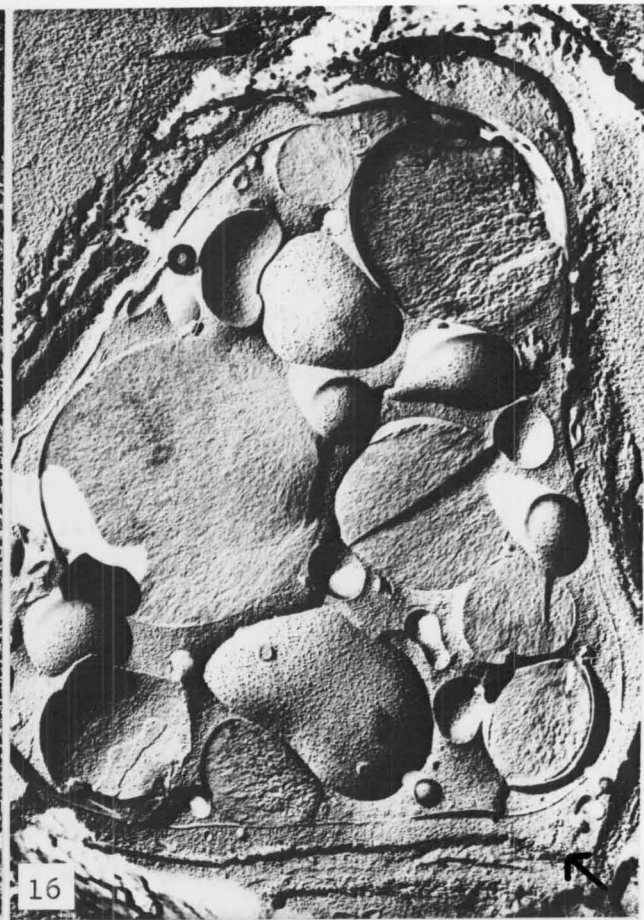
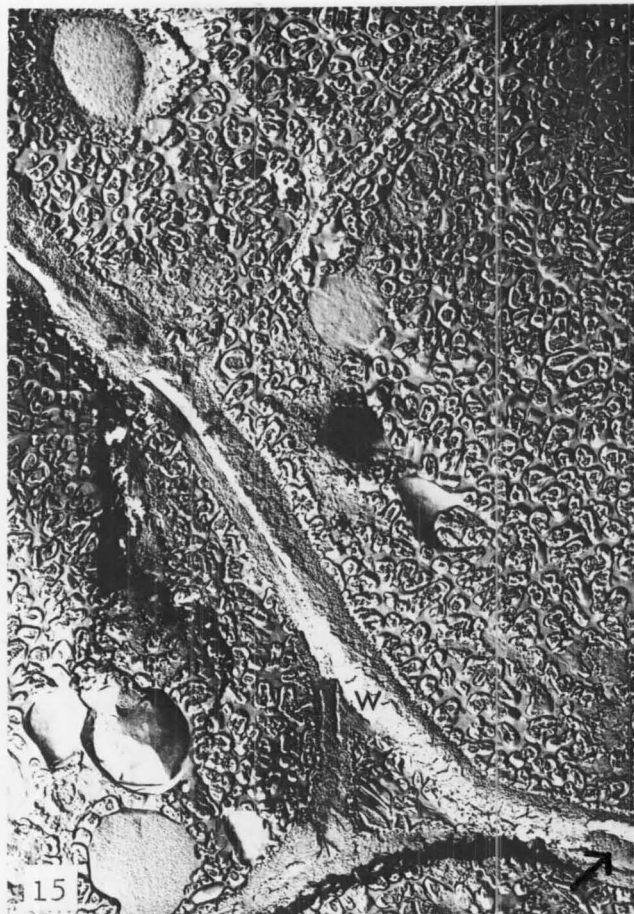
Fig. 15 Allium cepa root tip initiated in 15% glycerol.

Cell walls and a few organelles are visible otherwise the tissue exhibits considerable freezing damage (x 9,400).

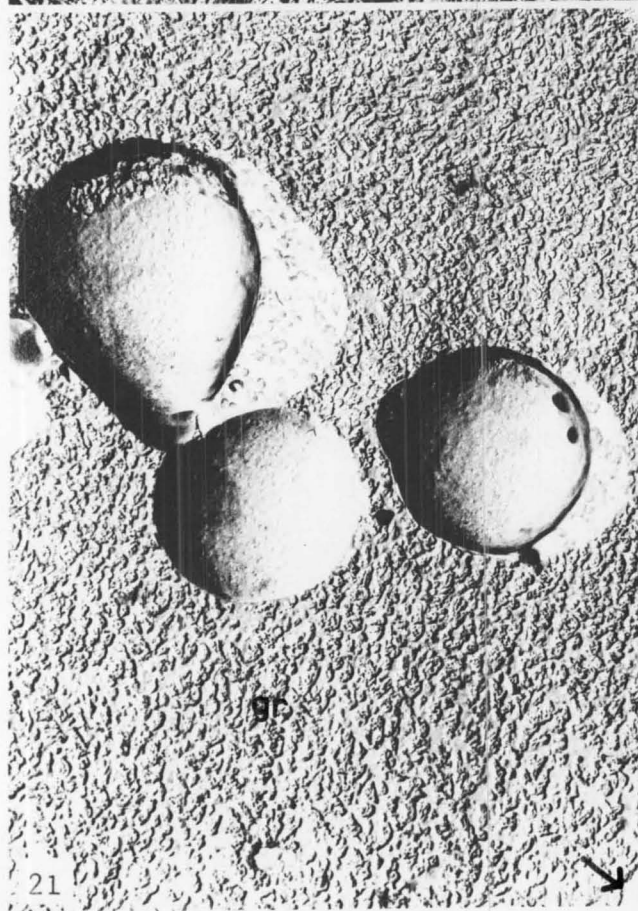
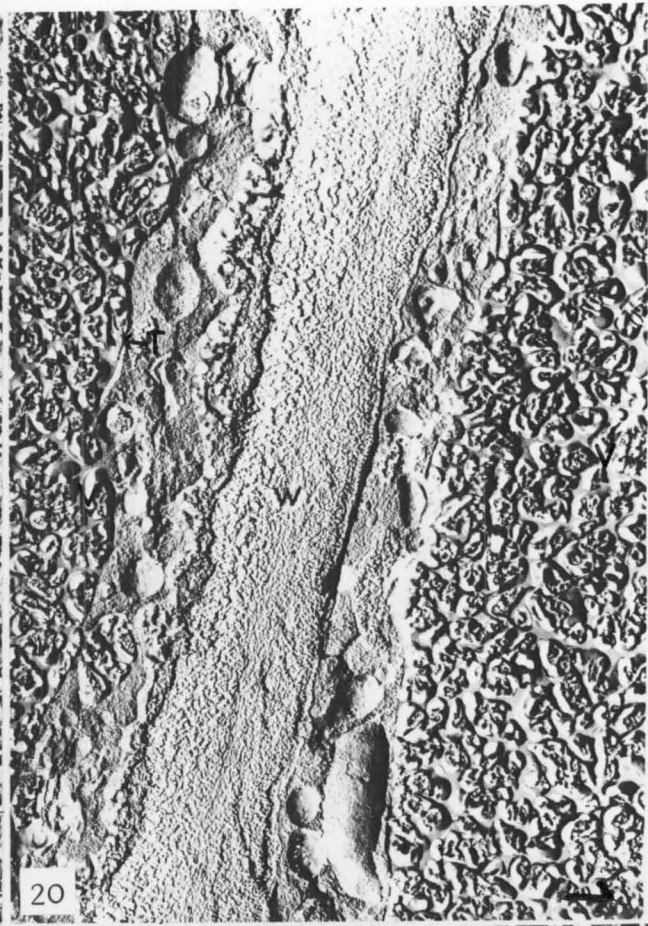
Fig. 16 Cross fracture of a fungal contaminant growing on roots of Mentha citrata placed in 15% glycerol for several weeks. The protoplast shows good freeze fixation compared with roots grown under these conditions (x 15,800).

Fig. 17 Cells from M. citrata root tips placed in 15% glycerol for several weeks and prepared for ultra microtomy (KMnO_4 fixation). Some cellular organisation is still evident after long exposure to glycerol (x 6,000).

Fig. 18 Degeneration of cell contents and walls in root tip cells of Lonicera nitida placed in 15% glycerol for several weeks. Material prepared for thin sectioning and fixed in KMnO_4 (x 10,000 appr.).

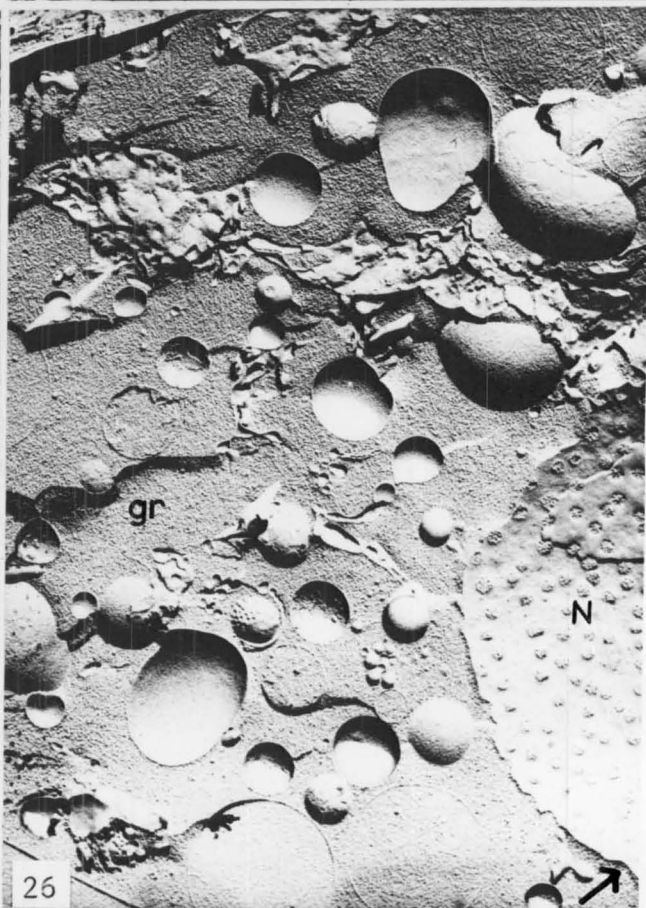
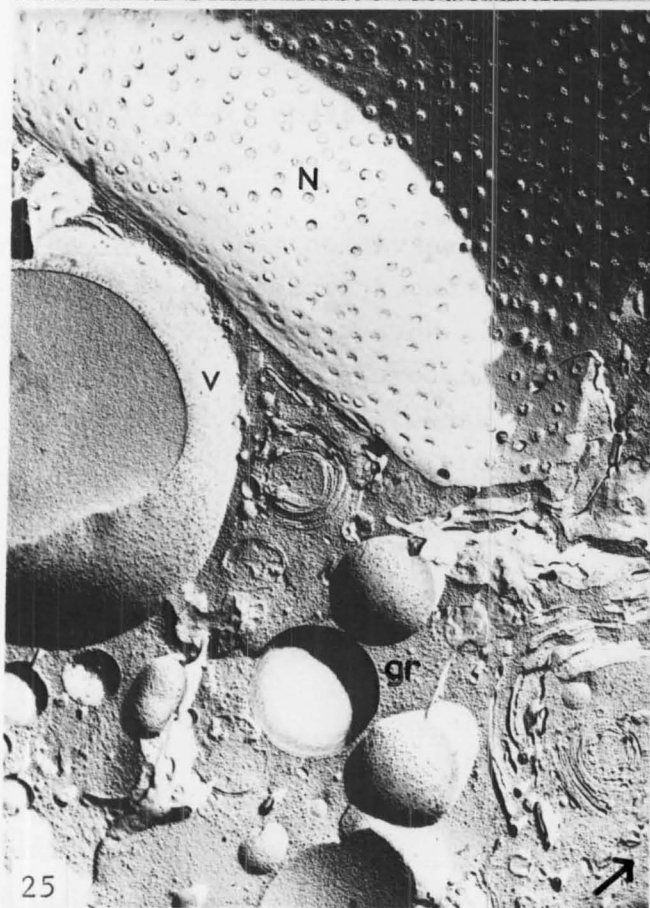
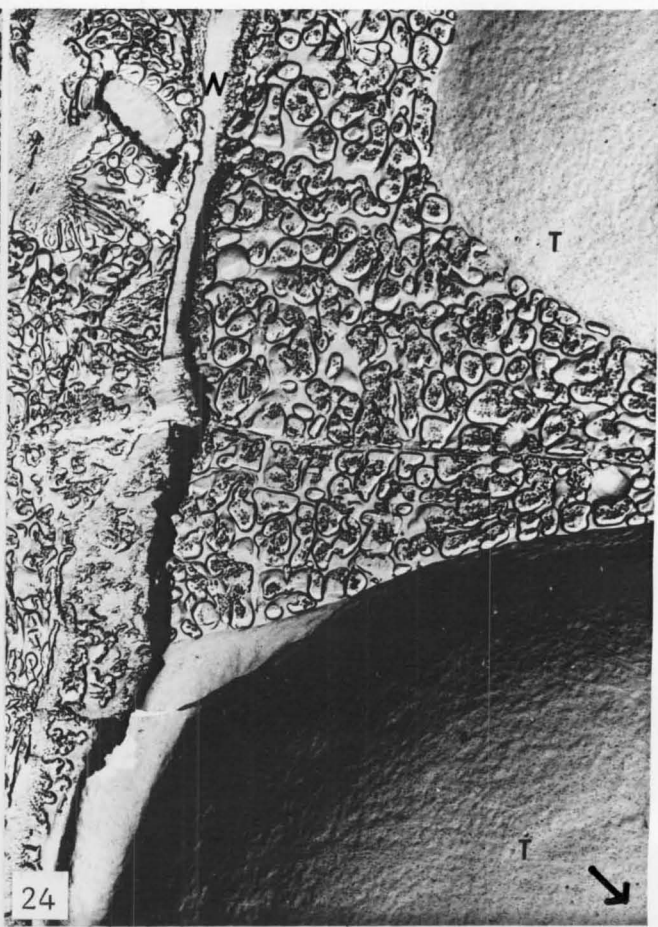
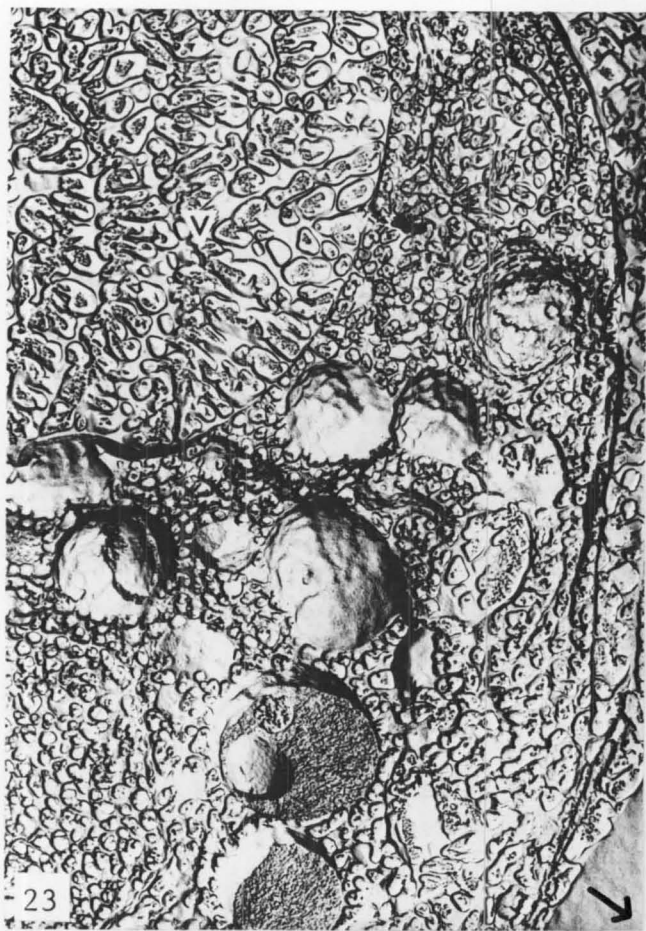


- Fig.19 Root tip of M. citrata placed in 20% glycerol overnight showing extensive freezing damage throughout the tissue (x 9,400).
- Fig.20 Portion of two root tip cells of M. citrata exposed to 15% glycerol for 3 days. The peripheral cytoplasm on each side of the cell wall shows good preservation but extensive freezing damage occurs in the vacuoles (x 33,000).
- Fig.21 Fair freeze fixation in portion of a root tip cell of L. nitida placed in 20% glycerol overnight. Some freezing damage is evident in the ground plasm. Note the platelet-like structures on the exposed faces of the membranes (x 38,500).
- Fig.22 A fungal contaminant growing on roots of M. citrata placed in 20% glycerol overnight. The cell shows good freeze fixation compared with root tips exposed to the same conditions (cf. Fig.19) (x 36,300).



Figures 23 - 34 Freeze-etch living root tip tissue.

- Fig. 23 Portion of a cell from an excised root of M. citrata placed in 20% glycerol for 6 hours. Extensive freezing damage is present but many organelles remain visible. Structures containing a high concentration of water, as in the large vacuole (V), show poor freezing in comparison with organelles of a lower water content (bottom) (x 9,400).
- Fig. 24 Cortical cell of M. citrata from the above material showing tonoplasts (T) in face view surrounded by badly frozen cytoplasm. Note the slightly dimpled appearance of the membrane. This is also apparent in Fig. 23 and is characteristic of poorly frozen cells (x 22,800).
- Fig. 25 Good freeze-fixation in meristem cell of Avena sativa grown in 20% glycerol for 7 days. Note the fine textured appearance of the ground plasm and contents of vacuole (V) (x 15,800).
- Fig. 26 Portion of a meristem cell of Triticum vulgare grown in 20% glycerol for 7 days showing good preservation of cellular detail. (x 12,000).



- Fig. 27 Zea mays grown in 20% glycerol for 7 days showing good freeze fixation. The specimen is weakly shadowed (x 27,600).
- Fig. 28 Portion of a cell of Zea grown in 20% glycerol for 7 days showing fair preservation. The specimen is weakly shadowed (x 15,800).
- Fig. 29 T. vulgare grown in 20% glycerol for 5 days illustrating good freeze fixation (x 91,000).
- Fig. 30 An example of fair preservation found in A. sativa grown in 20% glycerol for 3 days followed by 25% glycerol for 3 days. Note the effect of freezing in the vacuoles and in some areas of immediate groundplasm where a vacuole appears to have ruptured (arrow) (x 33,000).

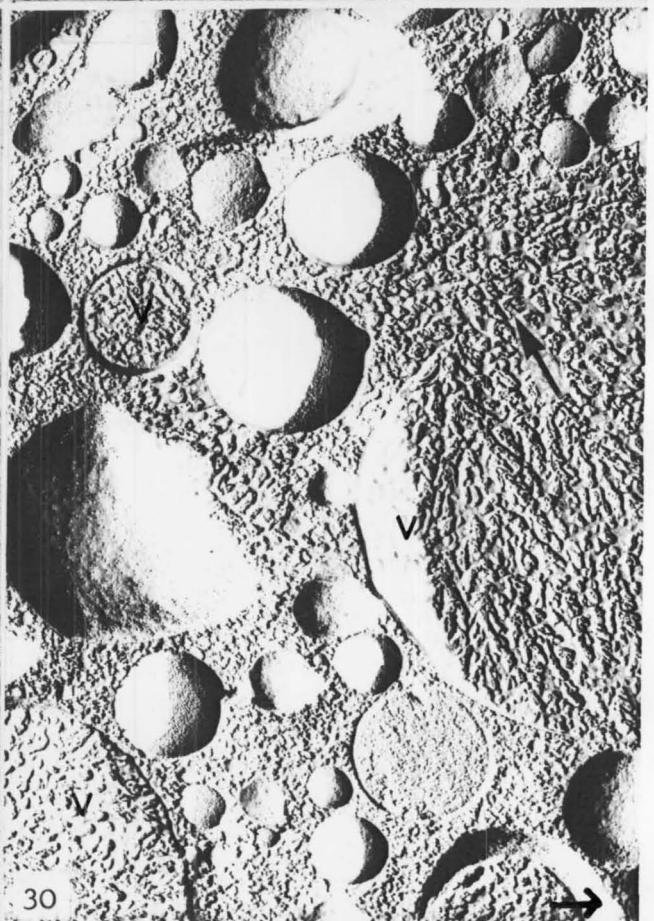
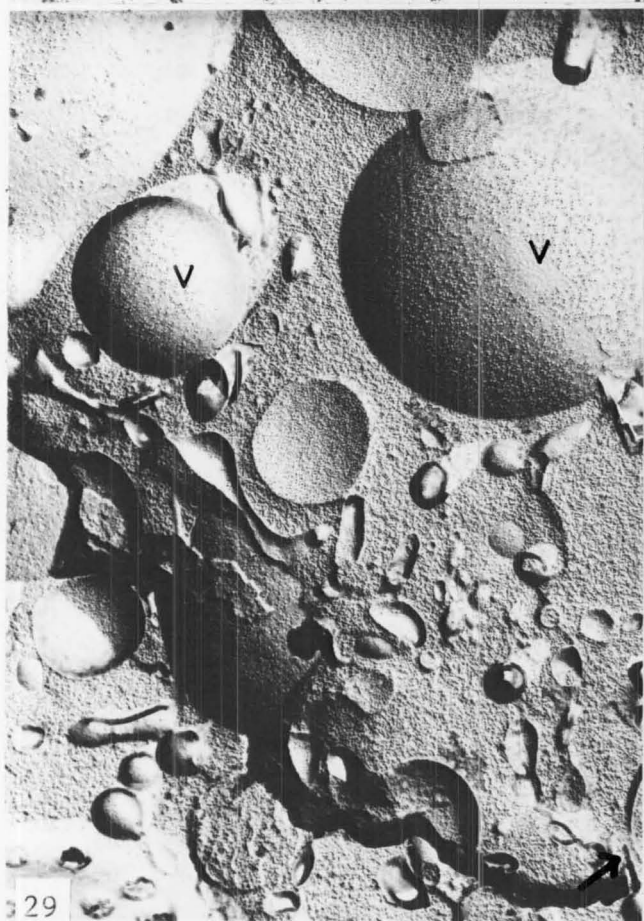
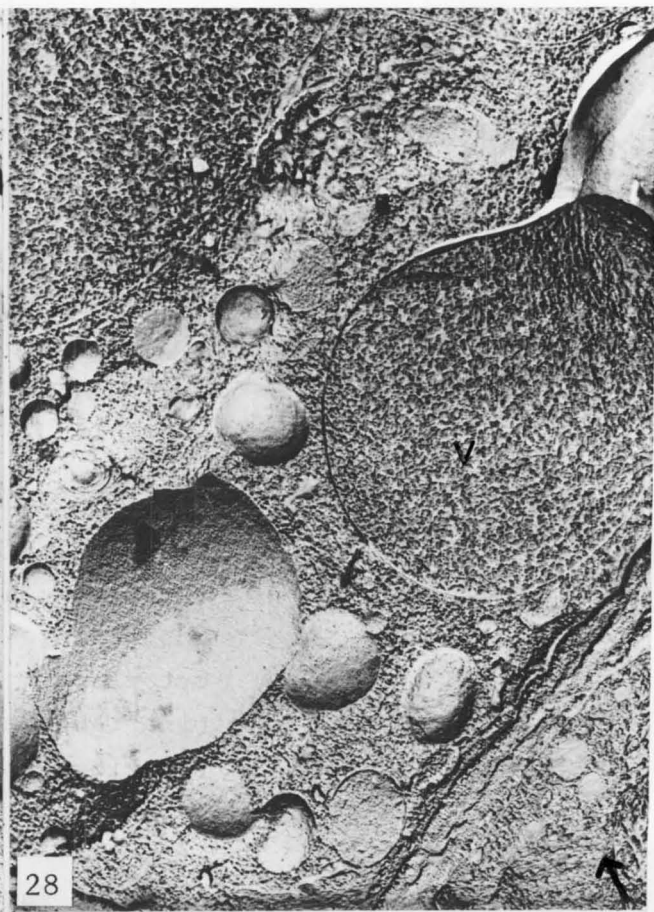
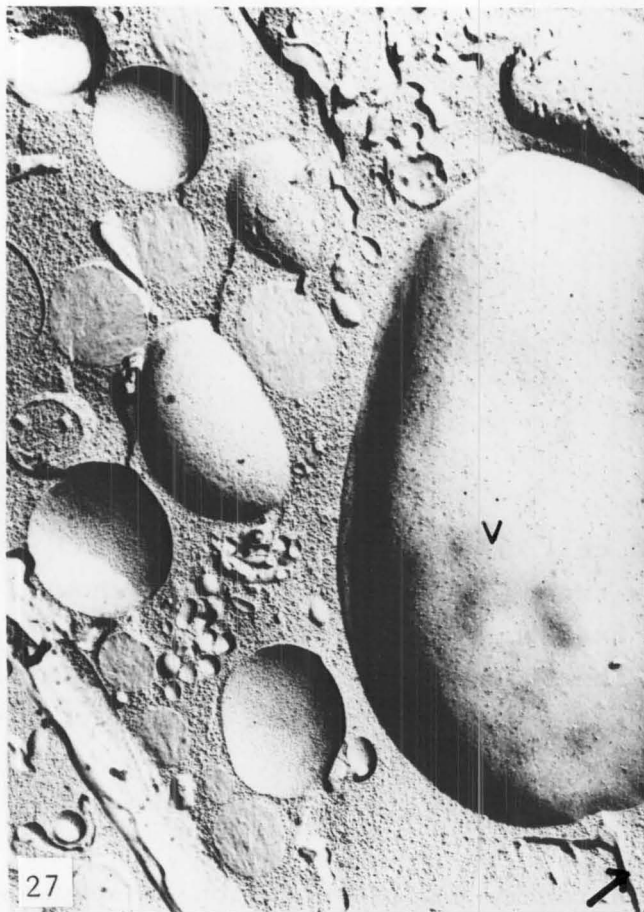
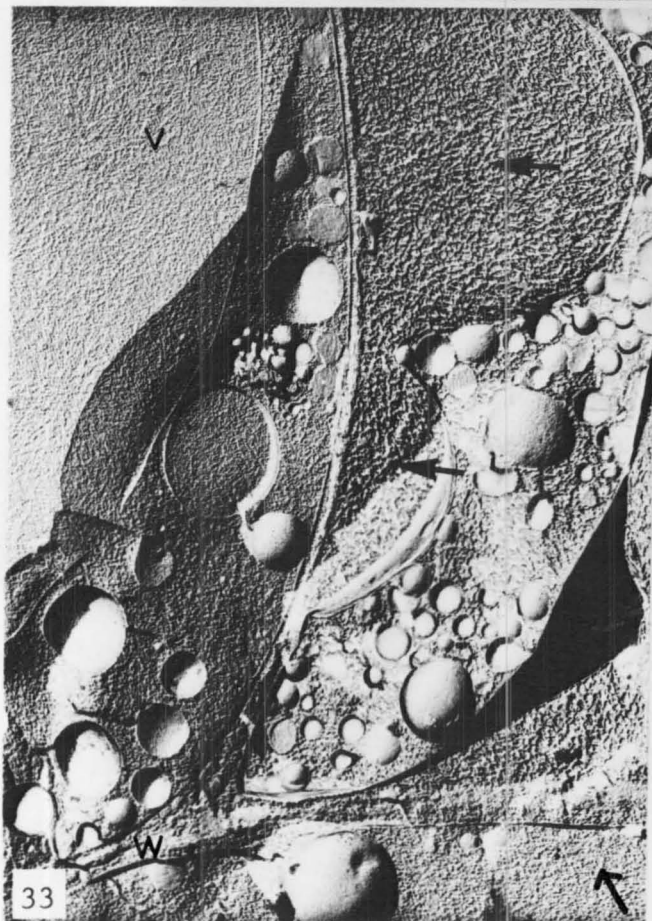
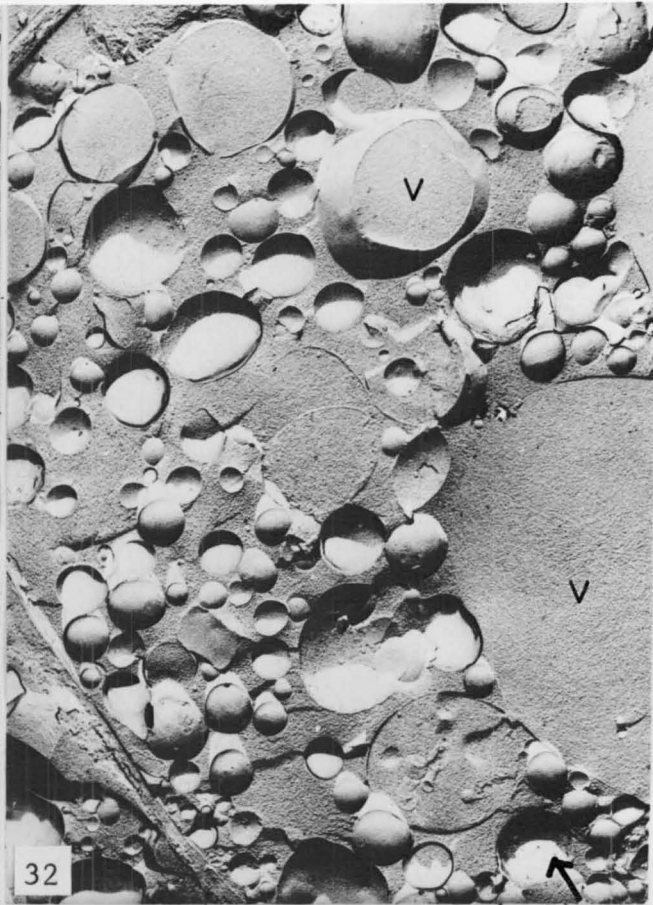


Fig. 31 Portion of a cell of A. sativa grown under the same conditions as those cited for Fig. 30 but showing good freeze-fixation in the vacuole and adjacent cytoplasm. The contents of the vacuole display a fine texture and a slightly radiate pattern of freezing. (x 15,800).

Figs. 32 and 34 Variation in the preservation of specimens of Z. mays roots grown in 20% glycerol for 3 days followed by 25% for 3 days. Fig. 32 shows good freeze-fixation; that in Fig. 34 is poor. (Fig. 32, x 7,200; Fig. 33, x 13,500).

Fig. 33 An example of the variation in preservation within a specimen. The cell on the left shows good freeze-fixation but the other is poorly preserved with a large plasmolysed area (arrows) (x 12,000).



Figures 35 - 44 Root tips prefixed in glutaraldehyde/prior to freeze-fixation.
glycerol

Fig. 35 Cortical cell of L. nitida with good preservation of contents. The specimen is weakly shadowed. (x 9,400).

Fig. 36 General preservation in tissue of Z. mays (x 12,000).

Fig. 37 Higher magnification of Zea cytoplasm. Note how the overall preservation is similar to that in frozen viable roots (cf. Fig. 27). (x 22,800).

Fig. 38 An example from T. vulgare illustrating the irregular appearance of organelles in some prefixed material. (x 37,100)

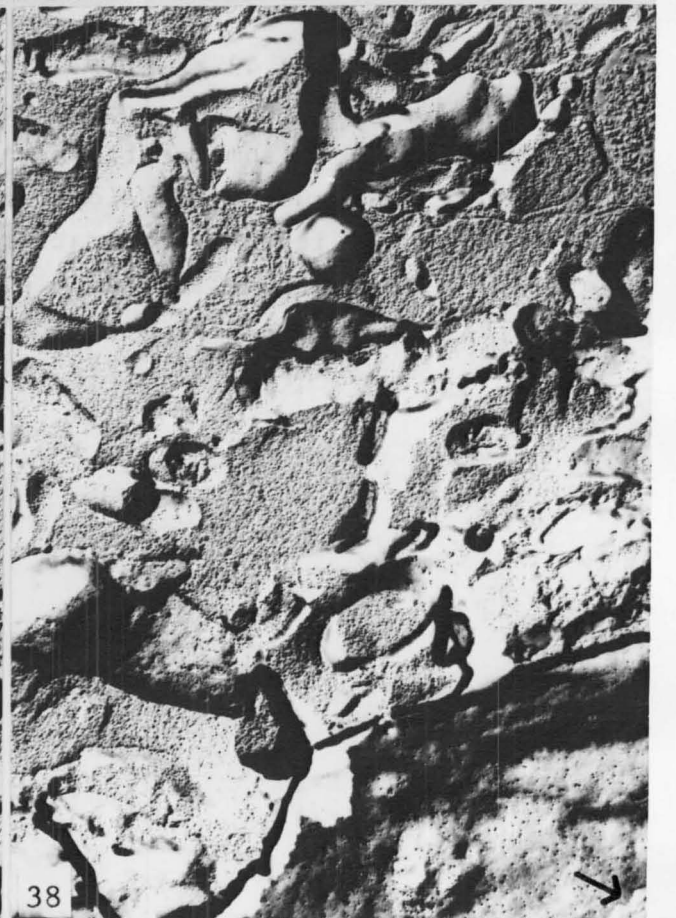
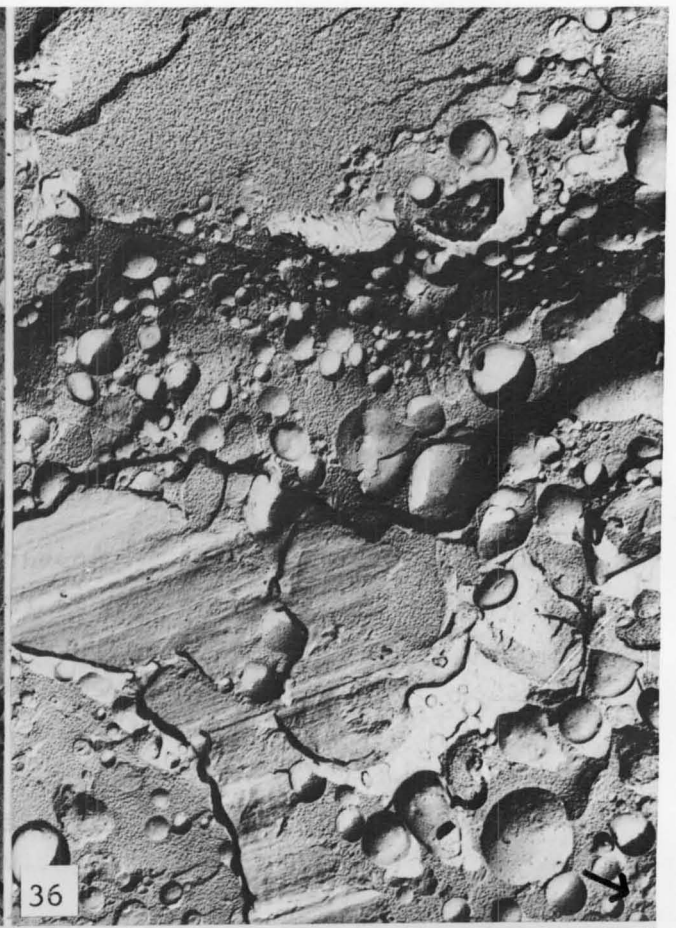


Fig. 39 T. vulgare. Appearance of the groundplasm as it is commonly seen in prefixed material. Note the clustered radiate pattern of freezing interspersed with non-etched areas. (x 55,000).

Fig. 40 A cell of L. nitida showing portion of a vacuole (V) with a slightly indented surface (x 53,600).

Figs. 41 and 42 Cells of Z. mayi showing the rather irregular appearance of cellular components found in some prefixed material. Fig. 41 shows a nuclear envelope (arrows) with a wavy outline and irregularly fractured membrane faces in some organelles (double arrow). Fig. 42 depicts an irregularly shaped organelle (arrow) probably a vacuole. (Fig. 41, x 15,800; Fig. 42, x 23,100).

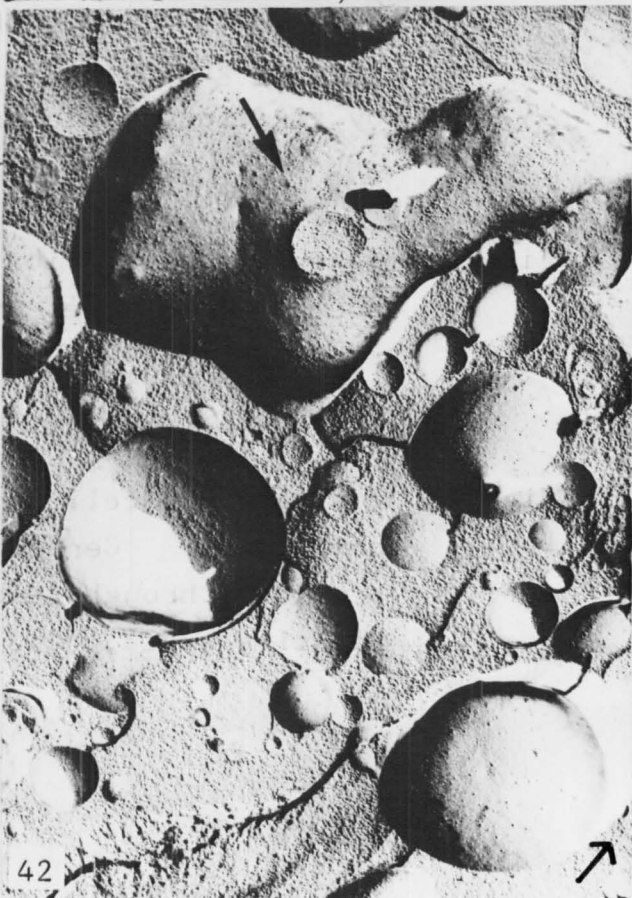
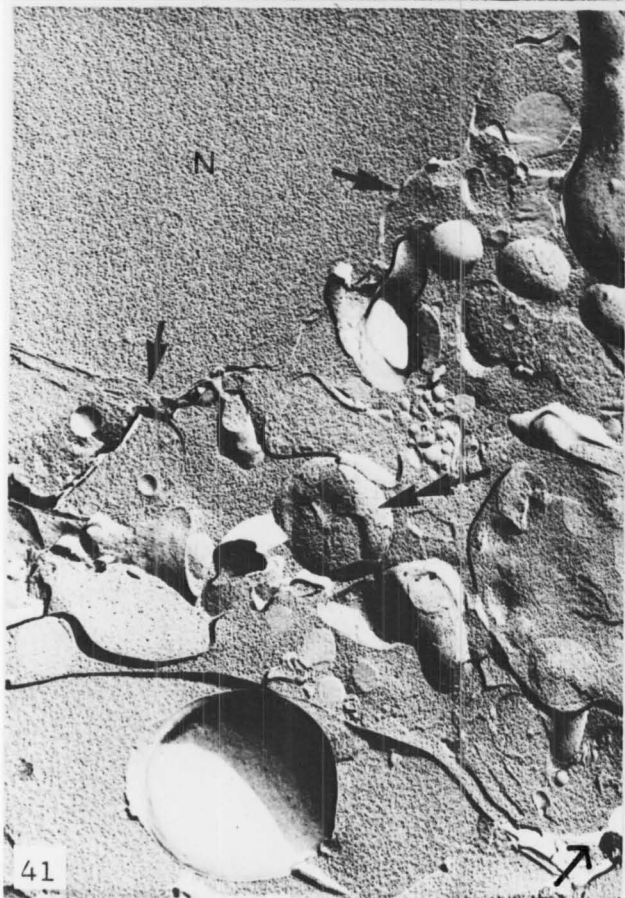
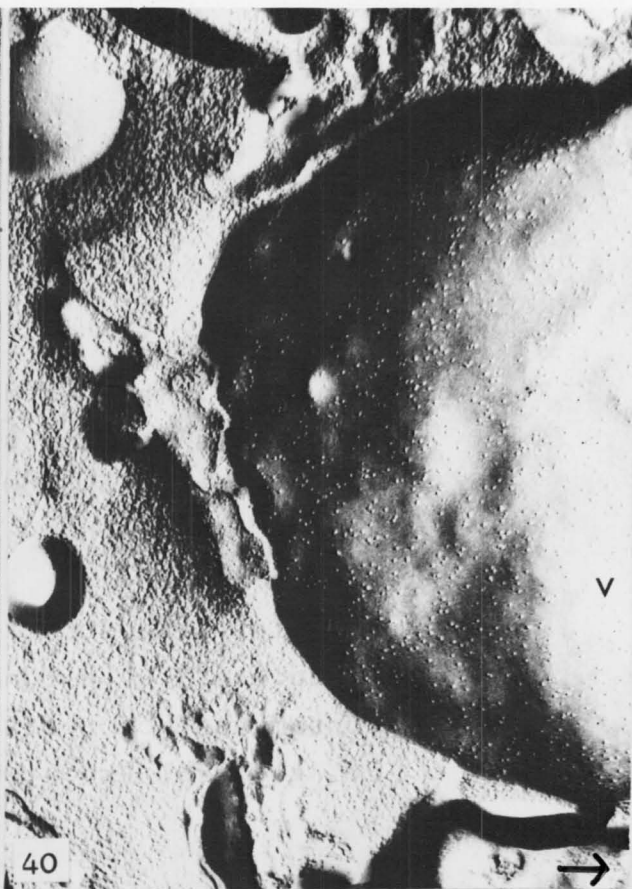


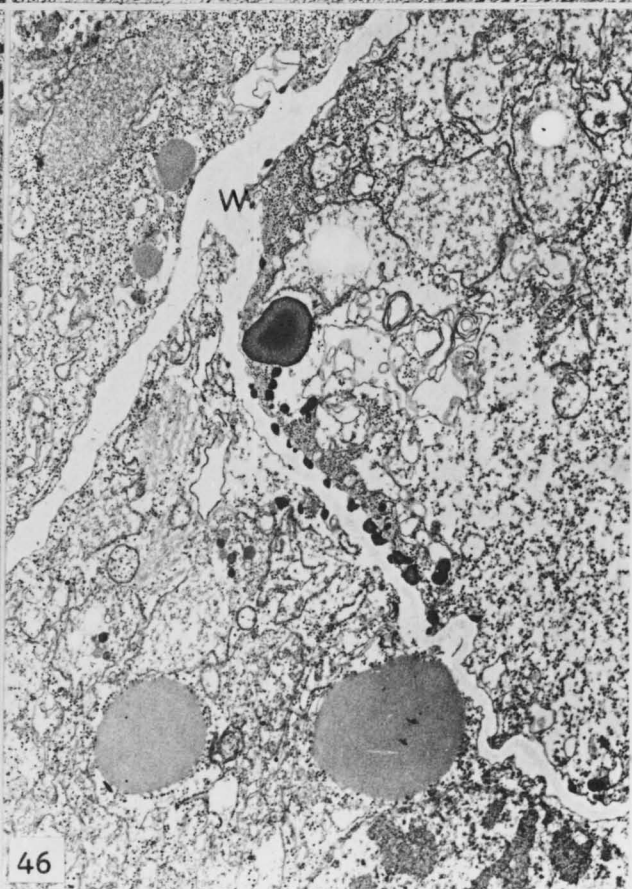
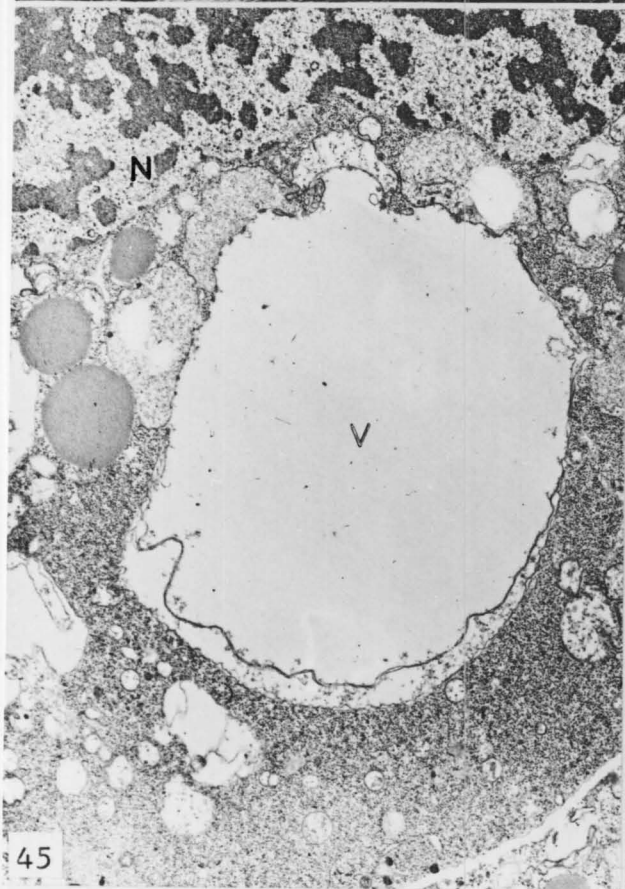
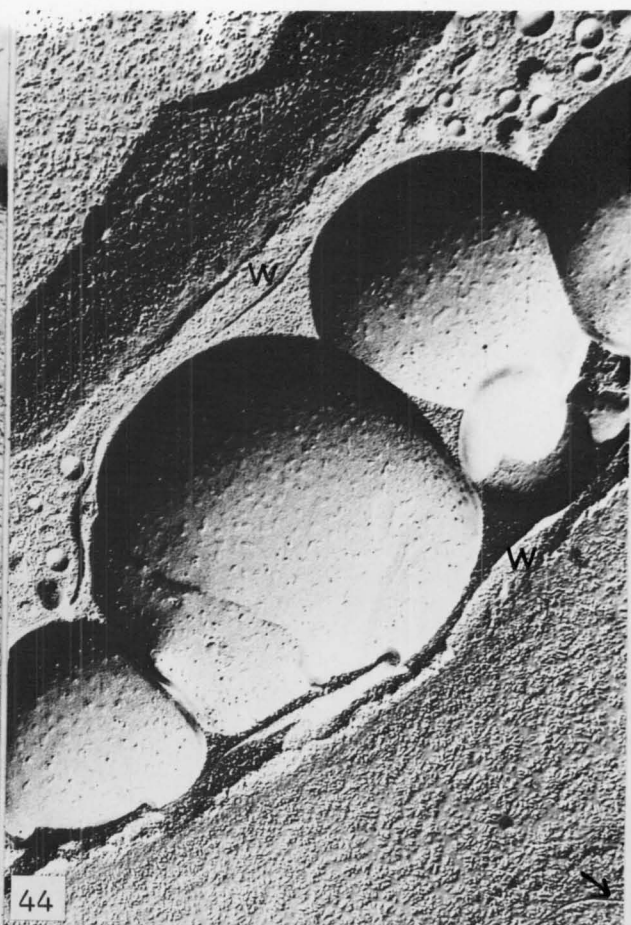
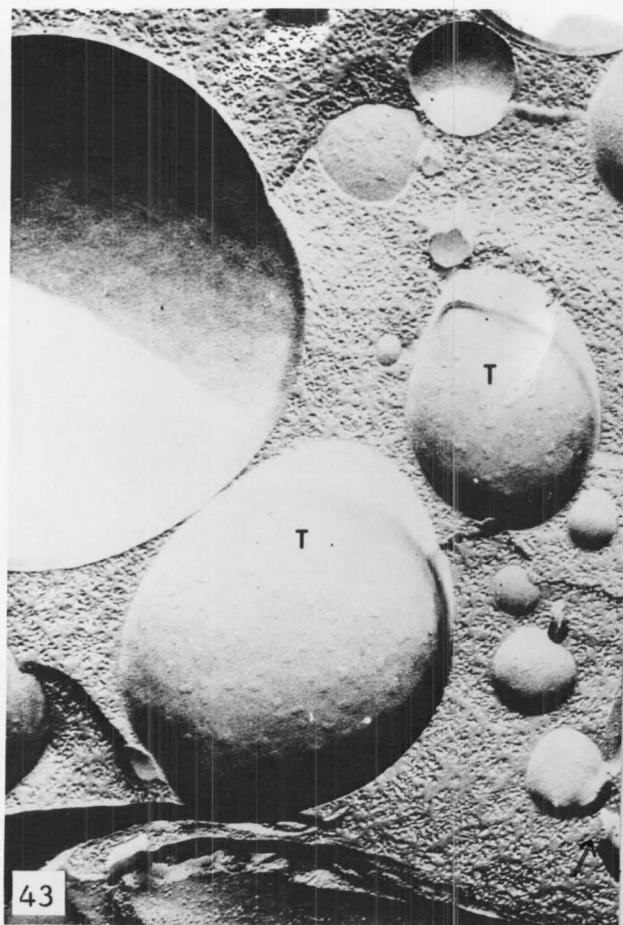
Fig. 43 Vacuoles in prefixed cells of L. nitida showing tonoplasts bearing slightly raised platelet-like structures (x 54,600).

Fig. 44 Platelet-like structures on the membrane of a vacuole (?) of a fungal hypha growing as a contaminant on roots placed for several weeks in 20% glycerol (x 84,000).

Figures 45 - 46 Root tip cells of Triticum vulgare grown in 20% glycerol for 7 days followed by fixation in glutaraldehyde/OsO₄.

Fig. 45 General preservation. Most organelles show a normal appearance; the cytoplasm contains abundant ribosomes (x 19,800).

Fig. 46 Portion of a cell showing detailed preservation of components. Certain organelles show irregularities, probably through reaction between glycerol and the fixative (x 23,100).



4. AN EVALUATION OF THE FORM OF VACUOLES IN ULTRATHIN SECTIONS AND FREEZE-ETCH REPLICAS

4.1 Introduction

A feature typical of many vacuoles in thin sections is their irregular appearance. Vacuoles of irregular form have been illustrated since thin sections were first prepared for electron microscopy. They are shown in a wide range of organisms: algae (Ueda, 1966; Pickett-Heaps, 1967), fungi (Vitols *et al.*, 1961; Hawker & Hendy, 1963; Peyton & Brown, 1963), bryophytes (Manton, 1961, 1962; Diers, 1965) and pteridophytes (Michaux, 1968). In seed plants, irregularly shaped vacuoles have been described in various cells and tissues at different stages of development. Examples may be seen in papers on: embryology (Jensen, 1963, 1965; Maruyama, 1965), differentiating xylem (Cronshaw & Bouck, 1965) and phloem elements (Bouck & Cronshaw, 1965; Esau, 1965; Evert & Murmanis, 1965; Evert *et al.*, 1966), apices of the root (Buvat & Mousseau, 1960; Porter & Machado, 1960; Bouck, 1963; Barton, 1965; Hřsel, 1966; Buvat, 1968b; Mesquita, 1968) and shoot (Buvat, 1958; Lance, 1958; Hohl, 1960; Marinos, 1963a & b; Nougarede, 1963b; Bowes, 1965a & b; Hřsel, 1965a; Gifford & Stewart, 1967), and in a diversity of other tissues (e.g. Sun, 1964; Wardrop & Foster, 1964; Srivastava & O'Brien, 1966). Irregular vacuoles occur in tissues fixed in osmium tetroxide, aqueous and buffered $KMnO_4$, GA/OsO_4 and in other fixative combinations. Many of these irregularly shaped vacuoles have an angular appearance, often with fine projections extending into the surrounding cytoplasm. This is particularly common in cells fixed in $KMnO_4$.

Several interpretations have been advanced to account for the presence of vacuoles of irregular form in thin sections. Some believe

that the narrow extensions represent continuity with ER. This was suggested by Buvat (1957, 1958) to explain the origin of vacuoles from rough elements of ER; later it was extended to include smooth membrane profiles (Buvat & Mousseau, 1960; Poux, 1961, 1962). These investigators still accept the concept (Buvat, 1960, 1961, 1963a). It has been adopted also by other workers for a variety of plant material (e.g. Hršel, 1961b, 1965a & b, 1966; Bowes, 1965b; Pickett-Heaps, 1967).

Other workers consider that the irregular form of vacuoles represents a stage in their development (Whaley et al., 1960a; Barton, 1965; Gifford & Stewart, 1967) and that ER is not involved in their origin (Mühlethaler, 1960; Manton, 1962). The irregular vacuoles found in meristem cells are sometimes regarded as "amoeboid" stages of growth. In Anthoceros, Manton was unable to explain satisfactorily the occurrence of tentacle-like outgrowths in her preparations. As the tonoplast is a living membrane capable of independent growth, she suggested that once vacuoles are formed the tonoplast could grow rapidly so that closely apposed extensions developed. Other investigators adopt similar views but suggest also that the extensions are formed in anticipation of vacuolar expansion; the vacuole then enlarges by distending the space within the extensions (Barton, 1965). Gifford and Stewart (1967) suggest that angular vacuoles may represent active stages of enlargement and spherical vacuoles a less active condition. This concept recalls the light microscope studies of Bailey (1930) and Zirkle (1932). In root tip cells of various species, Zirkle found that vacuoles were normally spherical but could be drawn into rods, threads and anastomosing reticula on the initiation of cyclosis.

Some investigators associate irregular vacuoles with an origin from the golgi apparatus. According to Marinos (1963a), individual cisternae in shoot apices of barley expand into young vacuoles characterized by tentacle-like protrusions. Presumably, the extensions represent unexpanded portions of the original cisterna, or outgrowths developed by the enlarging tonoplast. The origin of vacuoles from the golgi apparatus in association with ER has recently been suggested by Ueda (1966) in the alga Chlorogonium. According to his diagram, irregular young vacuoles would arise through fusion of golgi vesicles with already formed provacuolar bodies.

In contrast to the above views, my work indicates that many of the irregular vacuoles in thin sections are the result of specimen preparation. Other workers have also suggested recently that irregular vacuoles might be artifacts (Moor & Mühlethaler, 1963; Branton & Moor, 1964; Moor, 1966b; Gifford & Stewart, 1967; Hess, 1968; Matile & Moor, 1968), but little work has been devoted specifically to the problem. Whereas most cell components exhibit a fundamental morphology irrespective of the fixative used, vacuoles in contrast are often variable in shape. Their image changes according to the method of fixation, the state of development of the tissue and the condition of individual vacuoles. Observations on five species suggested that the fundamental form of vacuoles is round in section with an entire outline to the tonoplast. However, it was seldom possible to preserve all vacuoles as rounded structures. The possibility that some vacuoles of angular shape might represent stages of development could not therefore be discounted from work on sections alone.

To evaluate the form of vacuoles in thin sections, material needs to be examined by an independent technique. Although light microscope

observations of living root tips show spherical vacuoles (Zirkle, 1932), optical microscopy is unsuitable as an alternative method because it is difficult to resolve vacuoles approaching the size of mitochondria. And it is vacuoles of about this size that frequently appear irregular in thin sections. Furthermore, if living cells deep within the roots are to be studied sections must be cut and some means of enhancing the ^{contents} vacuolar/is necessary such as staining with neutral red (See Bailey, 1930; Guilliermond, 1941). The possibility of mechanical and chemical damage to the cells cannot therefore be discounted. Instead, a technique is required by means of which vacuoles can be studied at magnifications comparable to those used for thin sections. At the same time, the method must avoid chemical fixation, dehydration and embedding. The technique of freeze-etching (Steere, 1957; Moor et al., 1961; Moor & Mühlethaler, 1963; Moor, 1964, 1966b; Koehler, 1968) fulfils these requirements. Living tissues are rapidly frozen, fractured, etched and a replica made with minimal alterations to the specimen.

There are several advantages in adopting freeze-etch procedures to supplement work on vacuoles by sectioning. It is difficult to determine the overall form of vacuoles from sections except by preparing models from serial sections whereas a three-dimensional image of the structures is readily provided by freeze-etching. It is also easier to see the spatial relationship between different vacuoles of a cell in frozen-etched preparations. In sections it is often hard to determine whether adjacent vacuoles are separate or represent diverticula of a common structure. This applies particularly to situations where fixations induce vacuoles of irregular shape. Another advantage of freeze-etching is that it avoids dehydration. Vacuoles contain a high

proportion of water (see Drawert, 1955b), and because specimens prepared for electron microscopy must be thoroughly dried, we must always bear in mind the possibility that the form of the vacuole may be modified when conventional techniques of thin sectioning are used. Freeze-etching circumvents this difficulty.

In this chapter, the vacuolar images produced by "standard" fixatives are described and compared with those obtained from freeze-etching. The shape of vacuoles in thin sections and freeze-etch replicas of yeast cells (Moor, 1966b) and the hyphae of the fungus Pyrenochaeta terrestris (Hess, 1968) have been compared briefly, but similar comparisons have not been published for root tips.

4.2 Observations

4.2 1 Identification of vacuoles in thin sections and freeze-etch replicas

Vacuoles are distinguished from other organelles of the cell by their size, the structure of the bounding membrane (tonoplast) and the appearance of their contents. Apart from early stages of development, vacuoles are typically larger than most other membrane-bounded organelles.

In section, their single limiting membrane clearly distinguishes vacuoles from mitochondria, plastids and the nucleus all of which are bounded by a double membrane or envelope (Figs. 47, 52, 62, 66). Identification of small vacuoles approaching the size of spherosomes, golgi vesicles and ER vesicles is occasionally difficult because all these components are surrounded by a single membrane (Figs. 56, 58, 59, 63). Sometimes, the reaction of different fixatives with

the contents of vacuoles can aid identification. Vacuoles fixed in GA/OsO_4 , or OsO_4 , usually appear electron transparent with little precipitation of the contents (Figs. 47, 65-70). By contrast, vacuoles preserved in KMnO_4 , normally show precipitates (Figs. 51-64). The density of the precipitates varies in different species, in different tissues of a root tip and within a cell depending on the composition of the vacuoles. In actively differentiating cells, the contents are often dense (Figs. 51, 52-54, 57-60).

In freeze-etch replicas, vacuoles are exposed by fracturing the specimen. Since fracturing is a random process, various portions of vacuoles are seen. When a vacuole is cross-fractured it exhibits an appearance similar to that seen in thin section (Fig. 48). Small vacuoles are often distinguished from other cross-fractured organelles bounded by single membranes by the presence of large ice crystals within the vacuole (Figs. 73, 75, 78). The direction of shadowing and the position of shadow relative to the vacuole indicate whether vacuoles are represented by raised or hollowed-out structures (Figs. 49, 50, 71-75). The outer and inner surfaces of the tonoplast are established from these views.

4.2 2 Form of vacuoles in ultrathin sections

A. Fixation with aqueous KMnO_4 : The greatest diversity of vacuole shape is found in material preserved in aqueous KMnO_4 of the various fixatives tested (compare Figs. 51-60 with 61-70). Although the fundamental shape of vacuoles is round in section, only a small proportion exhibit an entire outline; most vacuoles have various irregularities.

This is found in all species studied irrespective of how the plants are grown. Between species some variation nevertheless occurs; for example, small vacuoles in differentiating cells of Triticum are usually less irregular than comparable vacuoles in Avena. The main biological factors which appear to influence the image of fixed vacuoles are the state of growth of the root tip and the physiological activity of individual vacuoles. Thus in actively growing roots vacuoles are usually more irregular than those in roots growing more slowly. Also, the larger the vacuole the less irregular is its outline (Fig. 53). This relationship of size and irregularity to state of activity applies to specimens fixed in either aqueous or buffered KMnO_4 , but irregularity is normally less with buffered solutions.

Figures 51-60 illustrate the typical appearance of vacuoles in cells from actively growing tissue fixed in aqueous KMnO_4 . In the promeristem the small vacuoles are irregular (Fig. 51) resembling those described elsewhere (Whaley et al., 1960a). Small irregular vacuoles also occur in actively differentiating tissues at some distance from the promeristem (Figs. 52 & 58). A few vacuoles show radiate patterns (Figs. 53 & 55) reminiscent of so called "stellate" vacuoles (Manton, 1961, 1962). Attenuated processes running into long narrow extensions are shown by other vacuoles (Figs. 53 & 54). In vacuoles where the contents are not electron dense these extensions superficially resemble ER cisternae (Fig. 55) but in no instance has

continuity been found between the tonoplast and ER. Other vacuoles occasionally show small villus-like protuberances (Fig. 57). Vacuoles of irregular shape, similar to those described above, are illustrated in many papers dealing with plant tissues fixed in aqueous KMnO_4 .

The irregular form of vacuoles is sometimes due to rupture of the organelle (Figs. 55 & 58). Rupture is particularly evident in vacuoles which contain dense precipitates after fixation with KMnO_4 . The groundplasm near such vacuoles becomes darkened through release of vacuolar precipitates (Fig. 58). Other vacuoles contain portions of cytoplasm in the form of small vesicles of groundplasm bounded by a membrane (Fig. 60), or larger masses containing various organelles (Fig. 58). Although vacuoles are known to engulf small portions of cytoplasm to form inclusion bodies (Gifford & Stewart, 1968; Matile & Moor, 1968; Chapter 7), the high frequency of these inclusions in poorly preserved material suggests that many have been artificially produced on collapse of the vacuoles during specimen preparation.

Vacuoles of irregular form are often closely associated. A common condition is that where an attenuated process of one vacuole protrudes into a neighbouring vacuole in the form of a delta-like indentation (Fig. 54). With some vacuoles, the protuberances extend towards each other (Fig. 59). Where a small vacuole abuts on to a larger one there is frequently a local invagination of the small vacuole by the larger. This small vacuole may assume a

crescentic form (Fig. 56) resembling that of certain peripheral vacuoles in cultured cells of Picea glauca (White, 1968).

Other components of the cell are occasionally associated with vacuoles of irregular shape. Organelles, particularly mitochondria and plastids, may exhibit a radiate alignment towards the vacuole. The ER may follow the contour of the vacuole (Fig. 60). However, in the immediate environs of large irregular vacuoles, organelles are sometimes sparse (Fig. 54).

- B. Fixation with buffered $KMnO_4$: Vacuoles preserved in buffered $KMnO_4$ usually exhibit a different appearance from those fixed in aqueous permanganate; a greater proportion of vacuoles are rounded with entire tonoplasts (Figs. 61 & 62). Both large and small vacuoles in a cell may be rounded (Fig. 62). While irregularly shaped vacuoles still occur (Fig. 64), the degree of irregularity is normally less than in vacuoles fixed in aqueous permanganate. Long profile-like extensions are seldom found and vacuoles with protrusions extending towards each other are infrequently seen. Vacuoles in promeristem cells fixed in buffered solution are slightly less irregular (Fig. 63) than those fixed with aqueous $KMnO_4$.
- C. Fixation with Glutaraldehyde/osmium tetroxide: Difficulty was sometimes experienced in handling this fixative combination. In some root tips the cells exhibited plasmolysis while the nuclear envelope and ER showed swellings. However, vacuoles were generally better preserved than those

fixed in KMnO_4 . This has also been noted by Gifford and Stewart (1967) and Hess (1968). Poorer preservation was experienced with seedling roots of Avena, Triticum and Zea than with root tips of Mentha and Lonicera.

In well preserved cells the vacuole is rounded with a smooth outline (Figs. 65-69). Even small vacuoles of promeristem cells often appear rounded, (Figs. 47, 67). Some irregularly shaped young vacuoles still occur but these are usually less irregular than small vacuoles of promeristem cells fixed in KMnO_4 .

In some root tips almost all vacuoles are irregular (Fig. 70), but in other specimens or individual cells irregularity is restricted to only a proportion of the vacuoles. Where rounded vacuoles are in close proximity the irregularity may take the form of a small inpushing of one vacuole by the other (Fig. 70); this is occasionally seen in published electron micrographs (e.g. Marinos, 1963a). The tonoplast may often have a wrinkled appearance (Fig. 70), a form illustrated in several papers (e.g. Bouck & Cronshaw, 1965). In general, vacuoles fixed in GA/OsO_4 are less angular in shape than those preserved in aqueous or buffered KMnO_4 .

A feature characteristic of several irregularly shaped vacuoles fixed in GA is the presence of variously arranged membranes within the vacuole. In some instances these comprise the tonoplast where it has pulled away from the boundary of the vacuole during fixation (cf Fig. 130). In other examples the internal membranes are not directly

connected to the tonoplast and appear to represent collapsed membranes surrounding vacuolar inclusion bodies.

4.2 3 Form of Vacuoles in Freeze-etch Replicas

In both frozen-etched fresh root tips and prefixed material vacuoles are mainly spherical (Figs. 71-77). The spherical shape is found in large and small vacuoles in cells from all parts of the root tip irrespective of the species studied. In cells at the surface of the cap the main vacuole has a regular contour in cross fracture and is smooth in surface views; small vacuoles in the cytoplasm surrounding large vacuoles are also spherical (Fig. 74). Before fusing to form the main vacuole, vacuoles in differentiating cells remain distinct and are spherical (Fig. 75 & 76). Even vacuoles approaching the size of mitochondria and smaller are spherical (Fig. 75). The identity of such vacuoles is confirmed when the aqueous contents freeze as large ice crystals (Fig. 27). The small vacuoles in cells of the promeristem are spherical.

In well frozen tissue the only departure from the spherical condition is where vacuoles have recently fused (Fig. 50) or where the form has been modified to accommodate other components of the cell. Occasionally slightly elongated vacuoles occur but these retain a rounded appearance (Fig. 73). Elongated vacuoles have also been shown in other frozen-etched root tips (Branton, 1966; Matile & Moor, 1968). Vacuoles forming diverticula, tentacle-like extensions or other outgrowths similar to these in sectioned material were not found. This applies to vacuoles at various stages of development and in all tissues of the root tip.

The few instances where vacuoles are not spherical and entire are those found occasionally in root tips fixed with GA at the time of glycerination prior to freezing (cf. Figs. 40, 42) and in frozen-etched fresh roots poorly impregnated with glycerol. Such vacuoles in prefixed material may show a slightly indented surface, sometimes with small projections. However, the modifications are small compared with those of vacuoles in "well fixed" sectioned root tips. In poorly frozen fresh roots vacuoles are sometimes ruptured and the contents released, causing a heterogeneous freezing of adjoining groundplasm (cf. Fig. 30). Infrequently, in poorly frozen specimens, vacuoles exhibit a slightly irregular appearance (Fig. 77). Occasionally small vacuoles in the peripheral cytoplasm appear crescentic in cross fracture (Fig. 56). Another feature of poorly frozen material is the dimpled appearance of the surface of the tonoplast. This is apparently caused through contraction of the membrane over underlying large ice crystals (cf. Figs. 23, 24).

4.3 Discussion

The form of vacuoles in freeze-etch replicas and in sectioned material shows striking differences. Whereas in frozen root tips vacuoles are predominantly spherical, those in thin sections present a diversity of shapes over the range of fixations used. In sections, vacuole shape ranges from predominantly angular (Fig. 55) to rounded (Fig. 47). The question arising is: which vacuole shape in electron micrographs most closely resembles that in the living cell?

4.3 1 Evaluation of the shape of vacuoles in thin sections

The angular shape seen in thin sections may be an artifact; this is shown by comparing vacuoles preserved under different

conditions. Most organelles retain their fundamental form irrespective of the fixative used. By contrast vacuoles assume various forms in different fixatives. Even with the one fixative e.g. $KMnO_4$, their shape may change depending on the condition of the root tip. On the other hand, when GA/OsO_4 is used root tips at various stages of development usually show vacuoles with a more consistent shape. These observations indicate that vacuoles are more sensitive to different fixatives than are other organelles; many of the irregular shapes are undoubtedly caused through reaction of the fixative with the vacuole.

The absence of a fundamental shape for angular vacuoles implies that these forms may not be normal. Apart from ER most other components of the cell have a fundamental form. In sections the only consistent shape of vacuoles is round and entire. Although this suggests that vacuoles are normally round, the possibility that some of the irregular forms might represent stages of development cannot be excluded. For example, light microscope studies indicate that vacuoles can be irregular at certain stages of growth (Bailey, 1930; Zirkle, 1932, 1937).

Non-miscible liquids in water tend to adopt the form of spherical droplets since this gives the most stable volume to surface area relationship. By analogy, irregular vacuoles may be unstable and may change to a spherical form. Observations on vacuolar fusion support this; the lobed shape of recently fused vacuoles (Figs. 65-69) soon rounds off into a single spherical entire vacuole. Although the tonoplast is a living system capable of independent growth - which may be localized

(see Chapter 6) - it is difficult to explain how the tonoplast could grow out into fine projections and at the same time maintain the vacuole as a turgid structure. If the vacuole grows by such extensions why should the surface area of the membrane be increased before the contents are able to fill the potential volume? Surely the surface area and volume of the vacuole would increase concomitantly as in many growing systems. However, vacuoles may assume irregular shapes when the turgor pressure is suddenly lowered. A reduction in volume results without a corresponding decrease in surface area of the tonoplast - although this probably occurs later. Irregularly shaped vacuoles of this kind are illustrated by White (1968) in living cells grown in culture media.

Evidence from thin sections suggests that the angularly shaped vacuoles are formed from spherical vacuoles which have collapsed. This condition is apparently due to shrinkage through extraction of water and/or other vacuolar contents. The degree of irregularity of a vacuole thus reflects the amount of shrinkage which has taken place. With extreme shrinkage portions of the tonoplast become apposed and form the characteristic fine extensions superficially resembling profiles of ER. The space within these vacuolar extensions is variable, however, and seldom corresponds to the dimensions of the intra-cisternal space of enchylema (Frey-Wyssling & Müllethaler, 1965) of ER membranes. This is noted also by Barton (1965).

If we accept that angular vacuoles are the result of shrinkage, how can this condition be explained? Gifford and Stewart (1967) and Hess (1968) suggest that it might be due to fixation and my

observations support this notion. For example, vacuoles of diverse form occurred when the fixative was varied and methods of dehydration and embedding kept constant. But this does not exclude the possibility that dehydration might not ultimately have caused shrinkage, as different fixatives probably vary in their ability to stabilize the vacuole against subsequent processing. However, proof that fixation rather than dehydration is involved is shown by frozen-etched prefixed root tips. In these, vacuoles occasionally exhibit slight irregularity although there has been no chemical dehydration during preparation. The other instance of irregular vacuoles in frozen-etched material is that found in living cells which have incompletely assimilated glycerol. Under these circumstances the glycerol tends to behave as a dehydrant causing the vacuoles to collapse slightly. This probably explains the situation shown in figures 77 and 78.

On comparing results from different fixatives it is evident that certain conditions of fixation cause greater collapse than others. A systematic evaluation of the various conditions of fixation affecting vacuolar preservation is outlined in Chapter 5. In the present chapter it is clearly shown that aqueous KMnO_4 causes greatest distortion of vacuoles. The vacuoles are less distorted when KMnO_4 is buffered but the best results, noted also by Gifford and Stewart (1967) and Hess (1968), were obtained using GA/OsO_4 fixation.

With any one fixative it is seldom possible to obtain consistent preservation of all vacuoles in root tips of a particular experiment, all cells within a single specimen or all

vacuoles in an individual cell. This variation appears to be influenced mainly by the physiological state of the tissue and of the individual vacuoles. For example, vacuoles in actively differentiating tissue are usually more collapsed than those from less actively growing root tips. It is probably for this reason that small vacuoles show greater shrinkage than large vacuoles. That physiological activity is more important than the size of vacuoles is demonstrated by comparing small vacuoles in meristem cells with structures of a comparable size in differentiating tissues. For instance, small peripheral vacuoles in root cap cells are usually rounded when fixed in buffered $KMnO_4$, whereas those from the promeristem are irregular. Since vacuoles of a cell differ physiologically (see Bailey, 1930; Zirkle, 1937; White, 1968) this probably explains why various shapes are obtained under the same conditions of fixation. While aqueous $KMnO_4$ gives poor preservation of vacuoles in actively growing tissue compared with GA/OsO_4 , reasonable fixation may be achieved in dormant root tips. When roots are metabolically inactive vacuoles are usually less sensitive to fixation damage, possibly because the ionic strength of the vacuole more closely corresponds to that of the fixative.

The distribution of cell components surrounding vacuoles is also an indication that the angular shapes may be artifacts. The paucity of organelles near badly shrunken vacuoles (Fig. 54) implies that when the vacuole collapsed the distribution of organelles nearby was unaffected, the volume originally occupied by the vacuole being filled by groundplasm flowing into the space. In $KMnO_4$ fixed material little difference in density of groundplasm

surrounding collapsed vacuoles is usually noted compared with the rest of the cell but in GA/OsO₄ fixed specimens ribosomes are sometimes reduced in density near shrunken vacuoles. Sometimes empty spaces occur which suggest that the vacuole probably collapsed suddenly. In other instances the radiate alignment of mitochondria and plastids around shrunken vacuoles indicates that the organelles have been drawn towards the vacuole when it collapsed. Profiles of ER following the irregular outline of the vacuole (Fig. 60) have undoubtedly arisen in a similar manner.

4.3 2 Evaluation of the shape of vacuoles in frozen-etched root tips

Freeze-etching provided the most convincing evidence that the fundamental shape of vacuoles is spherical. Recently several workers using this technique have suggested that the irregular forms seen in section might be artifacts of specimen preparation (Moor & Mühlethaler, 1963; Branton & Moor, 1964; Moor, 1966b; Hess, 1968; Matile & Moor 1968), but the problem has not received systematic attention.

In early studies using frozen-etched yeast cells, Moor & Mühlethaler (1963) found only spherical vacuoles which lacked the irregular processes described in chemically fixed material (Vitols et al., 1961; Wallace et al., 1968). From such observations Moor and Mühlethaler implied that the irregular form might be due to shrinkage. Branton and Moor (1964), working with frozen-etched root tips of Vicia faba, also noted that although vacuoles occasionally showed "bump-like" surface irregularities vacuoles were conspicuously rounded by comparison with structures seen in most chemically fixed material.

Collapsed protrusions which in fixed material might be confused with membranes of ER were not seen in these freeze-etch replicas. Recently, spherical vacuoles characterized as lysosomes have been described in yeast cells (Matile & Wienken, 1967) and Zea root tips (Matile, 1968; Matile & Moor, 1968). Apart from modifications in shape caused through fusion, these vacuoles were found to be mainly spherical. The ballooning of ER repeatedly seen in sectioned material (e.g. Buvat, 1961; Poux, 1962; Bowes, 1965b; Buvat 1968b) was not found in the preparations. Comparisons of the form of vacuoles in frozen-etched replicas and sectioned material of a fungal protoplast led Hess (1968) also to consider that the irregular shapes in chemically fixed material are due to specimen preparation. Spherical rather than irregular vacuoles are shown in illustrations by other workers using freeze-etching (Moor, 1964, 1965, 1967; Jost, 1965; Hess et al., 1966; Northcote, 1967; Remsen et al., 1967b; Sassen et al., 1967; Northcote & Lewis, 1968).

The present work demonstrates that vacuoles in frozen-etched root tips are basically spherical. Since vacuoles as well as other cell components retain a fundamental shape this indicates that freeze-etching is a more reliable method for obtaining consistent preservation of vacuolar form than thin sectioning.

However, before results from freeze-etching can be fully accepted the validity of the method must be evaluated. Since freeze-etching involves a physical process artifacts caused by chemical fixation, dehydration and embedding are avoided (Steere, 1957; Moor et al., 1961; Moor & Mühlethaler, 1963; Moor, 1964,

1965, 1966a & b). Because substances are not washed away shrinkage is prevented. Although freezing introduces potential artifacts it has been shown that these do not present a major obstacle (Sakai, 1966) and should at least be different from those caused by chemical fixation (Steere, 1957). Also subsequent steps in the freeze-etch procedure do not seriously modify the specimen (Moor & Mühlethaler, 1963; Moor, 1964, 1966b; Koehler, 1968). The most important evidence demonstrating the validity of freeze-etch results is that frozen cells remain viable on thawing (Moor & Mühlethaler, 1963; Moor, 1964; Moor, 1966b; Nanninga, 1968). While this has been established for frozen yeast cells and bacteria, it is usually difficult to prove survival in animal and plant tissues unless the objects can be cultured (Moor, 1966b). The presence or absence of ice crystal images in the replica nevertheless provides a basis for judging the quality of preservation.

One of the chief criticisms of results obtained from freeze-etching is that an anti-freeze agent such as glycerol must be employed to prevent formation of ice crystals in tissues containing above 75% water (Moor, 1966b). While the root tips of many plants absorb glycerol only with difficulty (Branton & Moor, 1964; Moor, 1966b; see Chapter 3) those of certain species will grow, albeit slowly, in a 20% solution. Furthermore, it has been found that various cultures of animal and human tissues impregnated with glycerol survive after freeze-fixation (for references see Moor, 1966b). It has been shown also that glycerol does not significantly affect the dimensions of chloroplast membranes (Dilley et al., 1967). Recent work by

Richter (1968a & b) has demonstrated that epidermal cells of various species of Campanula will withstand high concentrations of glycerol and remain viable provided that the glycerol is introduced gradually. Nanninga (1968) has found that bacteria grown in glycerol and then frozen resume growth on thawing. From these studies it is reasonable to assume that the presence of glycerol in root tip tissue does not appreciably alter the fundamental form of cell components.

It may be argued that the spherical shape of vacuoles in frozen viable cells need not be typical of roots grown under natural conditions because root growth is retarded in the presence of glycerol. According to some light microscope studies spherical vacuoles are characteristic of inactive cells, the irregular forms appearing on initiation of cyclosis (see Voeller, 1964). Since the presence of glycerol inhibits cyclosis (Richter, 1968a) this might explain the occurrence of spherical forms in frozen-etched preparations. Although vacuole shape undoubtedly changes during periods of cellular activity, it seems unlikely that all the diverse forms found in thin sections represent such phases of activity. Proof that the spherical vacuoles in frozen-etched cells resemble those of roots grown without glycerol is obtained by comparing frozen-etched fresh material grown in glycerol with prefixed actively growing root tips. In both fresh and prefixed frozen-etched roots spherical vacuoles abound. This clearly demonstrates that spherical vacuoles are equally characteristic of inactive and active root tips. However, vacuoles may be modified in shape to accommodate other organelles, during stages of fusion and during phases of cell activity (e.g. cyclosis).

The shape of vacuoles in promeristem cells has been clarified by freeze-etching. In sectioned material these are typically irregular (Whaley et al., 1960a; Fineran, 1966). During the present study various fixatives were used in an endeavour to preserve the true image of these vacuoles but no consistent shape could be obtained. When freeze-etching was first applied to the problem results were disappointing as fracturing seldom revealed the promeristem. To overcome this difficulty, the promeristem was isolated as small cubes about 1 mm³. Root tips of Zea were used as these are known to have a large promeristem (Clowes, 1961). In both frozen-etched living tissue and prefixed specimens promeristem cells showed only spherical vacuoles. It is therefore concluded that vacuoles in promeristem are fundamentally spherical as in other tissues of the root tip.

4.3 3 Evaluation of irregular vacuoles from optical studies

A fundamental spherical shape for vacuoles in the root tip has been demonstrated. How may the irregular forms seen with the light microscope be explained?

Zirkle (1932) found that the shape and size of vacuoles in living and fixed root tips was conditioned primarily by the activity of the cytoplasm. When the cytoplasm was quiescent vacuoles tended to be spherical in the smaller cells whereas in cells with active protoplasmic streaming the vacuoles became rod-like, were drawn out into "Holmgren canals", or formed a reticulate apparatus. Vacuoles of irregular shape are figured also by Küster (1927). Stages in the development of vacuoles from filamentous structures have been illustrated in the growing leaflet

of rose by Guilliermond (1941).

Irregularly shaped vacuoles seen with the light microscope have been taken as evidence for the validity of irregular vacuoles in thin sections (Whaley et al., 1960a). However, examination of published illustrations indicates that the structures are not usually comparable in form. Apart from vacuoles with narrow projections drawn by some workers (e.g. Küster, 1927) most of the irregular vacuoles in optical preparations are entire rather than angular and lack fine projections. Instead elongated rounded bodies are usually shown (e.g. Bailey, 1930; Zirkle, 1932; Guilliermond, 1941). Possibly some of these correspond to chains of fusing vacuoles which in thin sections may be difficult to interpret. The conditions shown in figures 65 and 69 may correspond to thread or rod shaped vacuoles seen at the light microscope level of observation. Nevertheless, there still remains the possibility that some vacuoles expand from spheres into elongated bodies. However, there is no evidence to suggest that this occurs by prior formation of membrane apposed extensions. Elongated vacuoles appear to develop mainly through fusion of spherical vacuoles. The reticulate vacuoles seen with the light microscope may in some instances represent superimposed chains of fusing or closely contiguous vacuoles which eventually develop into the main central vacuole of the cell.

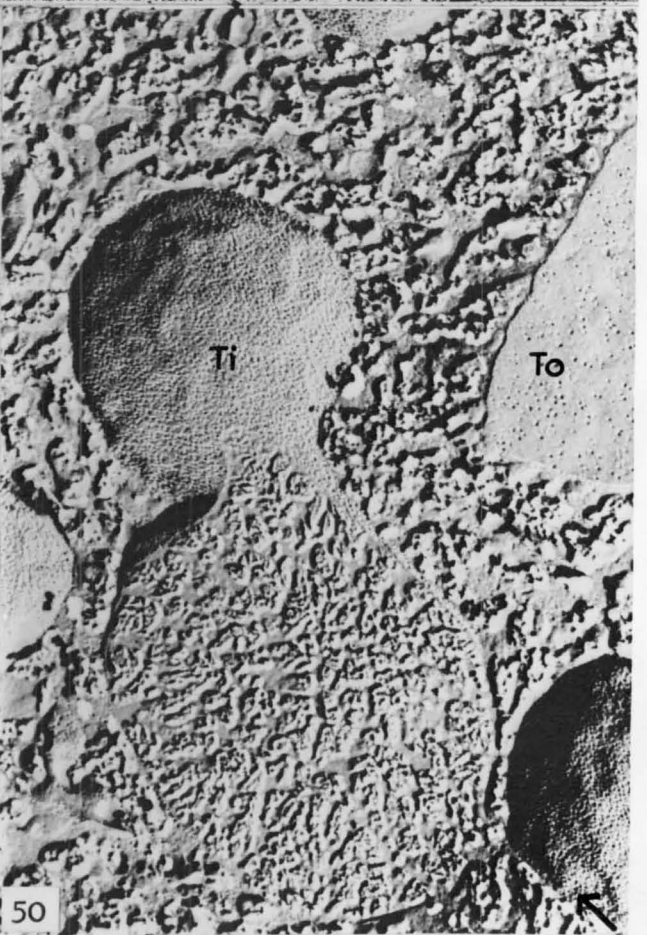
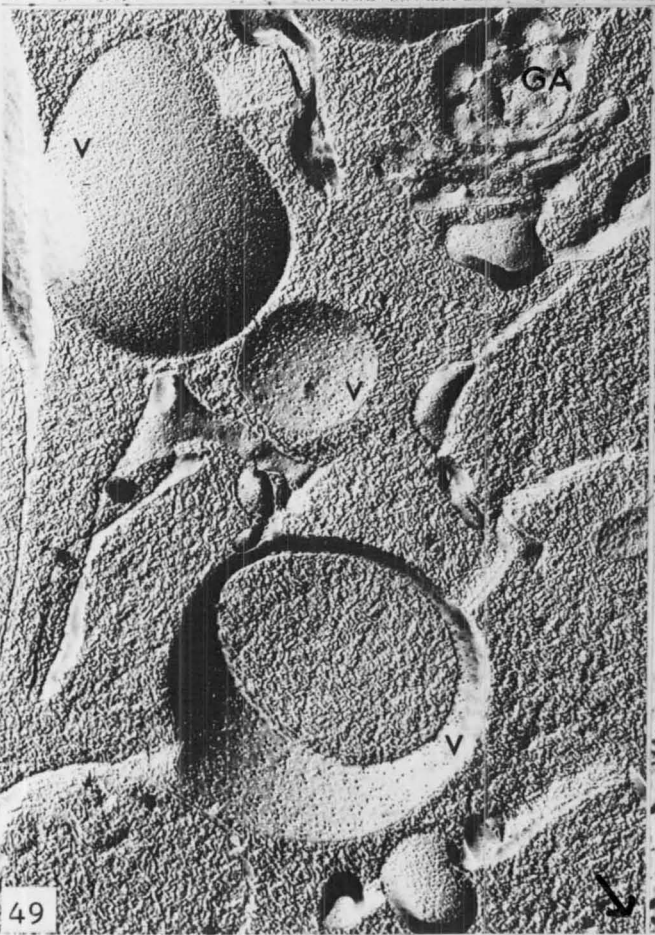
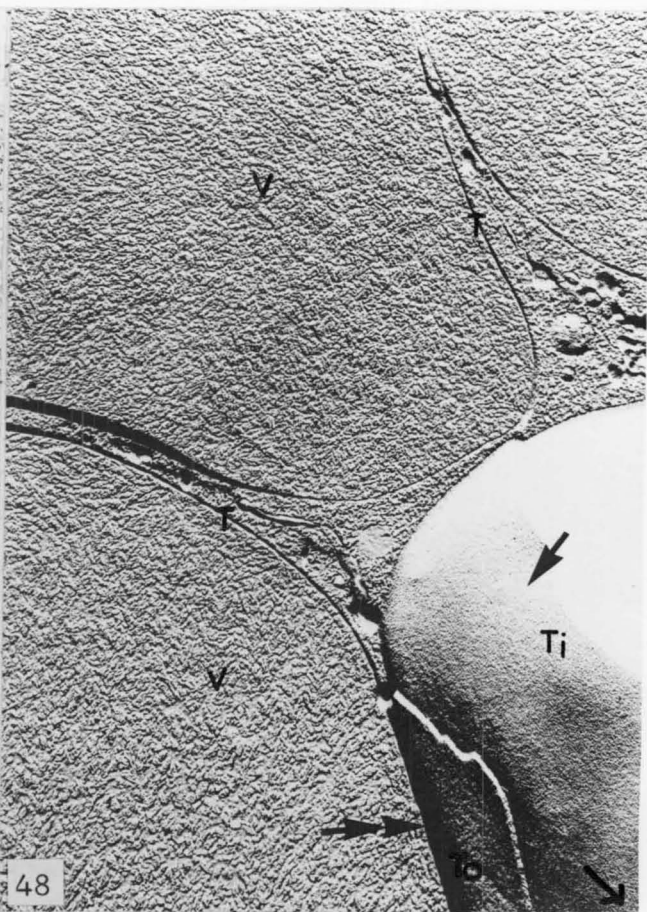
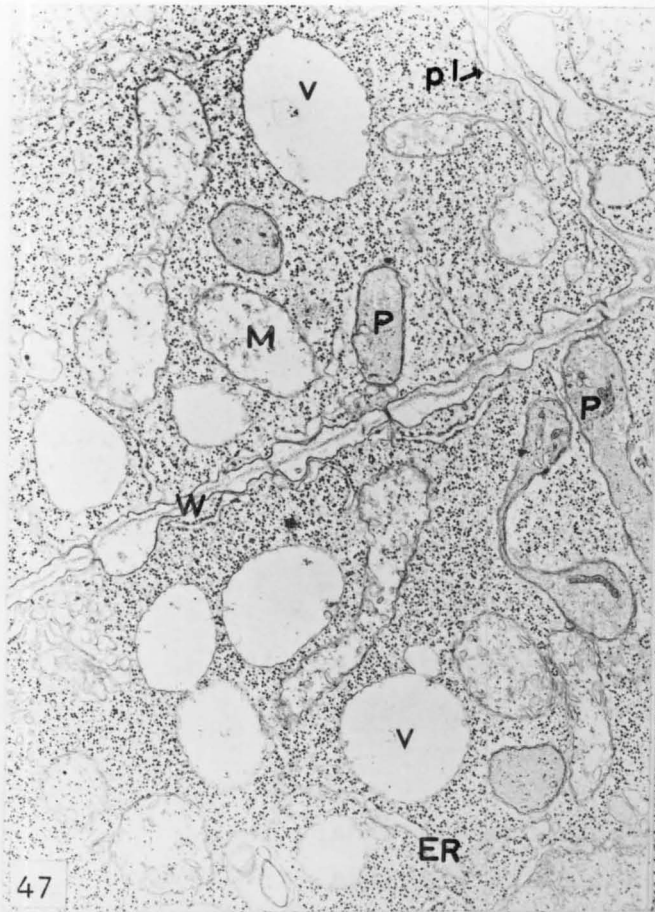
4.4 Summary

A comparison is made of the form of vacuoles in thin sections and freeze-etch replicas of root tips. In sections vacuoles exhibit a diversity of shapes, the greatest irregularity being found with aqueous $KMnO_4$ fixation. In contrast, vacuoles of

frozen-etched roots are spherical or occasionally elongated. Vacuoles were not found with profile-like extensions or other irregularities but retained a turgid appearance with a smooth contour to the tonoplast, except in some poorly frozen cells. As freeze-etching avoids the artifacts of chemical fixation, it is considered that the shape of vacuoles in frozen-etched cells more closely resembles than in a normal living cell than does than shown in sectioned material. In sections, vacuoles of irregular shape are apparently caused during fixation through the shrinkage of mainly spherical vacuoles. When shrinkage is severe portions of the tonoplast become apposed and superficially resemble profiles of ER.

Figures 47 - 50 Identity of vacuoles in section
and frozen-etched replicas of root tips.

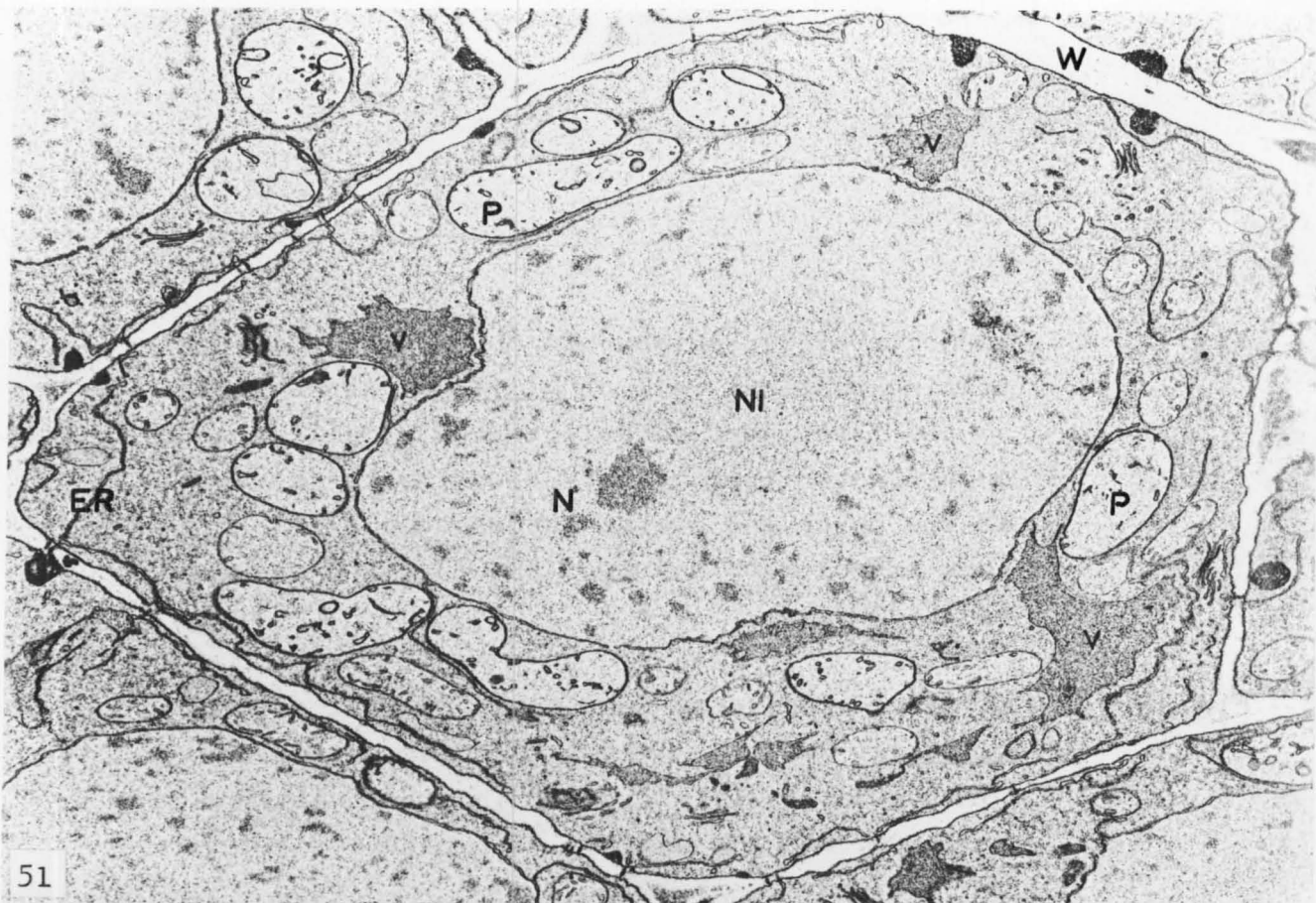
- Fig. 47 Vacuoles in thin section of promeristem cells of M.citrata fixed in glutaraldehyde/OsO₄. The single limiting membrane distinguishes vacuoles from the other organelles visible. The electron-transparent contents are also characteristic after this fixation. (x 26,800).
- Fig. 48 Surface and cross-fractured views of a group of vacuoles in A.sativa. The three upper vacuoles are cross-fractured and show a single limiting membrane. The inner surface of another vacuole is visible at bottom right; part of the outer surface of the adjoining vacuole is shown here also. (x 37,500).
- Fig. 49 Portion of three fractured vacuoles in T.vulgare. The lower organelle is identifiable as a vacuole from the appearance of contents in cross fracture and presence of a single bounding membrane. As the shadow is on the right and extends slightly beyond the vacuole (shadowing is from left to right) the structure is raised above the surrounding groundplasm; the surface fractured portion shown therefore represents the outer face of the tonoplast. The surface shown by the middle vacuole is also an outer face. In the top left vacuole the inner surface of the tonoplast is visible. (x 40,000 approx.).
- Fig. 50 A dumb-bell shaped vacuole in A.sativa formed by fusion of two spherical vacuoles. In the lower portion contents bounded by a single membrane are visible in cross fracture; in the upper half contents have been removed exposing the inner surface of the tonoplast. The upper half of the vacuole represents a hollow; this is shown by the position and direction of shadowing (right to left). (x 45,600).



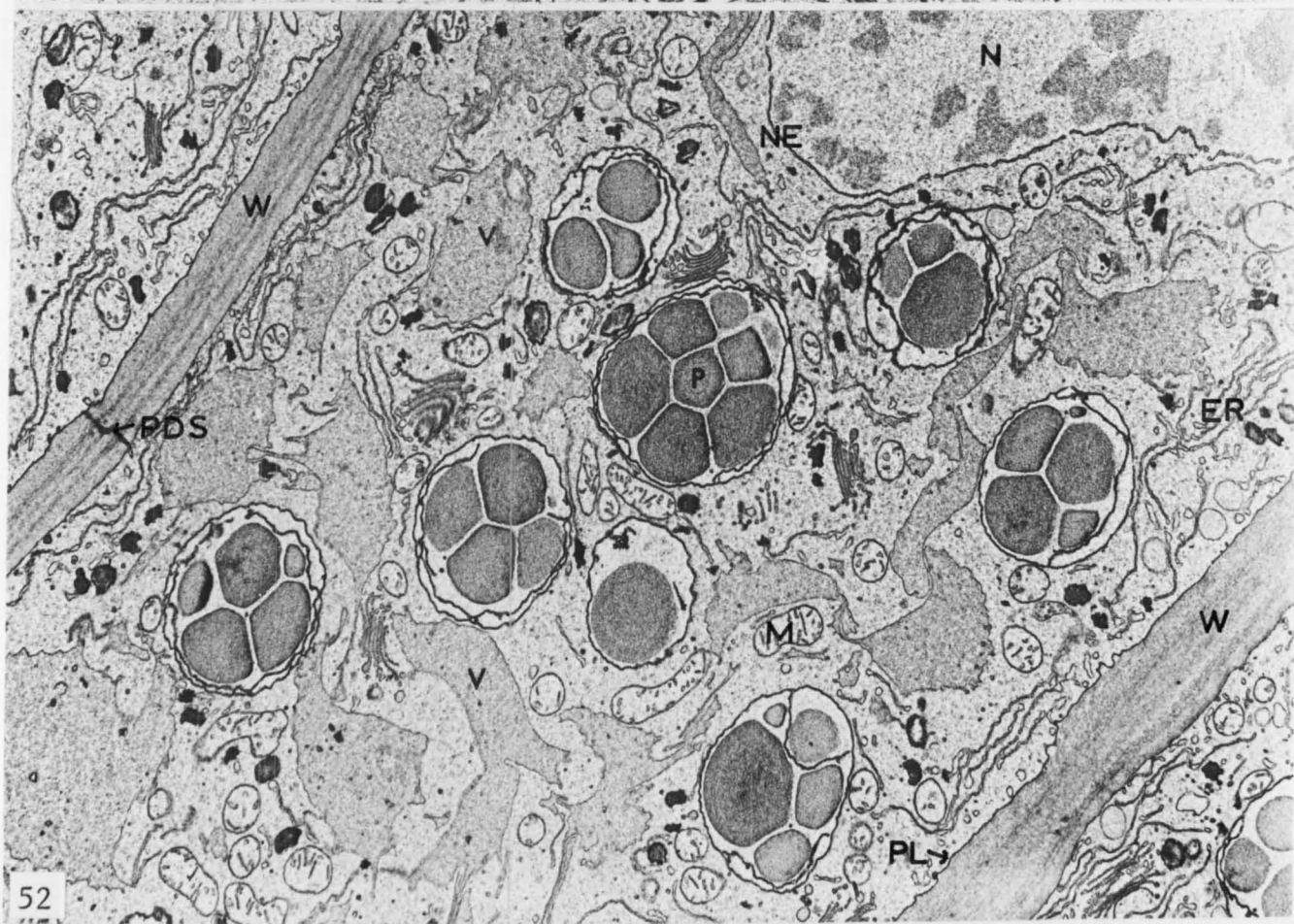
Figures 51 - 60 Root tip cells fixed in 2%
aqueous KMnO_4 .

Fig. 51 Small vacuoles (V) of irregular shape in a
promeristem cell of Lonicera nitida (x 15,600).

Fig. 52 Portion of a root cap cell from an actively
growing root of Avena sativa. Numerous small
angular-shaped vacuoles are distributed through-
out the cytoplasm. Well developed amyloplasts
are also present (x ca. 18,000).

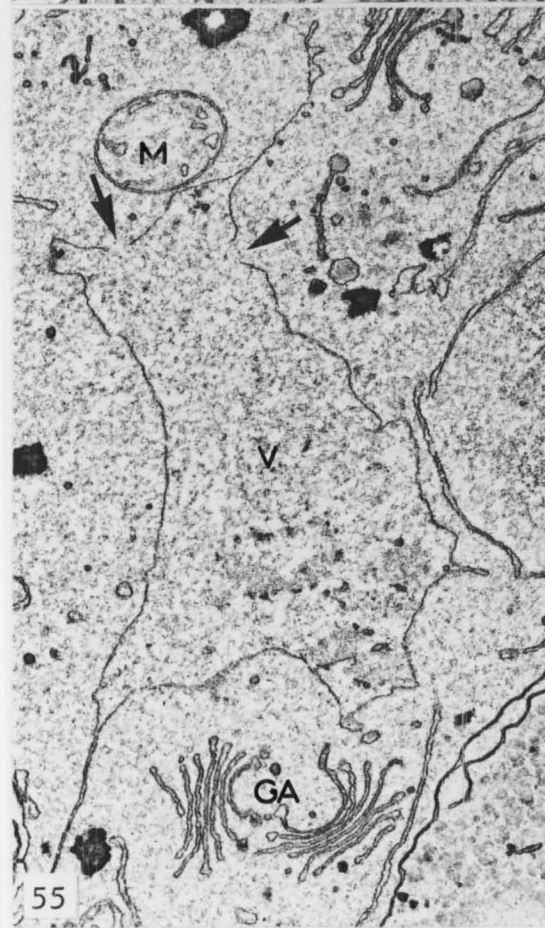
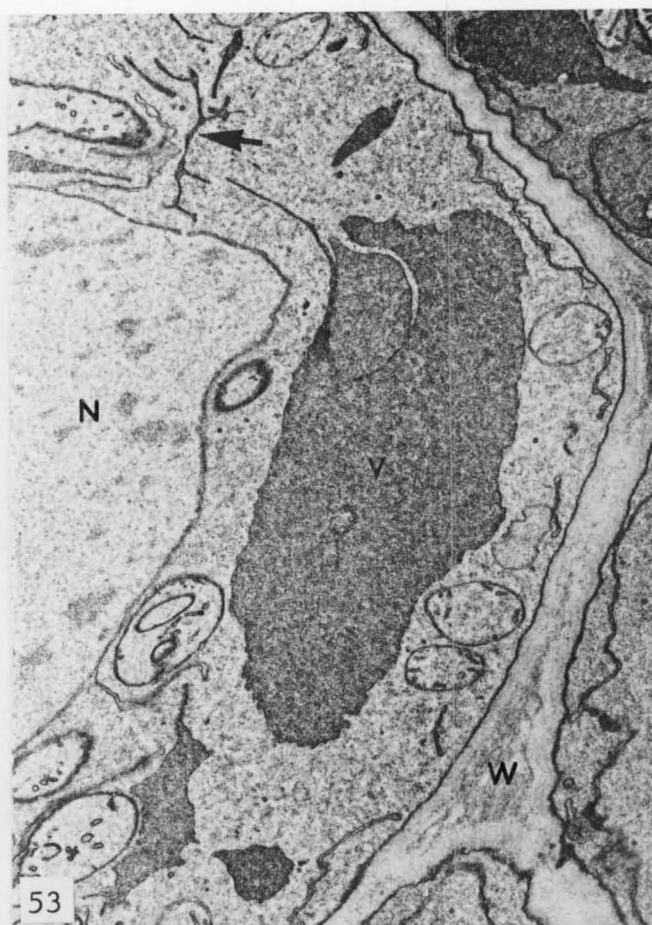


51

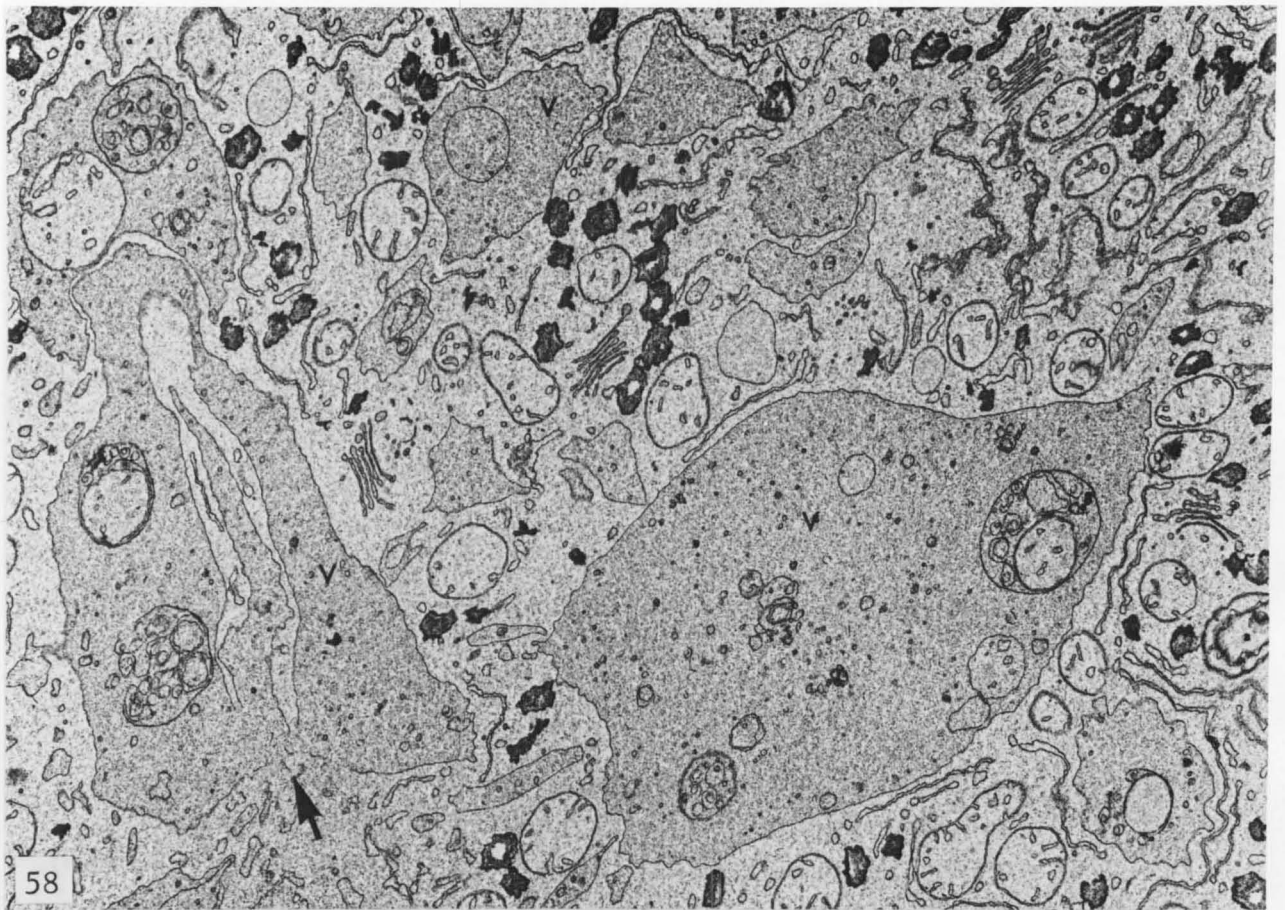


52

- Fig. 53 Vacuoles of irregular form in an early differentiating cortical cell of L.nitida. As depicted by the "stellate" vacuole (arrow) small vacuoles show greater irregularity than the large vacuole. At the upper end of the large vacuole fine projections extend both out from and into the vacuole (x 22,500).
- Fig. 54 Vacuoles of diverse form in procambial cell of L.nitida. Note the narrow vacuolar extensions forming "delta-like" in-pushings into neighbouring vacuoles, and the paucity of organelles between the vacuoles (x 18,400).
- Fig. 55 Portion of a root cap cell of Z.mays. The collapsed vacuole shows narrow extensions which superficially resemble profiles of ER. Ruptured areas are also visible (arrows) (x 38,400).
- Fig. 56 A small crescentic-shaped vacuole (arrow) bordering a larger vacuole in an outer root cap cell of T.vulgare. The tonoplast of the large vacuole evaginates towards the smaller one (x 20,900).
- Fig. 57 An enlarging vacuole in a root cap cell of L.nitida showing portion of the tonoplast with villus-like processes (x 26,400).



- Fig. 58 Numerous vacuoles of irregular shape in a root tip cell of A.sativa near the surface of the cap. Masses of cytoplasm and fragments of organelles are contained within the vacuoles. Some of these appear to have been sucked into the vacuole on collapse of the organelle during fixation. Ruptured areas are visible in some vacuoles (arrow) (x 18,400).
- Fig. 59 Vacuoles with processes extending towards each other in a root cap cell at A.sativa (x 26,400).
- Fig. 60 A collapsed vacuole in a root cap cell of A.sativa. The adjoining ER follows the contour of the partially shrunken vacuole (x 43,200).



Figures 61 - 64 Root tip cells fixed in buffered
KMnO₄.

Fig. 61 A root cap cell of Z. mays showing vacuoles more rounded in outline than vacuoles from similar cells fixed in aqueous KMnO₄ (cf. Figs 52 & 60) (x ca. 18,000).

Fig. 62 Portion of a root tip cell of Z. mays containing both large and small vacuoles (arrows) round in section. Dense lipid bodies are also present (x ca. 23,000).

Fig. 63 Small vacuoles of irregular form in a promeristem cell of A. sativa. The young vacuoles are less irregular than corresponding vacuoles fixed in aqueous KMnO₄ (x **26,400**)

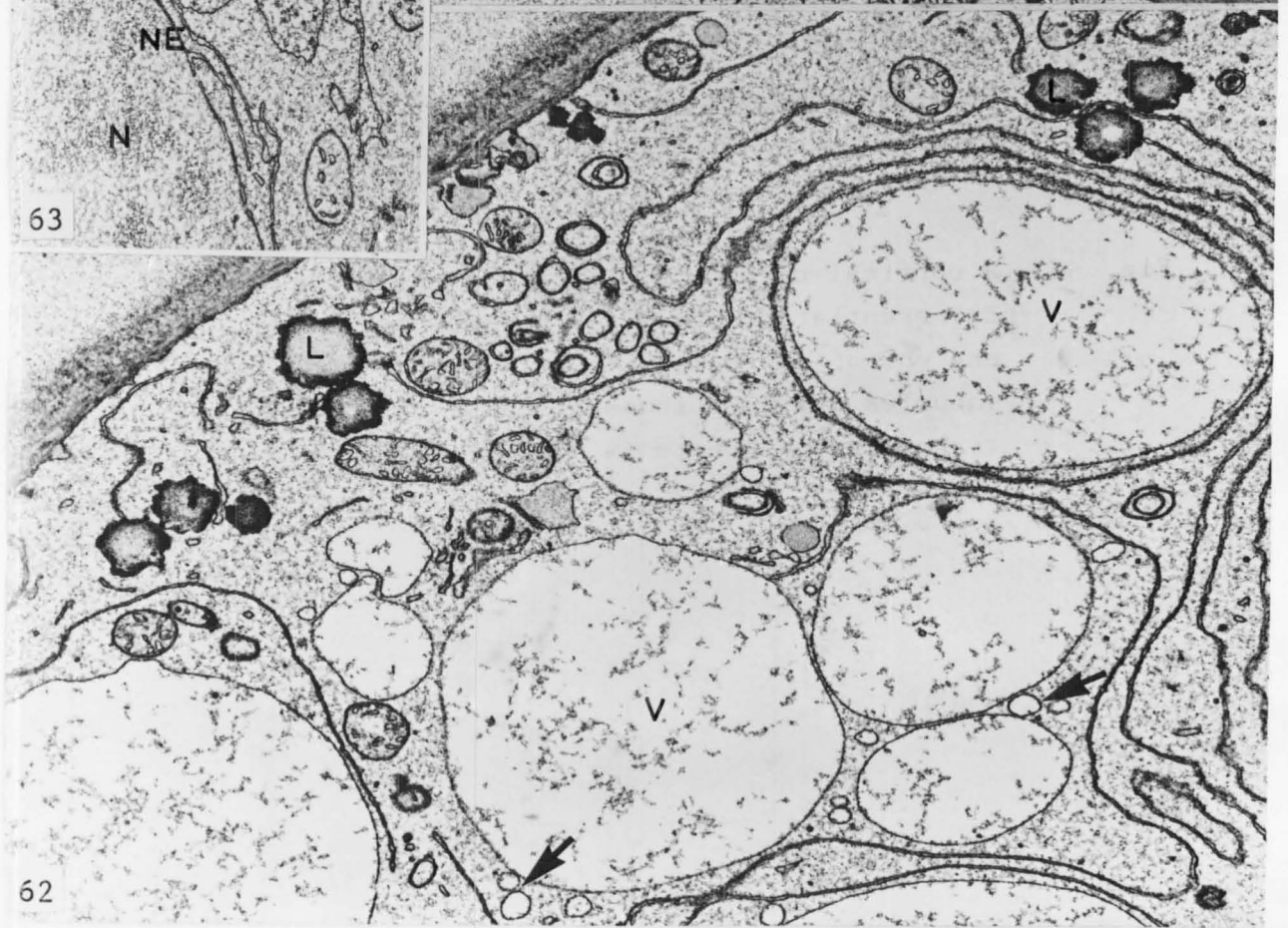
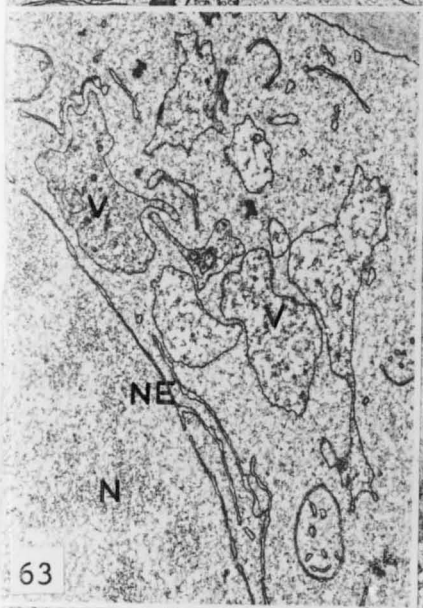
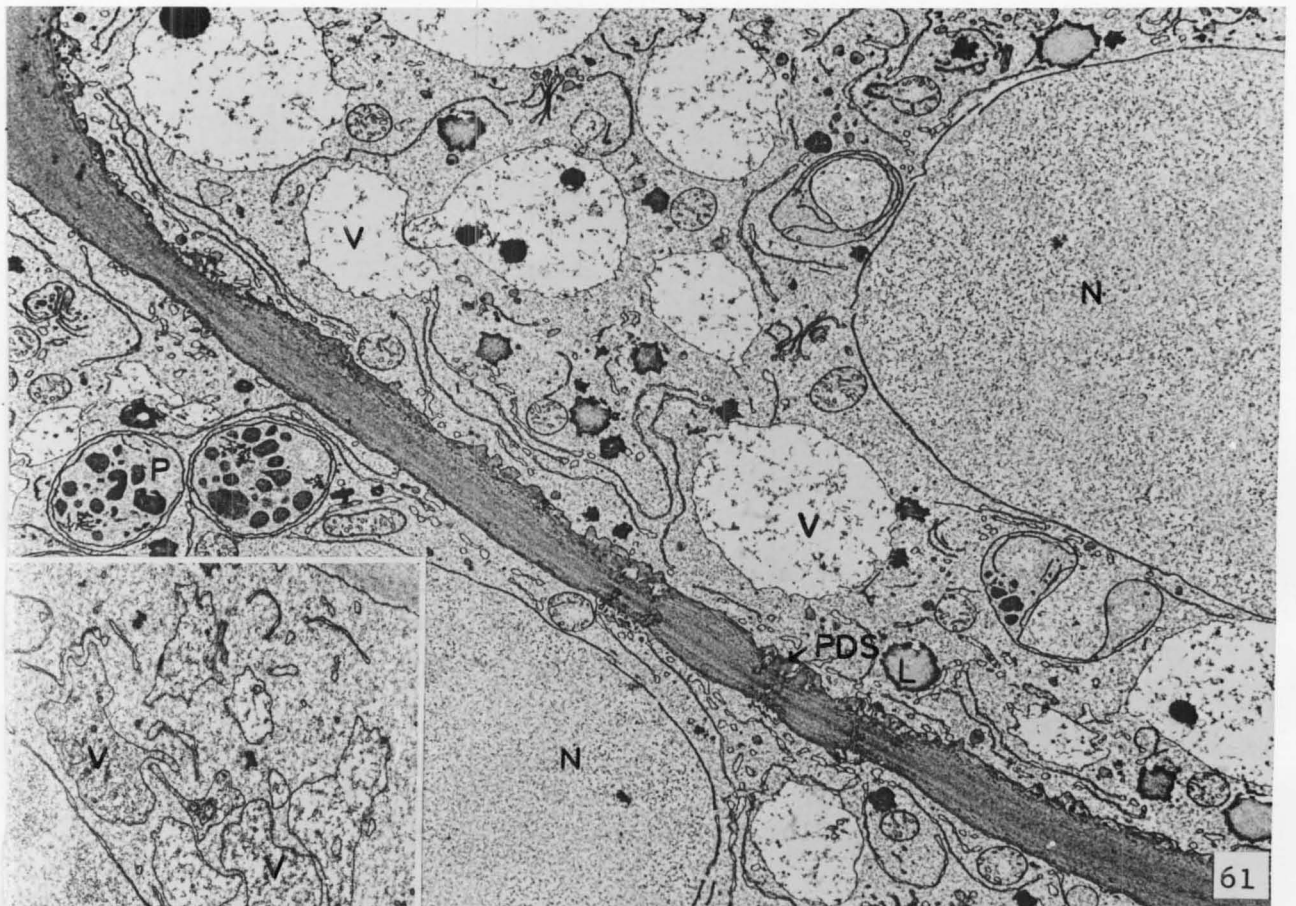
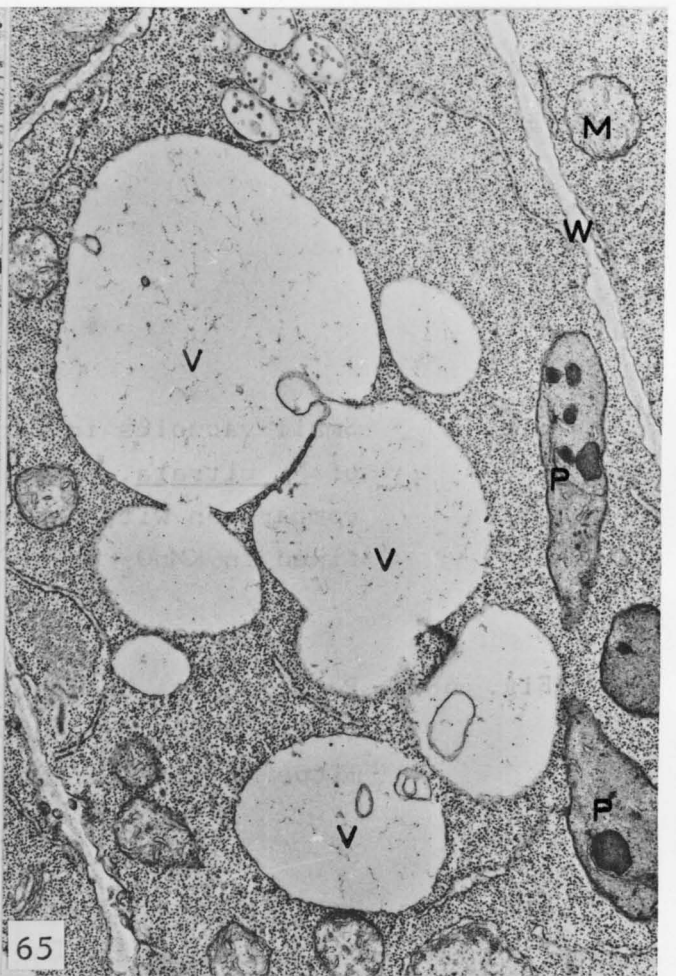
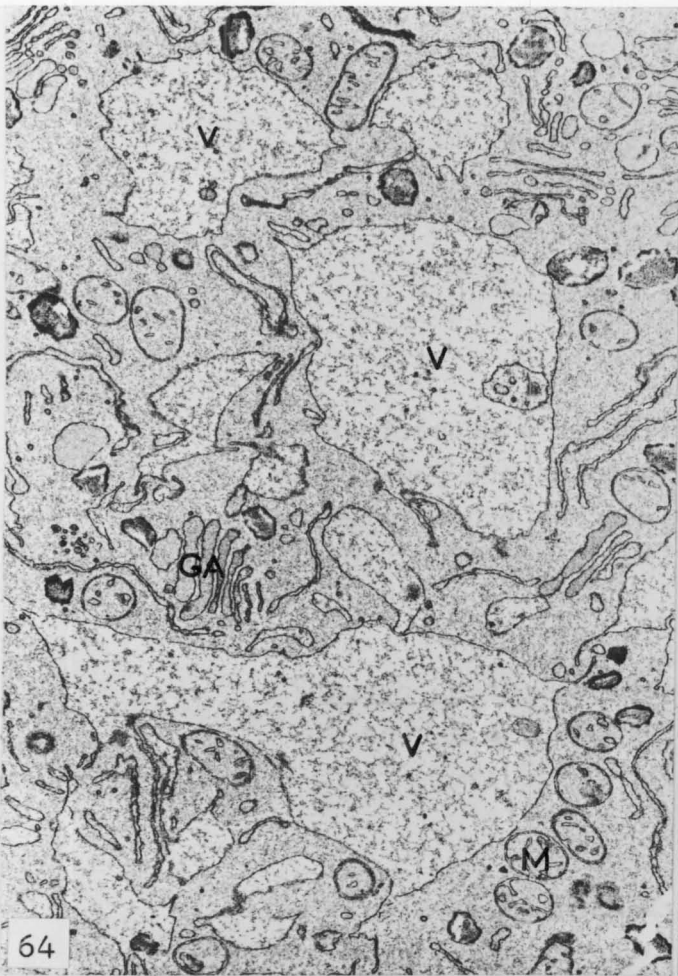


Fig. 64 Portion of a root tip cell of A. sativa showing vacuoles of angular irregular shapes as found in some buffered preparations of KMnO_4 (x 20,100)

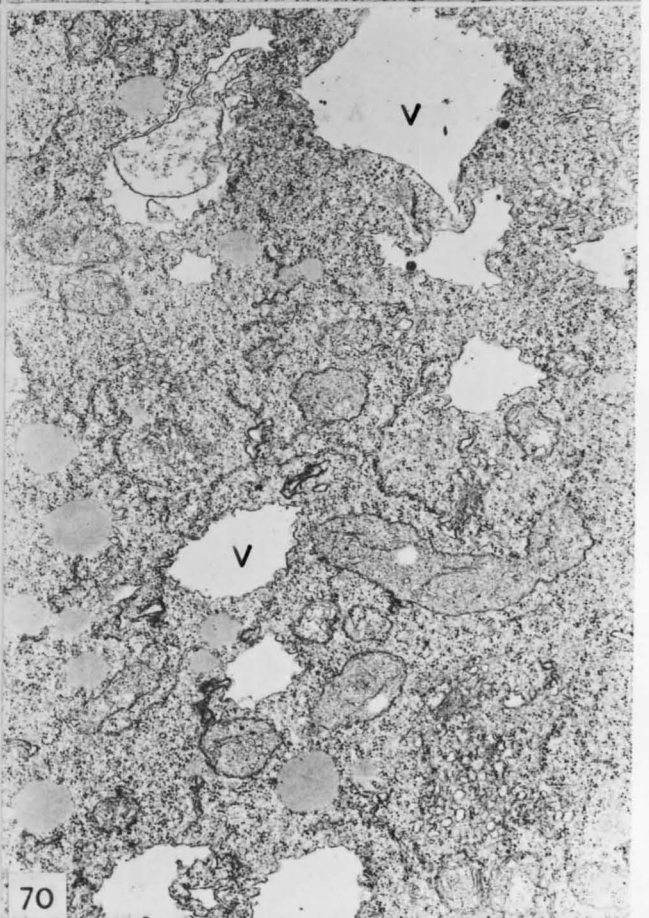
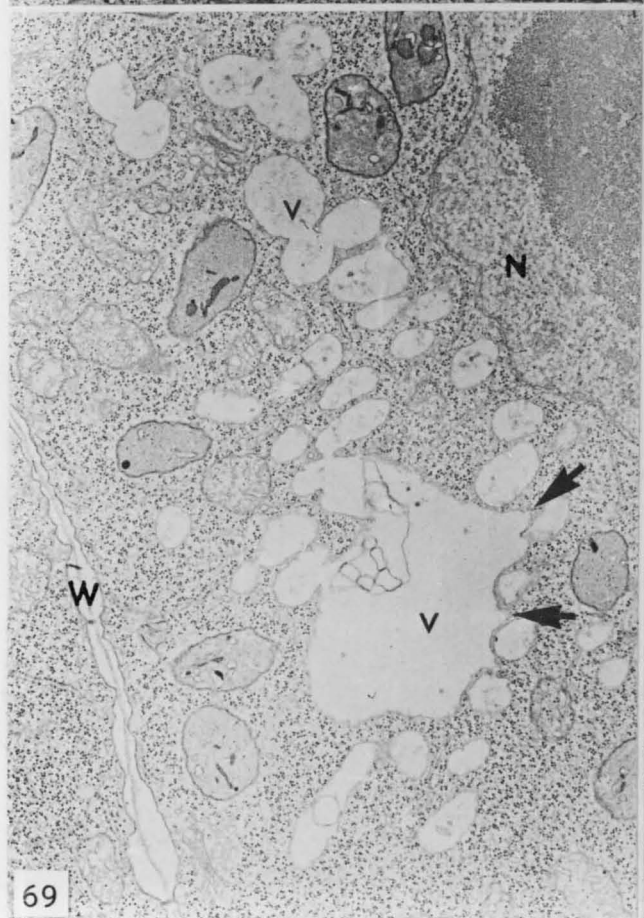
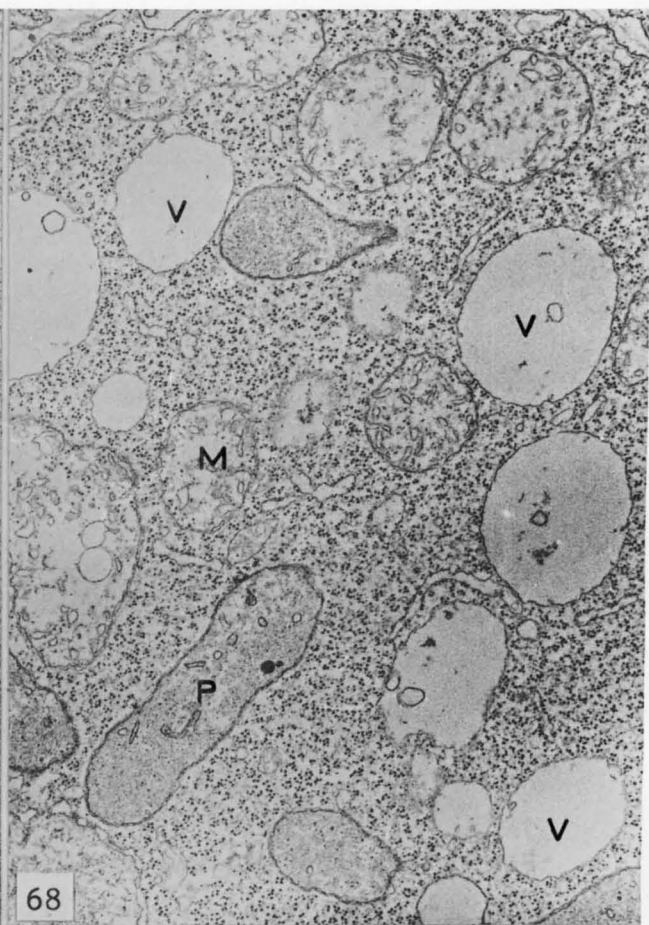
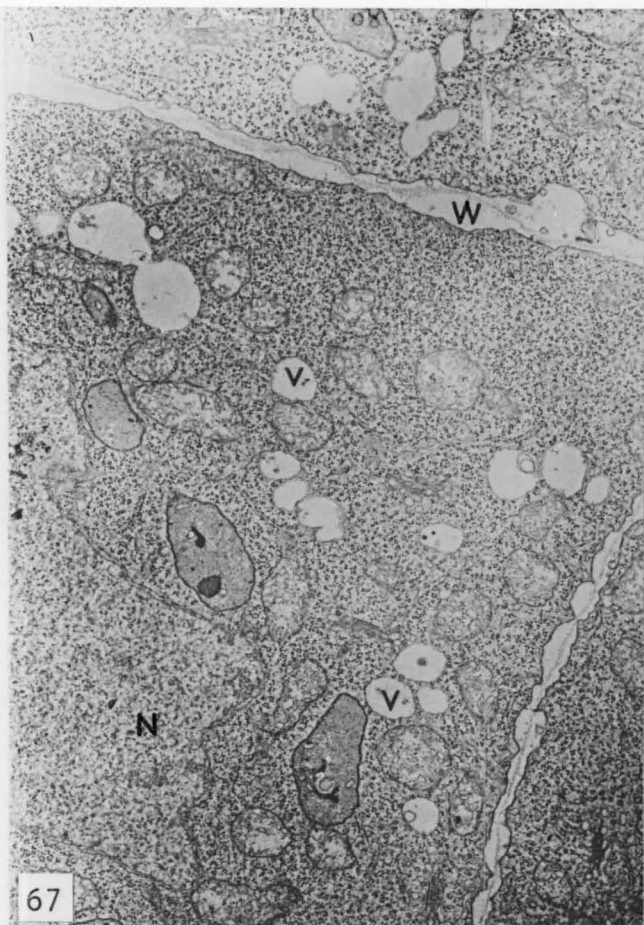
Figures 65 - 70 Root tip cells fixed in
Glutaraldehyde / OsO_4 .

Fig. 65 Vacuoles at one end of a differentiating procambial cell of Mentha citrata. The vacuoles are round in section except where they have fused (x 20,000)

Fig. 66 A cortical cell of M. citrata at an early stage of differentiation showing a large rounded vacuole at one end of the cell. This and the other small vacuoles are conspicuous because of electron transparent contents (x 15,600).



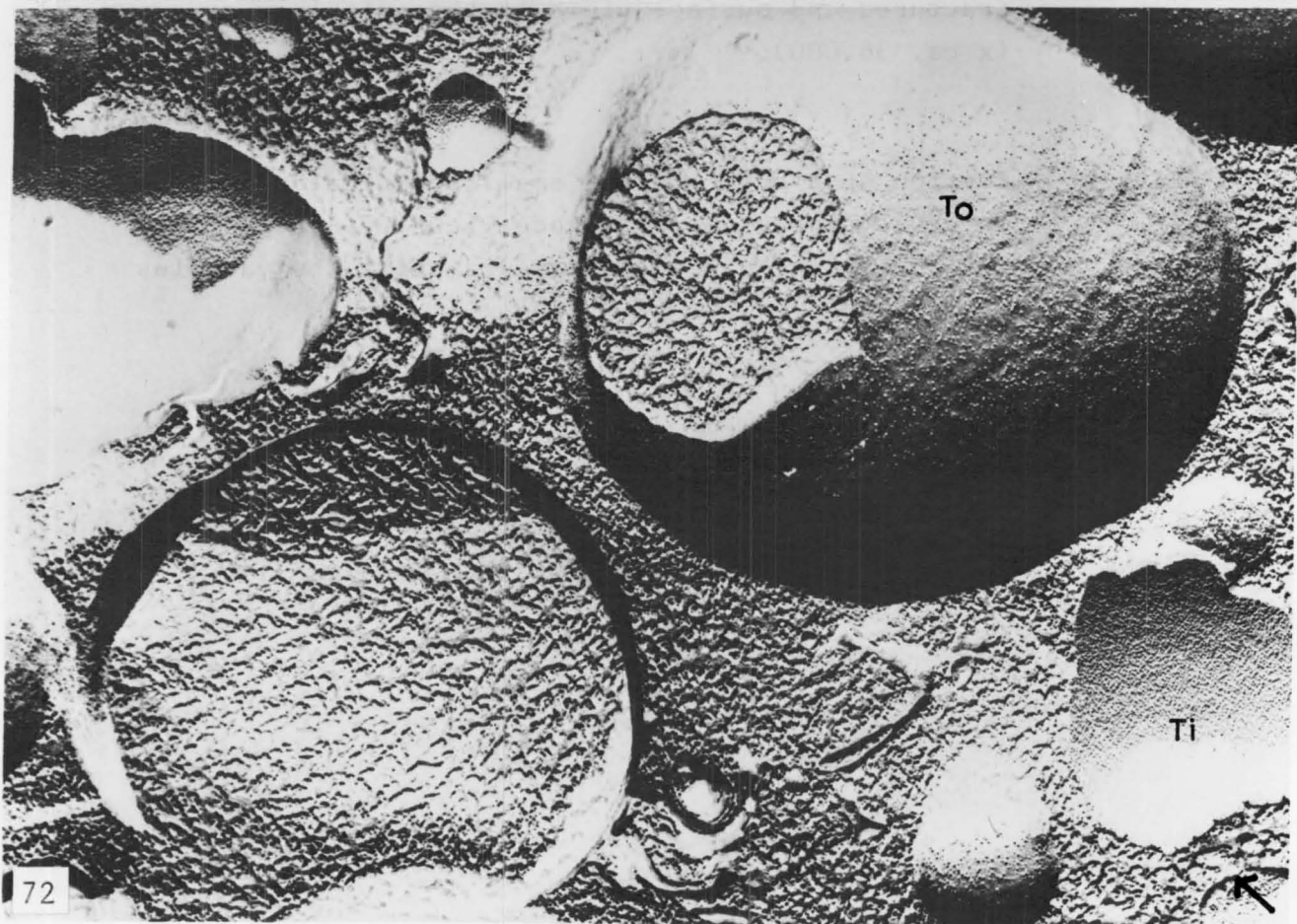
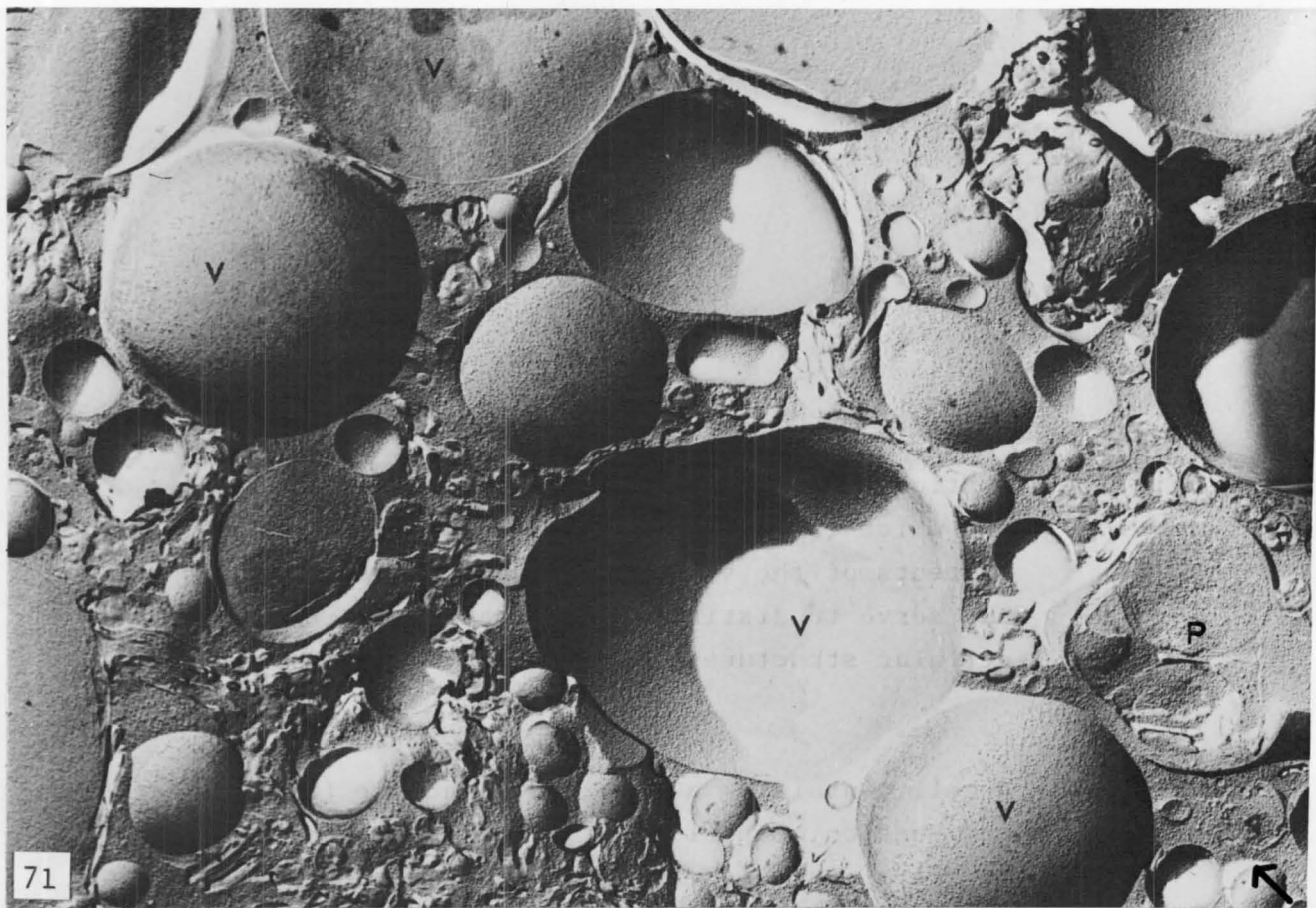
- Fig. 67 Small vacuoles in portion of a promeristem cell of M. citrata. The vacuoles are rounded in comparison with vacuoles from promeristem cells fixed in KMnO_4 (cf. Fig. 51) (x **20,200**)
- Fig. 68 Portion of a promeristem cell of M. citrata showing rounded vacuoles about the size of mitochondria and smaller (x **28,300**)
- Fig. 69 Vacuoles at various stages of fusion in a cell of M. citrata. The vacuoles are rounded, mainly entire and lack fine projections characteristic of many vacuoles preserved in KMnO_4 . The large vacuole shows slight irregularity through shrinkage (arrows) (x 23,000).
- Fig. 70 Root tip cell of A. sativa showing vacuoles shrunken as a result of poor preservation (x 23,000).



Figures 71 - 74 **Frozen**-etched vacuoles in root
tip cells of Avena sativa.

Fig. 71 Cell showing several spherical vacuoles (V) in
surface view and cross fracture (x 17,400).

Fig. 72 A spherical vacuole (upper right) showing both
surface and cross fractured views in the same
structures. The cross fractured portion reveals
the frozen contents of the vacuole bounded by a
single membrane. The lower left vacuole is seen
in cross fracture (x **28,500**)



- Fig. 73 Portion of a cell showing spherical vacuoles and one which is slightly elongated. The aqueous contents of the vacuoles show large ice crystals and serve to distinguish the vacuoles from other cellular structures (cf also Fig. 75) (x16,500)
- Fig. 74 A cell containing a large main vacuole (right) and portion of peripheral cytoplasm. Small spherical vacuoles (V) are visible in both cross fractured and surface views in the cytoplasm (x ca. 36,000).
- Fig. 75 Portion of a cell showing small spherical vacuoles in cross fracture and surface views. A few mitochondria (M) are scattered amongst the vacuoles (x 16,500)
- Fig. 76 A small spherical vacuole contained in the cytoplasm between three larger vacuoles of a root cap cell. Note the entire outline of the tonoplast in all vacuoles (x 23,300)

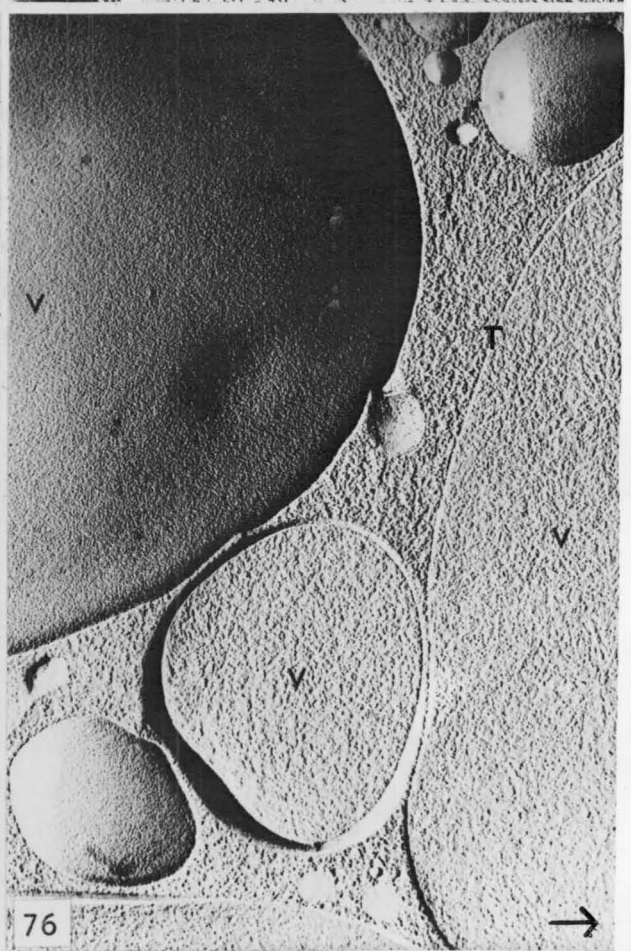
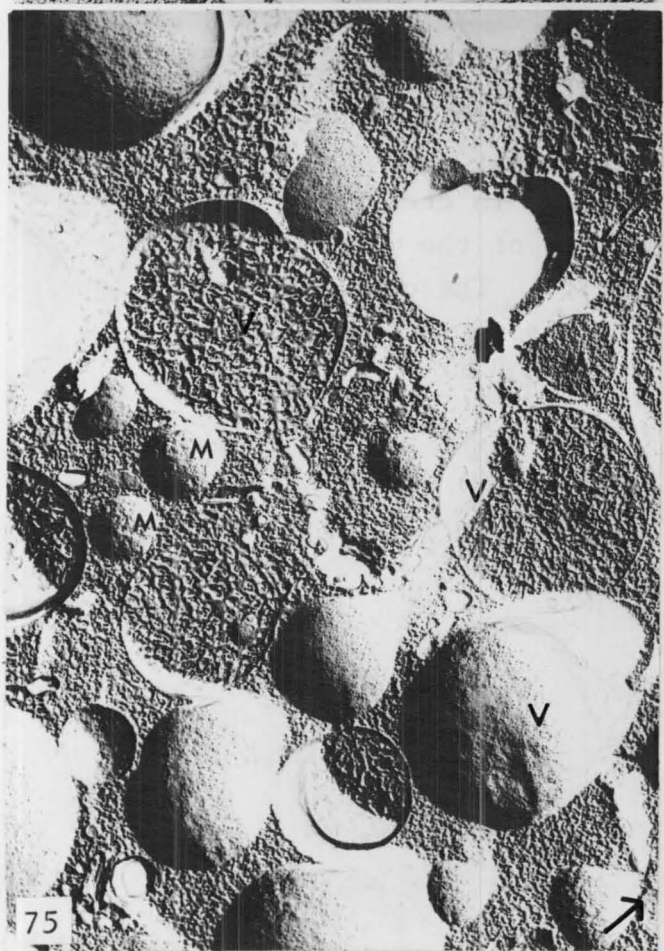
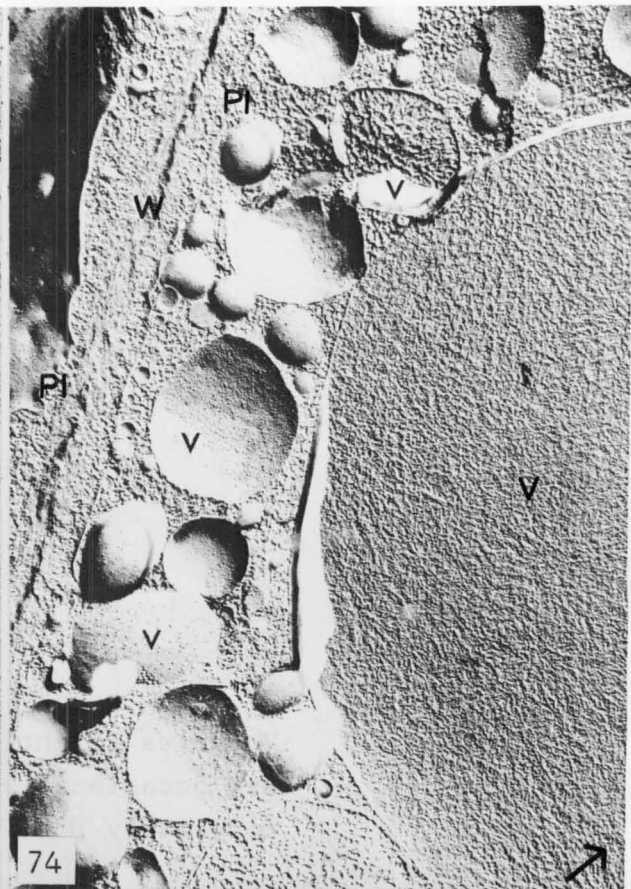
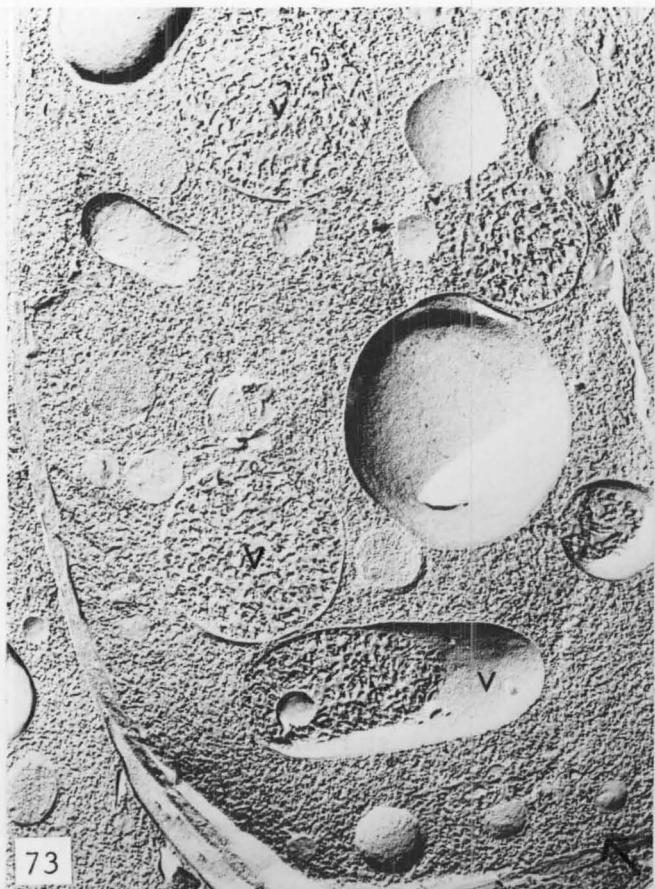
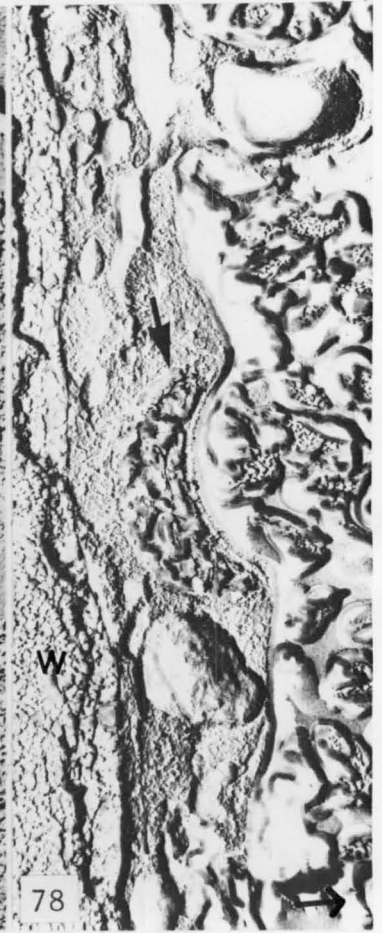
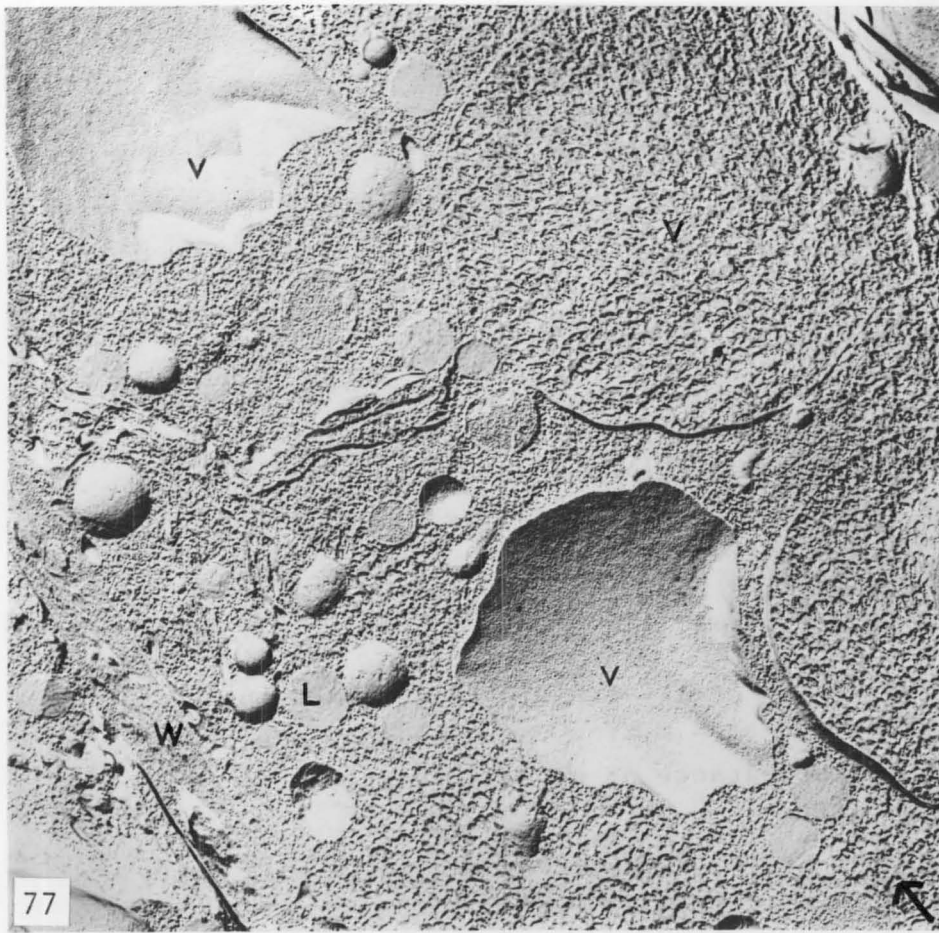


Fig. 77 Vacuoles slightly irregular in shape. These are occasionally found in viable cells with moderately poor freezing (x 24,900).

Fig. 78 Peripheral cytoplasm in highly vacuolated badly frozen cell of M. citrata. The small vacuole in the cytoplasm in crescentic in shape and the tonoplast of the main vacuole invaginates towards it. The condition resembles that seen in some sectioned material (cf. Fig.56) (x 20,900)



77

78

5. EFFECT OF VARIOUS CONDITIONS OF FIXATION ON
PRESERVATION OF VACUOLES FOR ELECTRON MICROSCOPY

5.1 Introduction

Methods of fixation of biological material for electron microscopy have been developed principally from work on animal tissue (see Pease, 1964; Trump & Ericsson, 1965; Glauert, 1966; Sjöstrand, 1967). Comparatively few fundamental studies have been made on plant material (e.g. Mollenhauer, 1959; McLean, 1960). Experiments on the fixation of plant tissues (e.g. Hairston, 1956; Caulfield, 1957; Ehrlich, 1958; Ledbetter & Porter, 1963; Sun, 1963; Esau, 1965c; Diers & Schötz, 1966; Hess, 1966; Rosen & Gawlik, 1966) have usually been incidental to other studies, few of which have been concerned with vacuoles.

No systematic study appears to have been made of how the various conditions of fixation affect the image of vacuoles in thin sections. The diverse interpretations of vacuole structure in electron microscopy (e.g. Buvat, 1957, 1958, 1961; Buvat & Mousseau, 1960; Roux, 1961, 1962; Manton, 1962; Bowes, 1965b) are probably the consequence of this lack of knowledge of vacuole behaviour during fixation. Recent observations on freeze-etch preparations suggest that many of the irregular forms seen in chemically fixed material are artifacts (Branton & Moor, 1964; Matile & Moor, 1968; Chapter 5).

In this chapter the results of investigations on preservation of vacuoles in root tips are outlined. A complete study of all possible parameters has not been made; instead certain factors commonly varied during fixation have been examined.

5.2. Literature Review

The effects on fine structure of various conditions of fixation have been studied by several workers and reviewed by Trump and Ericsson (1965).

Temperature: Opinion differs regarding the importance of temperature during fixation (See Pease, 1964; Sjöstrand, 1967; Wischnitzer, 1967). Some investigators find that temperature is unimportant (Millonig, 1962; Baker, 1965; Baker & McRae, 1966); others maintain that fixation should be at 4°C., because cellular activities and spontaneous post-mortem changes are decreased at this temperature. A few cytologists have found better preservation at temperatures above ambient (e.g. Manton, 1962). According to Glauert (1966), temperature is more important in fixing plant tissue than it is for animal material but the reasons for this are not given.

pH: Various views are held about the role of pH during fixation. Many light microscopists maintain that pH is important (see Zirkle, 1928; Gray, 1954; Baker, 1958) acid fixation being preferred for nuclear studies and alkaline fixation for cytoplasmic detail. Moss (1966) found a pH of 4.0 suitable for fixing various higher plant tissues in GA for light microscopy. Most work on the effect of pH on fixation for electron microscopy has involved animal tissue fixed in OsO₄. Palade (1952) obtained best results at pHs 7.2 to 7.4. He introduced Michaelis' veronal acetate buffer to prevent the wave of acidity which precedes the penetration of OsO₄. Claude (1961) has also found that pH is important in OsO₄ fixation. In contrast, Rhodin and Zetterqvist (see Sjöstrand, 1956b) found that pH could be varied from 5.1 to 9.5 without noticeable effect on the quality of preservation in OsO₄ fixation. Other workers similarly find that pH is unimportant during fixation (Malhotra, 1962a, 1963; Baker, 1965; Schultz & Karlsson, 1965). Sjöstrand (1956a, b, c, 1967) has concluded from these various studies that while a certain control of pH is desirable it is not as critical as Palade maintained.

Buffers: Several different buffers have been used for adjusting pH

during fixation (see Pease, 1964; Glauert, 1966; Sjöstrand, 1967). Veronal acetate - HCl is commonly used for this purpose because of its physiological properties. However, the use of veronal acetate has been criticised on the grounds that its buffering capacity falls off in extreme ranges (Malhotra, 1963; Baker, 1965; Schultz & Karlsson, 1965; see also West, 1963). Because of this, other buffers more effective in extreme pHs have been introduced for certain purposes. Phosphate buffers exist in living systems (Millonig, 1962; Glauert, 1966). It has therefore been found convenient to use them as buffers with OsO_4 (Millonig, 1961b, 1962), aldehydes (Holt & Hicks, 1961a & b; Sabatini et al., 1963), and KMnO_4 (Sedar, 1962). The effects on fine structure of various buffers have been studied in animal tissues (e.g. Palade, 1952; Millonig, 1962, 1966; Luft & Wood, 1963; Ericsson et al., 1965; Trump & Ericsson, 1965; Wood & Luft, 1965; Machado, 1967). Some workers attribute little importance to the particular buffer (e.g. Palade, 1952) but others find specific ion effects (e.g. Wood & Luft, 1965). The influence of different concentrations of buffer has also been examined (Vial & Orrego, 1966).

Concentration and Duration of Fixation: Several workers have examined the effects of fixative concentration and duration of fixation (e.g. Trump & Ericsson, 1965; Baker & McRae, 1966). The main result of increasing the concentration of KMnO_4 and OsO_4 is to enhance the contrast of the specimen (Palay & Palade, 1955; Palade, 1956; Afzelius, 1957, 1959; Pease, 1964). Within reasonable limits, variation in aldehyde concentration has little effect on fine structure preservation (Schultz & Karlsson, 1965; Baker & McRae, 1966; Maunsbach, 1966; Rosen & Gawlik, 1966; Machado, 1967). Studies with several fixatives indicate that duration of fixation is not critical (e.g. Palade, 1952; Manton, 1962; Baker, 1965; Baker & McRae, 1966; Paolillo et al., 1967), although prolonged exposure will cause extraction of some cell material (e.g. Falk & Sitte, 1963; Sjöstrand, 1967; Wischnitzer,

1967).

Addition of indifferent compounds: According to some researchers (Sjöstrand, 1953, 1956a, b, c, 1958; Rangan, 1960; Tahmisian, 1964) fixatives should be isotonic with respect to the tissue. This is achieved by addition of either salts (see Sjöstrand, 1967) or non-ionic substances such as sucrose (Caulfield, 1957). Zetterqvist's "balanced salts" (see Pease, 1964) and sucrose have been added to fixatives for plant tissues (e.g. McLean, 1960; Porter & Machado, 1960; Wooding & Northcote, 1965, 1966; Pickett-Heaps & Northcote, 1966) but the effect on preservation has not been evaluated.

5.3 Materials and Methods

In initial experiments roots of Lonicera nitida were used. In later trials Mentha citrata and Avena sativa were substituted, as material at the same stage of differentiation could be more readily selected. All root tips used were at comparable stages of growth.

Over 120 different fixations were made for the study. An average of 6 root tips were prepared per fixation. At least two blocks were sectioned per fixation and 2 to 6 grids examined from each block. Observations were confined to the root cap and the first 0.5 to 1.0 mm of basipetal tissues.

The following conditions were varied and the effect on vacuole preservation examined.

1. Evacuation during fixation: Roots of Lonicera were fixed in standard (see Section 2.3 1) buffered KMnO_4 , one group without evacuation, the other evacuated at 64 cm pressure of mercury for the first 30 minutes of fixation.
2. Temperature during fixation: Specimens of Lonicera were preserved in standard permanganate fixatives and in standard GA/OsO_4 at the following temperatures ($^{\circ}\text{C}$):-

(a) aqueous KMnO_4 : 4, 16, 20

(b) buffered KMnO_4 : 4, 16, 20, 25, 30, 37

- (c) GA/OsO₄: 4, 20
3. pH of fixative: Experiments using different buffers (see Vogel, 1946; Glauert, 1966; Sjöstrand, 1967) in 2% KMnO₄ for 2 hours at room temperature were conducted on Lonicera.
- (a) Veronal acetate - HCl buffer (0.14M) at pHs 2.0 to 13.5 at intervals of pH 0.5. Most pH levels were tested at least twice; 40 fixations were examined.
- (b) Sodium acetate buffer at pHs 2.0 to 5.0 (intervals of pH 0.5).
- (c) Sorensen's phosphate buffer (0.066M) at pHs 5.5, 6.0, 7.0 and 8.0.
4. Concentration of fixative:
- (a) Roots of Lonicera were fixed in standard aqueous and buffered KMnO₄ at % concentrations (W/V) of 0.25, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 and saturated KMnO₄ (6.4% at 20°C).
- (b) Roots of Lonicera and Avena were fixed in standard GA/OsO₄ (see section 2.3 1) but at % concentrations of GA of 0.78, 1.56, 3.12, 6.25, 12.5, 25.0 and 50.0. OsO₄ was kept constant at 1%.
- (c) Roots of Avena were fixed in standard GA/OsO₄ but at % concentrations of OsO₄ of 1.0, 2.0 and 5.0. GA was kept constant at 6%.
5. Duration of Fixation: Specimens of Lonicera were fixed in standard permanganate fixatives for durations of:-
- (a) aqueous KMnO₄: 15 and 30 minutes; 1, 2, 3, 4, and 8 hours.
- (b) buffered KMnO₄: 2, 15 and 30 minutes; 1, 2, 3, 4, and 8 hours.
6. Addition of Zetterqvist's "Balanced Salts": 3.4 ml of salt solution (see Glauert, 1966 or Sjöstrand, 1967) containing Na, K and Ca chlorides were added to 46.6 ml of standard aqueous and buffered KMnO₄ and to standard GA/OsO₄. The molarity of the balanced salts in each fixative was about 0.003M. The experiments used Lonicera and Mentha.
7. Addition of Sucrose: Root tips of Avena were fixed in 6% GA in 0.66M

phosphate buffer made up in 10% sucrose, washed in buffer containing 10% sucrose, and postfixed in 2% buffered OsO_4 in 10% sucrose. Duration, pH and temperature of fixation followed that of standard GA/ OsO_4 .

8. Different Concentrations of Buffer: Roots of Mentha were fixed in 3% KMnO_4 (0.189M) in veronal acetate - HCl buffer of various concentrations. Serial dilutions of buffer gave fixatives with buffer molarities of: 0.28, 0.224, 0.168, 0.14, 0.112, 0.056, 0.028 and 0.00. Fixations were for 2 hours at room temperature. Trials using 0.066M and 0.016M phosphate buffer in GA/ OsO_4 fixative were conducted on Avena and Mentha.
9. Different buffers at the same molarity and the same pH: Root tips of Avena were fixed with 2% KMnO_4 in 0.14M buffer. Veronal acetate - HCl, sodium cacodylate and Sorensen's phosphate buffers were used at pHs 6.8. Fixations were for 2 hours at room temperature.
10. Osmium tetroxide fixation: Root tips of Avena were preserved on 2% OsO_4 made up in water or in 0.28M veronal acetate - HCl buffer (pH 6.8) for 2 hours at room temperature. Molarities of the solutions were about 0.08M and 0.36M, respectively.
11. Buffering at Different Stages in GA/ OsO_4 Fixation: Root tips of Avena were preserved in standard GA/ OsO_4 . Phosphate buffer (0.066M) was present at stages indicated (x):-

| GA | Wash | OsO_4 |
|----|------|----------------|
| - | - | - |
| - | x | - |
| x | x | - |
| - | - | x |
| x | x | x |

12. GA/ KMnO_4 Fixation and Buffering at Different Stages: Roots of Avena were fixed at 4°C in 6% GA (4 hours) followed by a wash (3 hours), then post fixed in 3% KMnO_4 (3 hours). Phosphate buffer (0.066M) was

introduced at the following stages (x):-

| <u>GA</u> | <u>Wash</u> | <u>KMnO₄</u> |
|-----------|-------------|-------------------------|
| - | - | - |
| x | x | x |
| x | - | x |
| - | - | x |

5.4 Results

The effect on vacuolar preservation of the various conditions of fixation tested are outlined below.

5.4 1 Evacuation

Vacuoles fixed without evacuation (Fig. 79) exhibited a similar range of form to those preserved under vacuum (e.g. Fig. 61).

5.4 2 Temperature

Vacuoles of Lonicera showed the same form irrespective of temperature during fixation (compare Figs. 35 & 62 with 80, 81, 82). Other cellular organelles were also unaffected by temperature. In aqueous KMnO₄, irregular vacuoles were common (Fig. 80). Mainly rounded structures occurred in cells preserved in buffered permanganate (Figs. 81 & 82) and in GA/OsO₄.

These observations show that temperature is not critical. The comparison between aqueous and buffered material suggests that presence of buffer is more important in maintaining vacuoles as rounded structures than is temperature during fixation. Although Manton (1962) claimed good preservation at 25°C., and at higher temperatures, in meristem cells of Anthoceros, the vacuoles displayed considerable shrinkage typical of cells fixed in aqueous KMnO₄.

5.4 3 pH during KMnO₄ fixation

A. Veronal acetate - HCl buffer: Vacuoles were little affected by pH (Figs. 83-90) except at extreme levels. Schultz and Karlsson (1965) have also found adverse effects of high and low pHs in animal tissue fixation.

Between pHs 2.0 and 12.5 vacuoles were mainly rounded (Figs. 83-89). At high pHs (12.5 - 13.5) the structures were shrunken and pseudovacuaoles had developed within the cytoplasm and nucleus (Fig. 90). The vacuolar contents were not preserved at pH 2.0 (Fig. 83) but at pHs above this contents were usually precipitated as dense material (Figs. 84-90). The tonoplast was preserved over a wide range of pH; on the acid side of neutrality it was fixed above pH 3.0 while in the alkaline range the tonoplast was still visible at pH 12.5.

- B. Sodium acetate - HCl buffer: Vacuoles were mainly round at pHs 2.0 to 5.0 (Figs. 91 & 92), but the tonoplast and contents were not fixed. Other organelles were also poorly preserved at these pHs.
- C. Phosphate buffer: Vacuoles were fixed between pHs 5.5 and 8.0 (Figs. 91 & 92). Vacuoles were more irregular with the phosphate buffered fixative than with the veronal-acetate fixation at corresponding pHs (cf. Figs. 91 & 92 with 85 & 87).

These experiments with Lonicera indicate that pH is not critical for preserving vacuoles. Studies with animal tissues show that pH plays little part in fixation (e.g. Sjöstrand, 1956a; Malhotra, 1962a). The buffer seems to be more important in functioning as an "indifferent" salt (Baker, 1960) than in maintaining pH. This conclusion is further demonstrated by comparing vacuoles fixed in aqueous KMnO_4 with those in the buffered solution. In the aqueous fixative vacuoles are mainly irregular while in buffered KMnO_4 they tend to be more rounded.

General preservation of fine structure is better at alkaline pHs than on the acid side of neutrality. Studies on animal tissues support this observation (Palade, 1952; Baker, 1965; Bowes & Cater, 1966). In contrast, some workers maintain the pH of the fixative should correspond to that of the tissue (Millonig, 1962; Tahmisian, 1964); pHs around 6.8 to 7.4 are commonly selected for this reason. pHs between 5.0 and

6.5 are normal for higher plants (see Hurd-Karrer, 1939; Drawert, 1955a; Voeller, 1964), but this study shows that such pHs are unsuitable for preservation of fine structure. Optimal preservation in Lonicera was obtained at pHs 7.0 to 8.5. Observations on animal tissue confirm that alkaline fixation is preferable for hydrated tissues (see Palade, 1956; Trump & Ericsson, 1965).

The results with different buffers demonstrate that various ions have specific effects on preservation of vacuoles. Sodium acetate is obviously unsuitable as a "physiological buffer" (Wood & Luft, 1965). Sorensen's phosphate buffer is inferior to veronal acetate at the molarities used. Work with animal tissue fixed in OsO_4 has shown that phosphate buffer gives poorer results compared with veronal acetate (Palade, 1952).

5.4 4 Fixative concentration

- A. KMnO_4 : In Lonicera irregular vacuoles occurred at all concentrations of the aqueous fixative (Figs. 95-97). Vacuoles preserved in the buffered solution were predominantly rounded irrespective of concentration (Figs. 98-100). The tonoplast was poorly fixed below 1% but well preserved at higher levels (Fig. 100). With increasing concentration, the vacuolar contents become darker, optimal contrast lying between 2 and 4% KMnO_4 . At the same and different concentrations, however, the contents often varied in appearance depending on the composition of the vacuoles. In actively differentiating cells the contents were denser (Fig. 79) than in dormant cells (Fig. 250).
- B. GA: Only small differences were noted in the shape of vacuoles fixed at different concentrations. At 50% GA vacuoles were slightly more irregular than those fixed at lower concentrations. But the degree of shrinkage was small compared with vacuoles preserved in aqueous KMnO_4 .

C. OsO₄: Vacuoles exhibited a similar form irrespective of the concentration of osmium. The main effect of higher concentrations was an increased contrast of contents, where these had been precipitated.

These observations indicate that fixative concentration plays little part in preserving vacuoles as rounded structures. This confirms the work of Manton (1962) for vacuoles of Anthoceros fixed in KMnO₄. Studies with other plant (Rosen & Gawlik, 1966) and animal tissues (Palay & Palade 1955; Palade, 1956; Afzelius, 1957, 1959; Schultz & Karlsson, 1965; Maunsbach, 1966; Machado, 1967) preserved in different fixatives also show that fixative concentration is not critical for fine structure preservation.

5.4 5 Duration of KMnO₄ fixation

Vacuoles in Lonicera roots fixed for 2 minutes were indistinguishable in shape from those preserved for 8 hours (Figs. 101-107). In aqueous permanganate the structures were usually irregular (Figs. 106, 107) but in the buffered solutions rounded forms were dominant (Figs. 101-105). The vacuolar contents became progressively darker up to about 3 hours fixation, thereafter any further change was slight. Most cell structures were clearly visible after 2 minutes fixation.

The observations show that duration of fixation is unimportant in preserving vacuoles. The results agree with those obtained by Manton (1962) using KMnO₄. The irregular shapes found after 2 minutes fixation in aqueous solutions suggest that the vacuoles shrink almost immediately after the roots are placed in the fixative. Penetration of KMnO₄ must therefore be rapid. This is in contrast to results published by Bradbury and Meek (1960) for artificial gels fixed in KMnO₄.

5.4 6 Addition of indifferent compounds

In Lonicera and Mentha Zetterqvist's "balanced" salts added to GA/OsO₄ (Fig. 109), aqueous KMnO₄ (Fig. 108), and buffered KMnO₄ produced no

detectable effect on vacuolar preservation. The vacuoles showed a similar form to those preserved in the standard fixatives (compare Fig. 108 with 54).

Addition of sucrose to GA/OsO_4 produced variable results in Mentha. Some cells showed good preservation (Fig. 110) but in others irregular vacuoles were common (Fig. 111). Many cells of the root cap displayed severe shrinkage (Fig. 111). These observations suggest that the fixative was hypertonic for some cells but probably isotonic for others.

5.4 7 Different concentrations of buffer

- A. Veronal acetate buffer: The shape of vacuoles changed as the concentration of buffer was altered (Figs. 114-119). Differences were slight at successive concentrations but unmistakable over the whole range examined. At no concentration was it possible to preserve all vacuoles satisfactorily without shrinkage or rupture. Apart from large vacuoles in outer cap cells, most of the structures became progressively more irregular as the concentration of buffer was lowered. At high concentrations vacuoles tended to burst, especially in cells near the surface of the cap. Between 0.28M and 0.056M buffer vacuoles about the size of amyloplasts were mainly rounded. Below 0.056M buffer few vacuoles were preserved as rounded structures. Irregularity was greatest when buffer was absent (Fig. 119).
- B. Phosphate buffer: Roots of Avena fixed in GA/OsO_4 containing 0.066M phosphate buffer showed mainly rounded vacuoles (Fig. 112). At 0.016M buffer slight irregularity occurred (Fig. 113).

These experiments demonstrate that the concentration of veronal acetate buffer is important for fixing vacuoles in KMnO_4 . The presence of phosphate buffer is, however, less critical for vacuoles preserved in GA/OsO_4 . In KMnO_4 fixation, the buffer seems to behave as an indifferent

salt in raising the ionic strength of the fixative.

5.4 8 Different buffers at the same molarity and same pH in $KMnO_4$ fixation

Roots preserved in permanganate containing 0.14M veronal acetate buffer showed some rounded and some irregular vacuoles. With 0.14M phosphate buffer results were more variable. Some specimens had vacuoles resembling those obtained with veronal acetate buffered fixative but in other root tips few vacuoles were visible. In these the tonoplast was not fixed and the vacuolar contents merged with the groundplasm. Few vacuoles were preserved when 0.14M sodium cacodylate buffer was used. The groundplasm and membranes showed a coarse granular texture in both phosphate and cacodylate buffered material.

The experiments show that various buffers at the same molarity and the same pH may have different effects on preservation of fine structure. For fixation with $KMnO_4$, veronal acetate buffer gives better preservation of vacuoles, and other cellular components, than do either phosphate or cacodylate buffers at 0.14M.

5.4 9 OsO_4 fixation

Roots of Avena fixed in aqueous OsO_4 showed irregular vacuoles (Fig. 121) although shrinkage was less than in aqueous permanganate. When the fixative was buffered shrinkage was reduced (Fig. 124) but irregular vacuoles still remained common (Figs. 122, 123). In both fixatives, vacuoles about the size of mitochondria showed better preservation than larger structures (compare Figs. 120 & 124 with 122 & 123). Cell walls, plastids and mitochondria usually had wrinkled outlines suggesting hypertonic conditions during fixation. Difficulty in fixing plant tissue in OsO_4 is noted by other workers (Ledbetter & Gunning, 1964).

Although these experiments show that presence of buffer is advantageous when fixing vacuoles in OsO_4 , buffering is not as important as for $KMnO_4$ fixation. In studies with animal tissue some workers obtain better results

using buffered OsO_4 (e.g. Palade, 1952; Schultz & Karlsson, 1965), but others find little difference between the aqueous and buffered solutions (Claude, 1961; Malhotra, 1962a & b, 1963; Baker, 1965).

5.4 10 Buffering at different stages during GA/ OsO_4 fixation

Introduction of veronal acetate buffer at various stages during fixation produced only slight differences in the appearance of vacuoles (Figs. 125-128). In Avena, vacuoles preserved according to an entirely aqueous schedule were mainly rounded (Fig. 125). When buffer was present during washing, vacuoles were a little more irregular (Fig. 126) than those in the material fixed according to the aqueous schedule. With buffering at the GA stage, the OsO_4 stage, or at all steps in fixation vacuoles resembled those found when buffering was confined to the washing stage.

Buffer ions thus appear to be unimportant for preserving vacuoles in GA/ OsO_4 at any stage during fixation. In contrast to buffered KMnO_4 , the GA seems to stabilize the vacuole directly. When buffer ions are present they raise the ionic strength of the fixative vehicle which may lead to shrinkage of the vacuoles. Possibly the buffer ions penetrate faster than those of the fixative (Millonig, 1962), causing the vacuoles to collapse before the tonoplast and contents are properly fixed by GA. As slight shrinkage occurs when aqueous GA fixation is followed by stages which are buffered, this suggests that vacuoles are not completely stabilized by GA. Schultz and Karlsson (1965) have found that certain animal tissues are incompletely fixed by GA. Although aqueous GA give satisfactory fixation of vacuolar form it is unsuitable for general preservation because its lower pH causes some extraction of ground-plasm (Figs. 125, 126, 128).

5.4 11 GA/ KMnO_4 fixation with buffering at different stages.

Recently GA combined with KMnO_4 has been used for fixation of plant tissue (Gifford & Stewart, 1967; Tucker & Hoefert, 1968).

Root tips of Avena preserved in GA/ KMnO_4 retained some characteristics

of both fixative components, while others were modified (Figs. 129-133). With GA fixation the tonoplast sometimes becomes detached from the vacuole (Fig. 130) (Gifford & Stewart, 1967). The vacuolar contents are modified by GA in that they fail to produce the dense precipitates often found after standard permanganate fixation. However, general preservation of cellular detail in GA/KMnO₄ more closely resembles that of KMnO₄ than that of GA/OsO₄ fixation. The soft grainy appearance of groundplasm and membranes is typical of phosphate-buffered KMnO₄ (Figs. 129-133).

When phosphate buffer was introduced at various stages of fixation there was comparatively little effect on vacuolar preservation. Vacuoles were mainly rounded, with some irregular forms, when buffer was present at all stages in the schedule (Figs. 130, 131). The vacuoles, however, were slightly less irregular than those in standard buffered KMnO₄. With aqueous fixation throughout, vacuoles were less rounded than those in the buffered series and preservation of groundplasm was poor. When buffering was carried out at the GA and washing stages (Fig. 132), the vacuoles resembled those seen when all stages were buffered. When buffer was present only at the KMnO₄ stage similar results were obtained (Fig. 133).

These experiments indicate that fixation in GA prior to KMnO₄ is more important in maintaining vacuoles as rounded structures than is presence of buffer at any stage in the schedule. Post fixation in aqueous KMnO₄, for example, produces less irregular vacuoles than fixation in standard aqueous KMnO₄. As with GA/OsO₄, buffering in the GA/KMnO₄ schedule raises the pH of the GA and this appears to reduce extraction of groundplasm.

5.5 Discussion

The various shapes obtained under different conditions of fixation demonstrate the sensitivity of vacuoles to specimen preparation. By contrast, most other structures in root tip cells have a characteristic form irrespective

of the fixative used. Studies on animal tissues also indicate that certain cell components are more sensitive to fixation than others (Sjöstrand, 1967). The high water content and associated osmotic properties probably play an important role in the behaviour of vacuoles on fixation. It seems unlikely that shrinkage of vacuoles is primarily caused by dehydration after specimens have been fixed because throughout the project the schedule for dehydration was standardised.

Vacuoles are diverse structures (Zirkle, 1928, 1937; Bailey, 1930; Matile, 1968; Matile & Moor, 1968). It is perhaps not surprising therefore that difficulty is experienced in obtaining good preservation of all vacuoles within a cell, in different cells and in root tips at various stages of development.

Preservation of vacuoles involves a reaction between the fixative, the tonoplast and the vacuolar sap. The chief variable in this reaction is probably the composition and concentration of vacuolar contents; this may lead to differences in preservation. The various shapes and densities of contents obtained - especially in aqueous $KMnO_4$ - therefore give a visual indication of the diversity of vacuoles within and among cells.

When a cell is bathed in a fixative several changes may occur (Bernard & Wynn, 1965). Early stages of fixation probably destroy the differential semi-permeability of the tonoplast (McLean, 1960) so that a new osmotic relationship is established between the vacuole and fixative. Vacuoles might be expected to shrink, burst or maintain a rounded shape depending on whether hypotonic, hypertonic or isotonic conditions prevail. The difference in the osmotic pressures of the fixative and the vacuole will probably influence the behaviour of vacuoles on fixation.

5.5 1 Importance of the various conditions of fixation examined

The present experiments show that only a few of the conditions of fixation tested are directly important in stabilizing vacuoles as rounded

structures in thin sections. Evacuation during fixation, temperature, pH, duration of fixation and concentration of the fixative are relatively unimportant. In fact, variation in preservation is often greater as a result of the different physiological conditions of the specimen. The main consideration for good fixation of vacuoles seems to be the fixative itself. Fixatives based on GA are superior to those based on KMnO_4 or OsO_4 alone.

5.5 2 Effect of molarity

Although molarity is important in that it influences osmotic conditions, it seems that molarity alone is not the main factor involved in vacuolar preservation. Vacuoles assume various images in different fixatives of comparable molarity. When 2% KMnO_4 is buffered with different buffers of the same molarity preservation of vacuoles is also variable.

5.5 3 Effect of different ions

Variations in fine structure of animal tissues have been reported when different buffers are used (Palade, 1952; Ericsson *et al.*, 1965; Wood & Luft, 1965). While some workers favour a particular buffer, others place little importance on the nature of the buffer provided it is physiological and the tonicity of the fixative is maintained (Schultz & Karlsson, 1966). The importance of non-buffer ions in fixation has also been examined; for example, permanganates of different metals have been found to affect the dimensions of fixed membranes (Wetzel, 1961; Afzelius, 1962).

The present experiments suggest that the behaviour of specific ions in a buffer is more important in the preservation of vacuoles than is the total molarity of the buffer. It has been stressed by Millonig (1962) that it is erroneous to relate molarity to osmotic pressure because various substances dissociate differently. Only osmotic pressure - as determined by freezing point depression - should be used as a measure of tonicity. The most fundamental consideration for preserving vacuoles is probably the effective concentration of osmotically active ions in the fixative vehicle (see also

Schultz & Karlsson, 1965; Maunsbach, 1966). Fixatives isotonic to the vacuole might be expected to be desirable. However, it is doubtful whether this can be satisfactorily achieved owing to the diverse composition of vacuoles. In animal cytology, fixative osmolarity is stressed by some groups of workers (Sjöstrand, 1953, 1956a, b & c, 1967; Caulfield, 1957; Elfin, 1962; Maunsbach et al., 1962; Schultz & Karlsson, 1965; Maunsbach, 1966; Vial & Orrego, 1966) but considered to be overemphasised by others (Baker, 1958; Malhotra, 1962b, 1963; Fawcett, 1964). The relative importance of various components in a fixing solution will probably vary depending on the particular tissue and its physiological state at the time of fixation.

5.5.4 Relationship between molarity, osmolarity and tonicity

The relationship between these properties is complicated; it does not follow a simple linear sequence with increasing concentration (Powell et al., 1964; Maser et al., 1967). Furthermore, results do not always agree with theoretical predictions from physical considerations of the fixative. Glutaraldehyde may be cited as an example. The concentration of GA used by many workers is hypertonic (Pease, 1964), in some instances with a very high osmolarity (Karnovsky, 1965), yet tissues may exhibit little sign of shrinkage. This is shown also by the present experiments where increasing the concentration of GA from 0.78% to 50.00% produced relatively small changes in the appearance of vacuoles. But when a small quantity of buffer (0.066M) was added to 6% GA, shrinkage of some vacuoles invariably occurred. In working with animal tissue, Schultz & Karlsson (1965) and Maunsbach (1966) have found that fixative concentration can be changed over a wider range, without causing cellular damage, than can the buffer. Total osmolarity of the fixative may therefore be misleading. What should be known is the osmolarity of the various components in the fixative vehicle (Maunsbach, 1966).

The relative importance of buffer and fixative ions is further demonstrated by vacuoles fixed in KMnO_4 . By keeping the molarity of buffer constant while altering the concentration of KMnO_4 (0.015M to 0.379M) the appearance of vacuoles remained much the same (Figs. 98,100). Different molarities of aqueous KMnO_4 similarly produced little change in the appearance of vacuoles, except that the structures remained irregular (Figs. 95, 97). In contrast, when the molarity of buffer was changed (0.028M to 0.28M) and the concentration of KMnO_4 was kept constant, marked differences occurred in the shape of vacuoles (Figs. 114-119). This clearly demonstrates the role of different ions in fixation.

5.5 5 KMnO_4 fixation

Preservation of vacuoles in KMnO_4 involves reactions different from those occurring in OsO_4 and GA fixations. As a preservative for electron microscopy (Luft, 1956; Mollenhauer, 1959; Bradbury & Meek, 1960; Glauert, 1966), KMnO_4 differs from most other fixatives in that it is a strong oxidizing agent which destroys RNA but unmaskes lipo-protein. With sugars it interacts to form dense precipitates (Johnson, 1966) which resemble the contents of many vacuoles fixed in KMnO_4 .

The formation of vacuolar precipitates on fixation in KMnO_4 seems to lower the osmotic pressure within the vacuole. As a result, water apparently moves from the vacuole causing the vacuole to collapse. The degree of shrinkage will thus vary depending on the difference between the osmotic pressure of the vacuole after fixation and that of the preservative. In contrast to KMnO_4 , GA and OsO_4 usually cause little precipitation of vacuolar contents. This may explain, in part, why these fixatives give better preservation of vacuoles compared with permanganate.

Small vacuoles in embryonic cells and enlarging vacuoles of differentiating tissue appear to be less highly hydrated than fully enlarged vacuoles or small

vacuoles in root tips less active in growth. This probably explains why vacuoles in embryonic and actively differentiating cells shrink more (in aqueous KMnO_4) than those in other parts of the root tip. It would also account for the reasonable preservation of vacuoles in dormant roots compared with those active in growth.

When KMnO_4 is buffered the reaction between the vacuolar sap and the permanganate is modified. The buffer ions seem to function as an indifferent salt in raising the tonicity of the fixative (Tahmisian, 1964; Sjöstrand, 1967). However, the relationship between buffer and fixative is complicated owing to the strong reaction of the permanganate ions with the contents of the vacuole. Exactly how the buffer operates is not fully understood. Possibly the buffer replaces those ions and molecules rendered osmotically inactive through precipitation by KMnO_4 . In this way an osmotic balance might be established between the vacuole and the fixative and the volume of the vacuole maintained. The establishment of such a balance will probably depend on whether the buffer and the fixative ions reach the vacuole at the same or different times. Work with OsO_4 fixation has shown that ions of certain buffers reach deeper parts of a tissue before those of the fixative (Millonig, 1962). If the buffer entered the vacuole before the fixative, the osmotic pressure of the vacuole would presumably be raised through addition of ions. This might explain why some vacuoles are prone to burst in buffered KMnO_4 . On the other hand, if permanganate ions reached the vacuole first, or if the concentration of buffer was insufficient to replace all ions removed through precipitation, then some vacuolar shrinkage might be expected to occur.

Some workers consider that the buffer in KMnO_4 fixation contributes no important properties to the fixative (Pease, 1964). With plant tissue (e.g. Mollenhauer, 1959), buffer is frequently omitted with results that are nearly indistinguishable from those obtained from buffered solutions (Pease, 1964).

Although this applies to the preservation of most organelles, differences are apparent in the appearance of vacuoles; this feature seems to have been overlooked by most researchers. Another noticeable effect of buffering is an increased granularity of groundplasm and membranes compared with that in aqueous KMnO_4 .

5.5 6 Effect of buffering in GA and OsO_4 fixation

While buffer is important in functioning as an indifferent salt in KMnO_4 preservation, buffering is less critical for GA and OsO_4 fixation of vacuoles. The presence of buffer in GA fixative vehicles serves mainly to stabilize the pH near neutrality; this improves preservation of groundplasm. In fact, addition of buffer, or sucrose, seems to raise the osmotic pressure of the fixative; this may lead to greater shrinkage of vacuoles. Probably the most important factor in GA and OsO_4 preservation is the nature of the fixative itself. These fixatives, especially GA, are usually regarded as good preservatives (see Pease, 1964; Sjöstrand, 1967) in that they maintain cellular structure with minimal alteration. When vacuoles are first fixed in GA this seems to give them a greater degree of stability to withstand post fixation procedures.

5.6 Conclusion

In root tips, vacuoles are found to be the most sensitive of the organelles to fixation damage. The appearance of the vacuoles seems to be determined largely by the reaction of the preservative with the vacuolar sap. Since the composition and concentration of sap varies among vacuoles it is often difficult to achieve satisfactory preservation of all vacuoles using any one fixative. Because of the diversity of cells in a small mass of tissue, root tips possess certain disadvantages for experiments on the preservation of vacuoles. The ideal tissue for such work would be homogeneous but this may be unattainable in plant material.

Within the limits of this study, the most important conditions affecting

the preservation of vacuoles are the fixative itself and the concentration of other ions contributing to the osmotic pressure of the fixative. Further studies are required to evaluate more closely the role of the osmolarity of the various ions in vacuolar preservation.

The best preservation of vacuoles was obtained with standard GA/OsO₄ of the fixatives tried. Possibly this fixative could be improved if the buffer were omitted and the GA neutralized with some other compound that would not greatly alter the osmotic pressure of the fixative. Alternatively, neutral GA might be used. With GA preservation, the aldehyde itself apparently plays a direct role in maintaining vacuoles as rounded structures. The presence of buffer increases the ionic strength of the solution and this may cause some vacuoles to shrink. The main function of the buffer is apparently to stabilize the pH near neutrality and thus to minimize the extraction of ground-plasm and other cellular material.

OsO₄ and KMnO₄ used singly are poor fixatives for vacuoles. Aqueous KMnO₄ was least suitable because of its reaction with the vacuolar sap to form insoluble precipitates. The precipitates seem to lower the osmotic pressure of the vacuole causing the vacuoles to collapse. The presence of buffer, acting as an indifferent salt, appears to raise the ionic strength of the fixative; this helps to maintain an osmotic balance between the vacuole and the fixative. Although buffer ions were found suitable for this purpose, ions contributed by Zetterqvist's balanced salts proved ineffective. The main advantage of using KMnO₄ for fixing vacuoles is to demonstrate differences between vacuoles. With aqueous KMnO₄ these are shown by the degree of irregularity in shape and the differences in the density of contents among vacuoles.

The present study is a contribution towards our understanding of the preservation of vacuoles for thin sectioning. Some of the factors which influence the preservation of vacuoles in root tips have been established.

However, much fundamental work is still required to examine some of these conditions of fixation in detail and to determine other factors which might also be important in the preservation of vacuoles. The possible interaction of fixative variables might be investigated; for instance, the effects of buffer might well vary with temperature.

5.7 Summary

A systematic survey has been made of the preservation of vacuoles in root tips. KMnO_4 , OsO_4 and GA were used as fixatives singly and in combination, with and without buffer. Evacuation, temperature, concentration, duration, pH and addition of Zetterqvist's balanced salts had little effect on the preservation of vacuoles. Variation in fixation was often more marked as a result of the different physiological conditions of the vacuoles and the specimens. The main consideration for good preservation of vacuoles seems to be the fixative itself. With KMnO_4 , the presence of buffer ions functioning as indifferent salts is also important; these raise the ionic strength of the fixative. GA followed by OsO_4 or KMnO_4 is superior to fixatives containing only KMnO_4 or OsO_4 . Molarity apparently affects vacuolar preservation mainly through the contribution of individual ions. Changing the concentrations of fixative ions has less effect on quality of preservation than changing the concentration of the buffer. In KMnO_4 fixation the precipitation of vacuolar contents in many vacuoles seems to modify the osmotic relationship between the vacuole and the fixative; this usually leads to the shrinkage of the vacuole. The addition of buffer modifies the reaction between KMnO_4 and the vacuolar sap and apparently helps maintain an osmotic balance between the vacuole and fixative.

Figure 79 Effect of evacuation.

Fig. 79 Root cap cell prepared without evacuation during fixation. The vacuoles are mainly rounded though small ones show irregularity. (x 5,720).

Figures 80 - 82 Effect of temperature.

Fig. 80 Cortical cells near the promeristem fixed in aqueous KMnO_4 at 4°C . The vacuoles show the usual irregular appearance on aqueous fixation. (x 19,360).

Fig. 81 Procambial cells in buffered KMnO_4 at 4°C . (x 14,960).

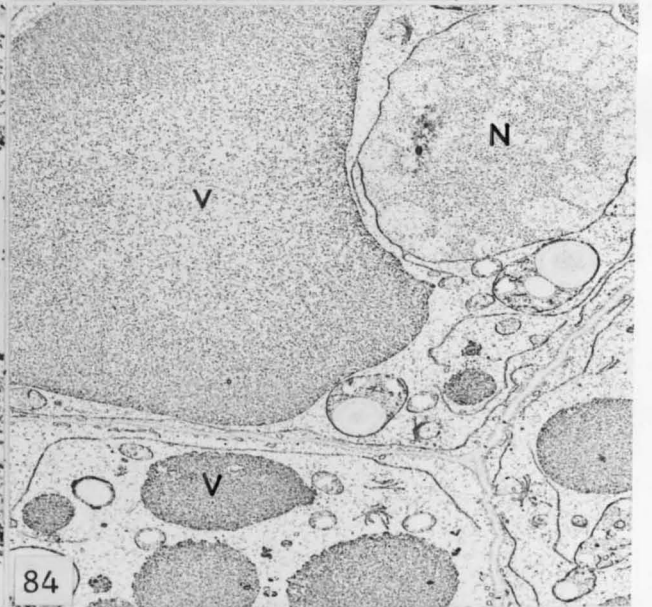
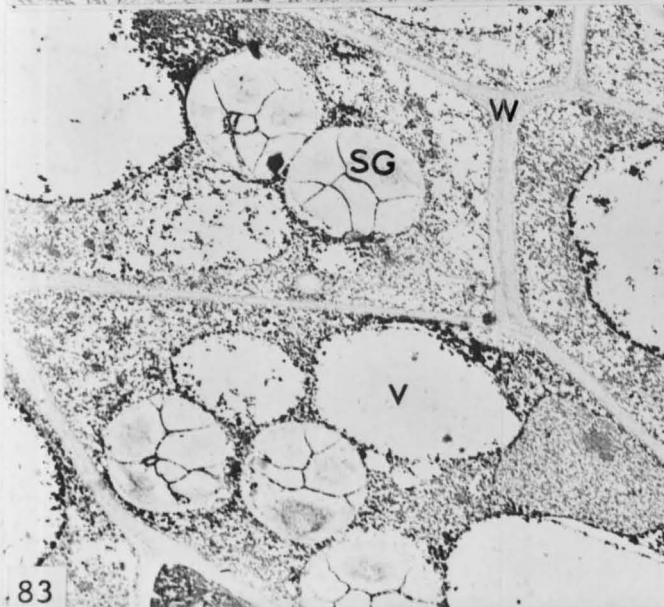
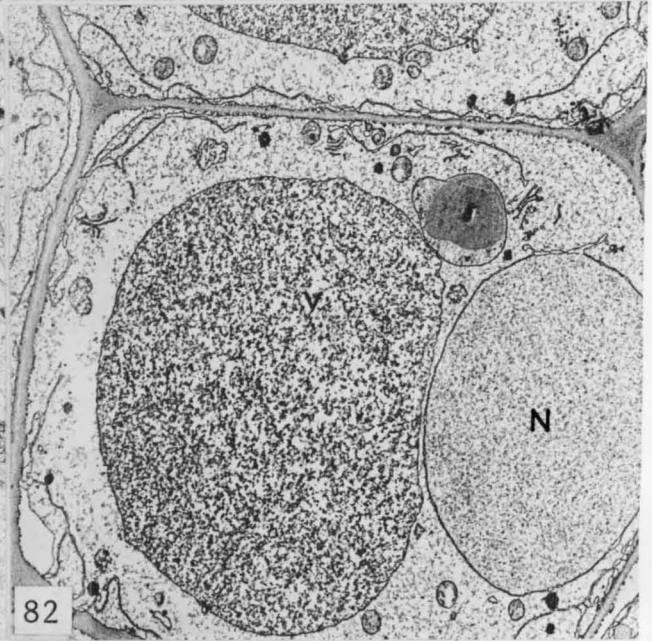
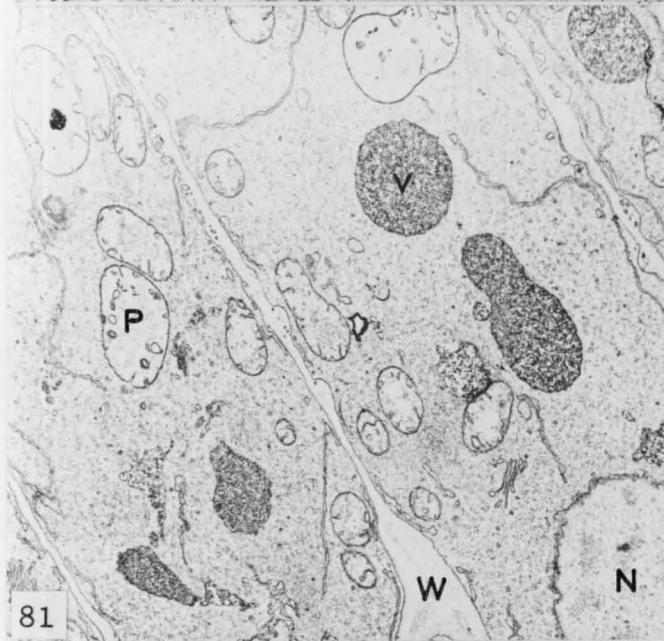
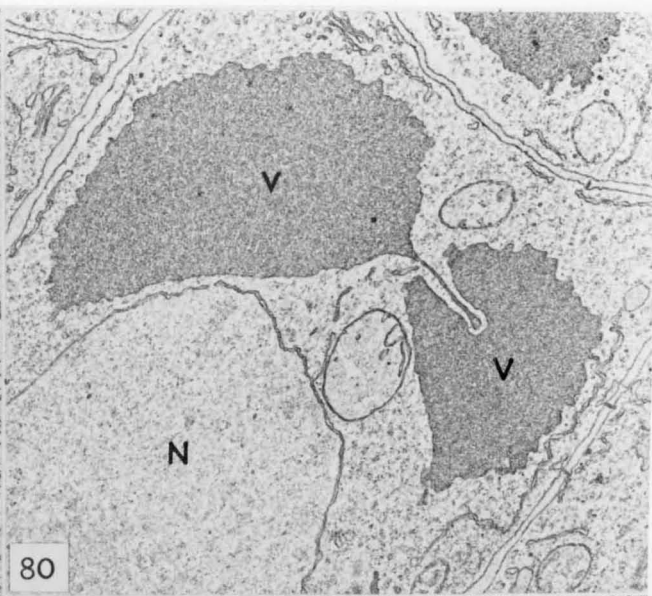
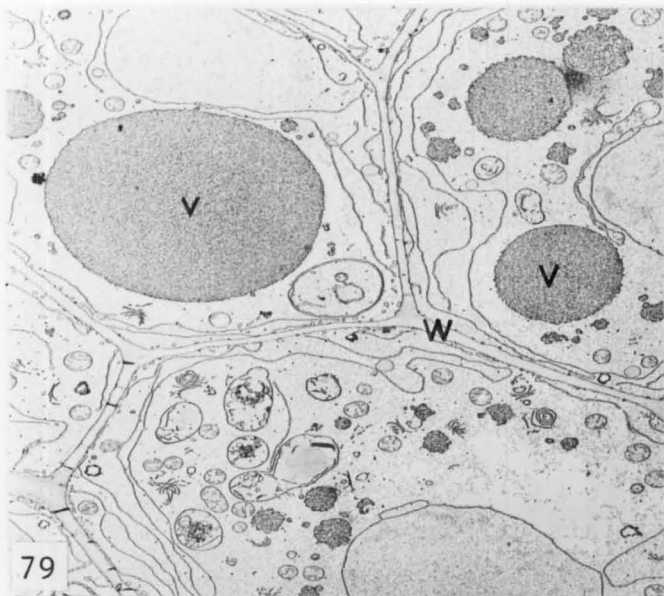
Fig. 82 Root cap cell fixed in buffered KMnO_4 at 37°C . (x 13,750).

Figures 83 - 94 Effect of pH.

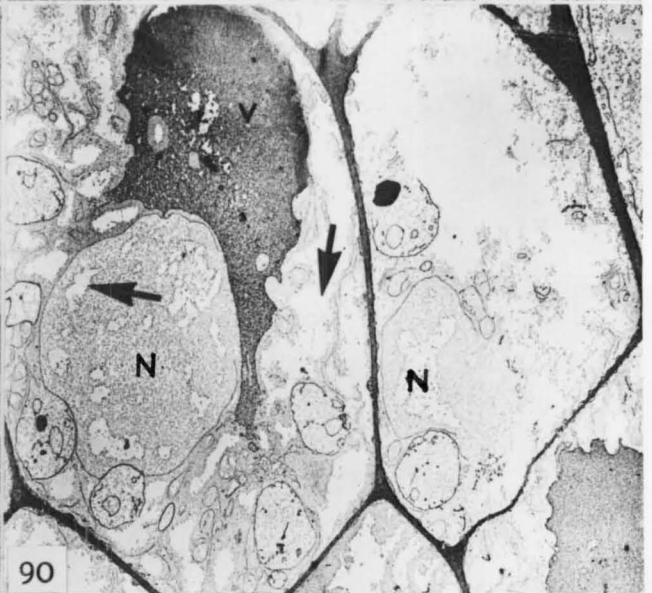
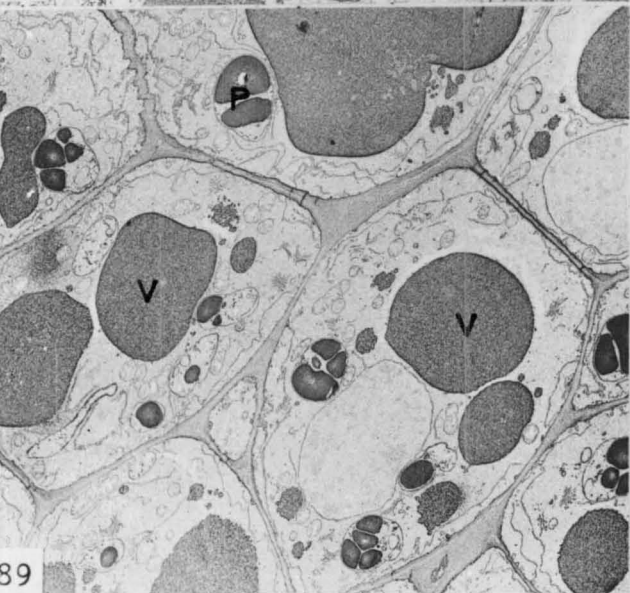
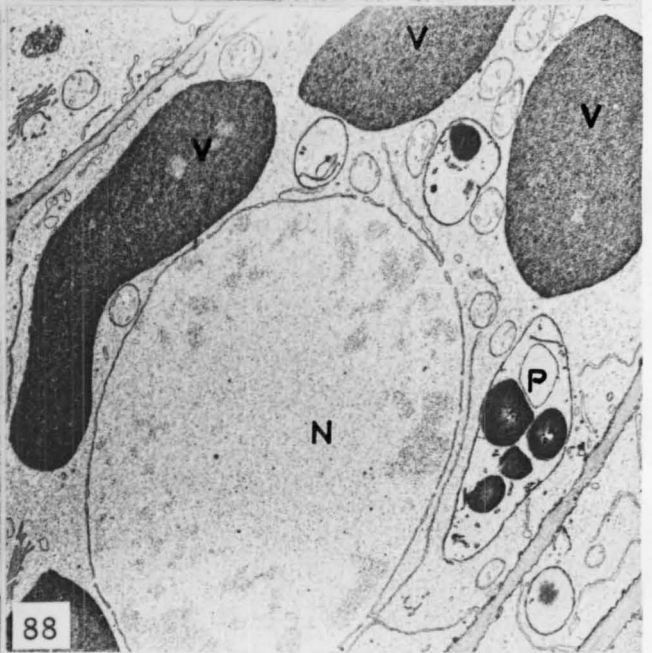
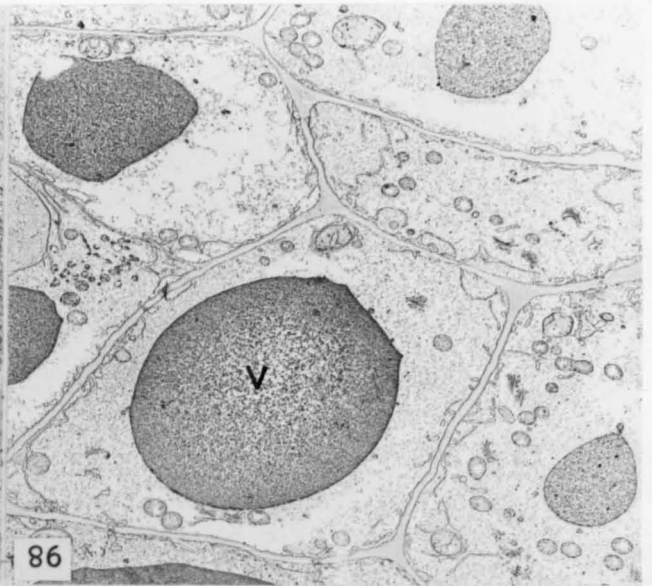
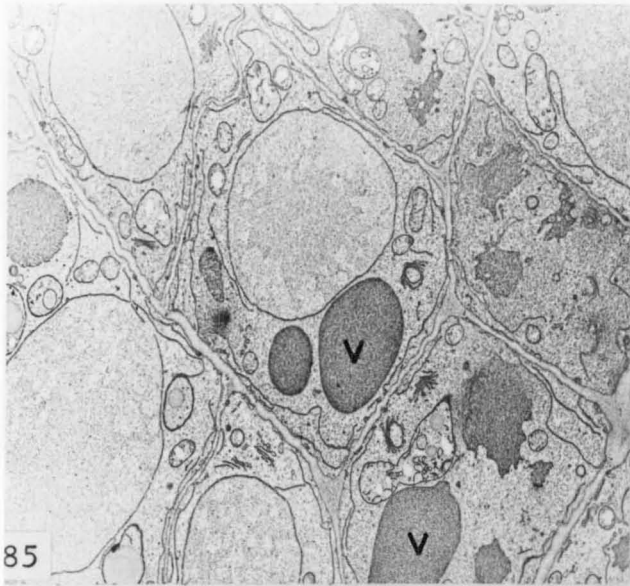
Figures 83 - 90 Veronal acetate-HCl buffered KMnO_4 .

Fig. 83 pH 2.0. Mid root cap cell. Apart from starch grains and the rounded outline of vacuoles little is preserved. (x 5,720).

Fig. 84 pH 2.5. Mid cap cell. Most cellular structures are preserved but the appearance of the protoplast is grainy. (x 9,240).



- Fig. 85 pH 4.5 Root cap initials (x 7,900)
- Fig. 86 pH 6.5 Root cap initials (x 5,700)
- Fig. 87 pH 8.0 Root cap cell (x 6,600).
- Fig. 88 pH 11.0 Outer lateral root cap cell (x14,900)
- Fig. 89 pH 12.5 Mid root cap cells (x 5,700).
- Fig. 90 pH 13.0 Mid root cap showing shrunken vacuoles.
The high pH has also caused the development
of pseudo-vacuoles within the cytoplasm and
nucleus (x 9,300)



Figures 91 & 92. Sodium acetate-HCl buffered.
KMnO₄.

Fig. 91 pH 2.0. Mid cap cells. Apart from starch grains and the outline of vacuoles little is preserved.
(x 5,700)

Fig. 92 pH 4.5. Cortical cells at an early stage of differentiation. Fixation is better than at pH 2.0 but membranes remain unpreserved. (x 6,600).

Figures 93 & 94. Phosphate buffered KMnO₄.

Figs. 93 & 94. Root cap cells showing vacuoles fixed at pHs 5.5 and 8.0 respectively. (Fig. 93 x 10,800; Fig. 94 x 14,900)

Figures 95 - 100 Effect of different concentrations of KMnO₄.

Figures 95 - 97 Aqueous KMnO₄.

Fig. 95 0.5% conc. Cortical tissue near the promeristem. Most organelles are preserved but contrast is low.
(x 13,200).

Fig. 96 1% conc. Young cortical cell showing small dense irregular vacuoles. At this concentration cellular components show optimal contrast. (x 8,500)

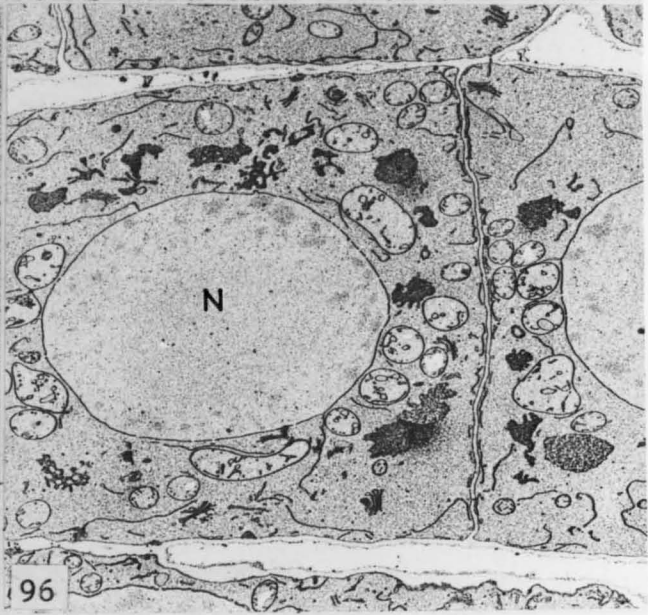
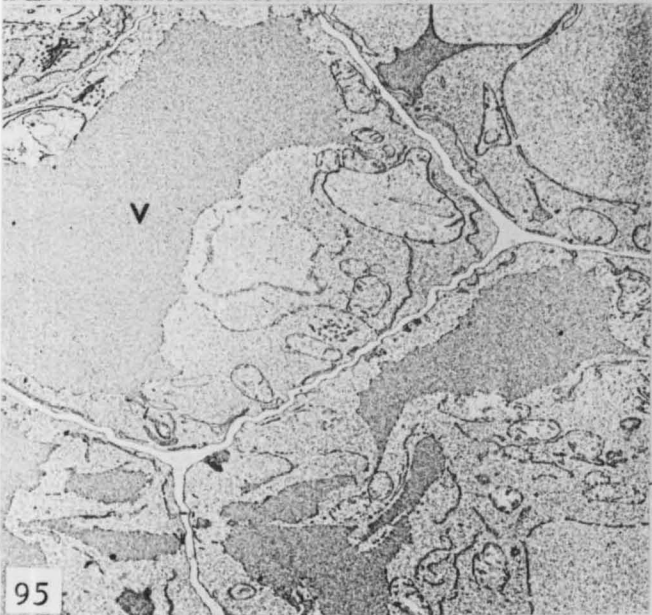
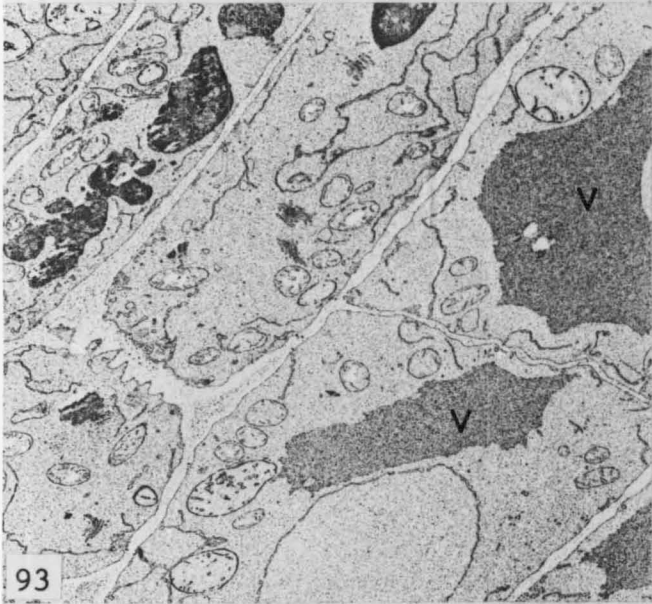
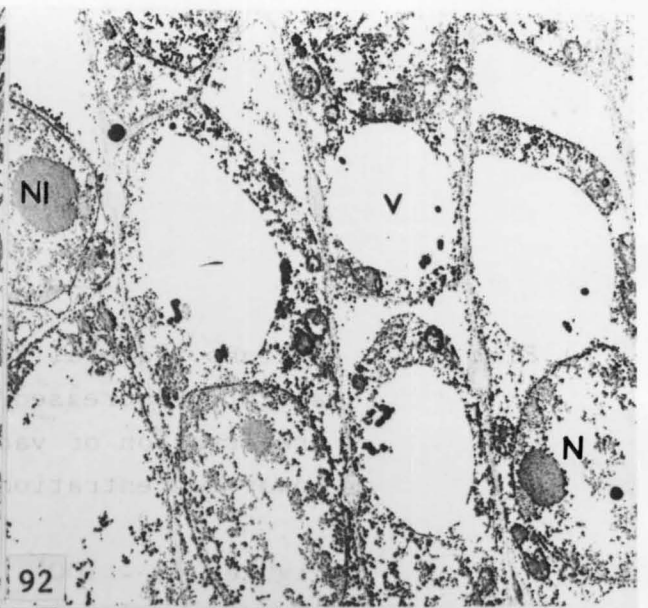
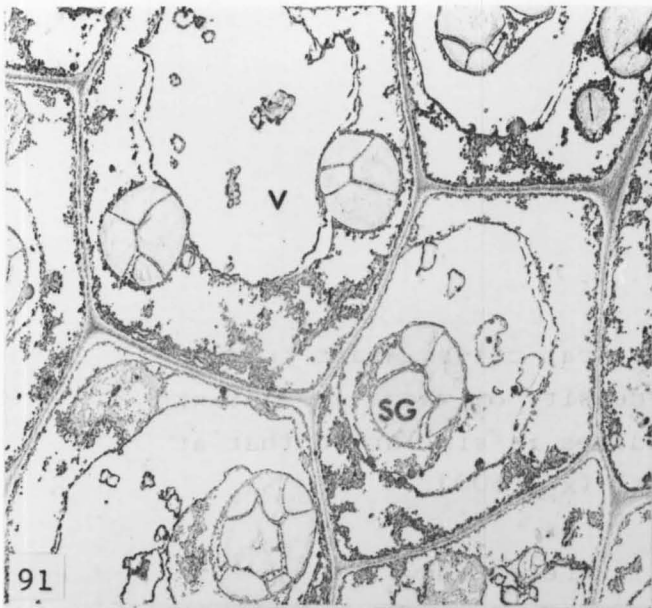


Fig. 97 5% conc. Lateral root cap cells. Apart from a slightly increased density of vacuolar contents preservation of vacuoles is similar to that at lower concentrations. (x 6,600).

Figures 98 - 100 Buffered KMnO_4 .

Fig. 98 0.25% conc. Procambial tissue. Few structures are preserved except for the outline of nuclei and some vacuoles. (x 10,500).

Fig. 99 0.5% conc. Root cap initial. Small vacuoles are fixed as rounded structures but the tonoplast is not preserved. (x **19,300**)

Fig. 100 Saturated KMnO_4 . Early differentiating root cap tissue showing rounded vacuoles with dense contents. (x 6,600).

Figures 101 - 107 Effect of duration of KMnO_4 fixation in L.nitida.

Figures 101 - 105 Buffered fixative.

Fig. 101 2 minutes. Mid cap. (x **10,100**)

Fig. 102 15 minutes. Mid cap. (x **9,200**)

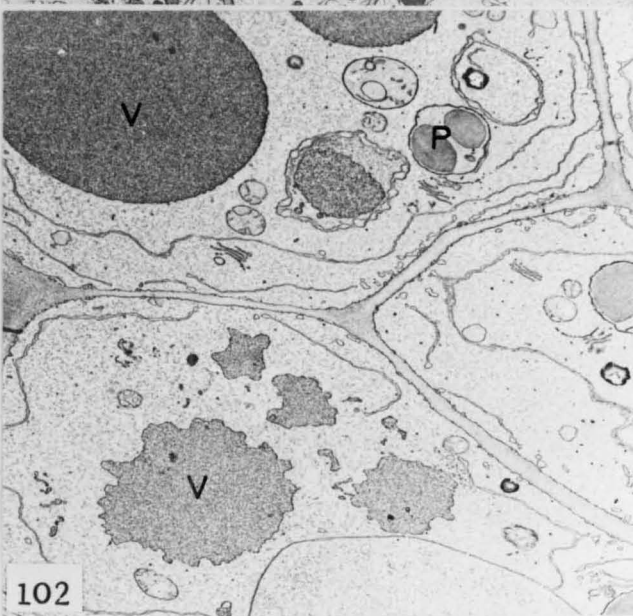
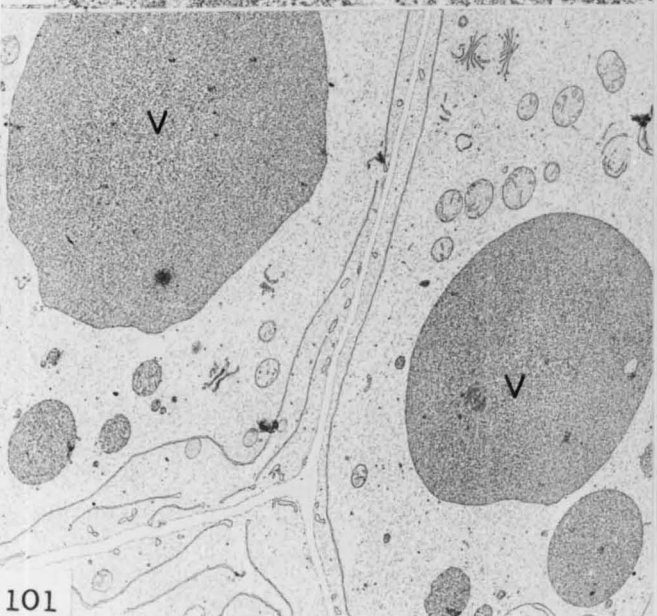
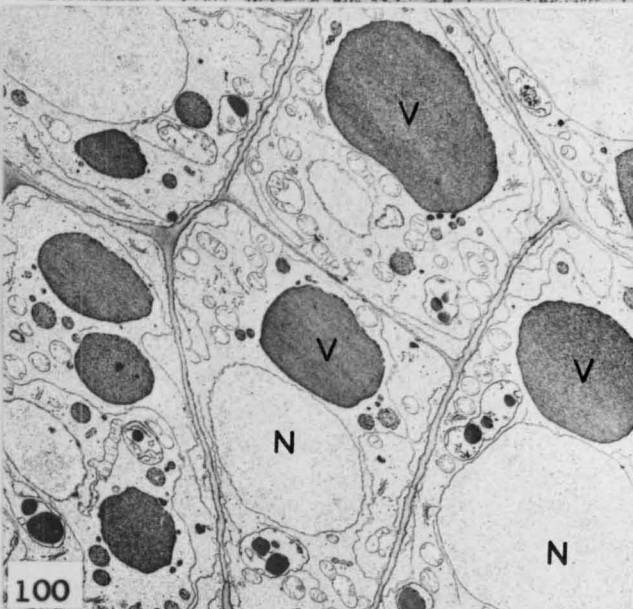
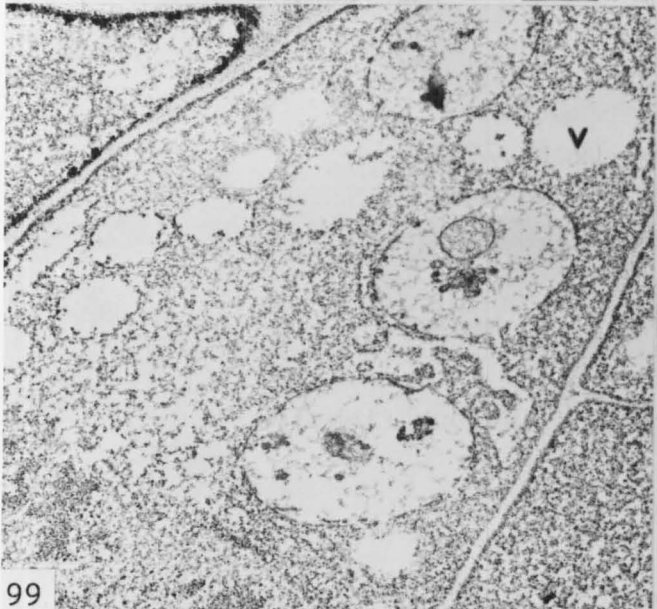
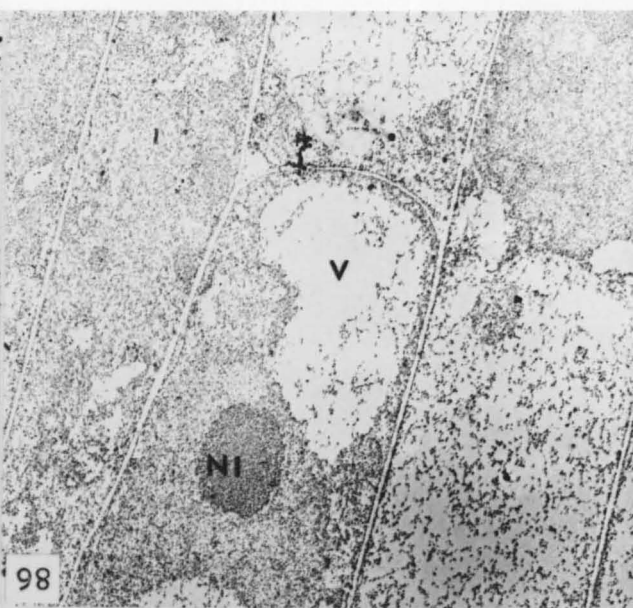
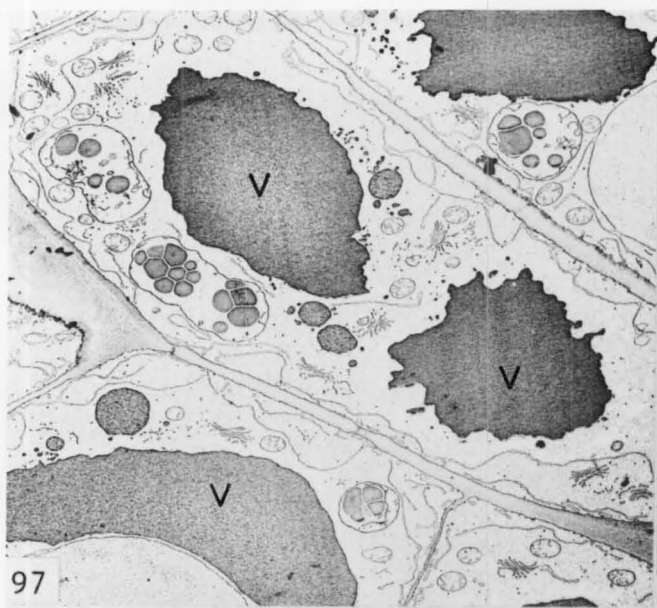


Fig. 103 1 hour. Mid cap. (x 10,700)

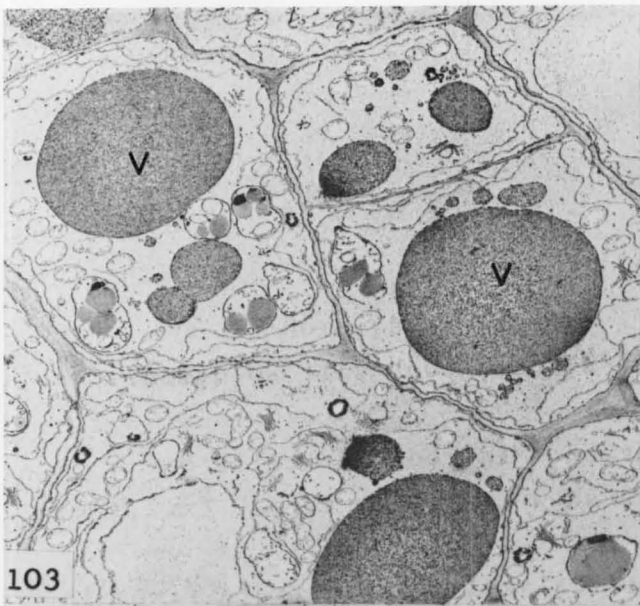
Fig. 104 3 hours. Early cortex. (x 7,000)

Fig. 105 8 hours. Outer cap. (x 9,700)

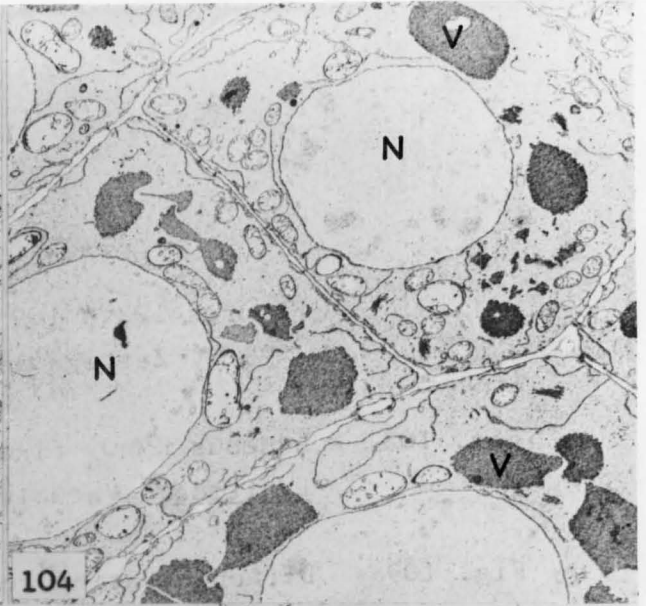
Figures 106 & 107 Aqueous fixative.

Fig. 106 15 minutes. Mid cap. (x 10,700)

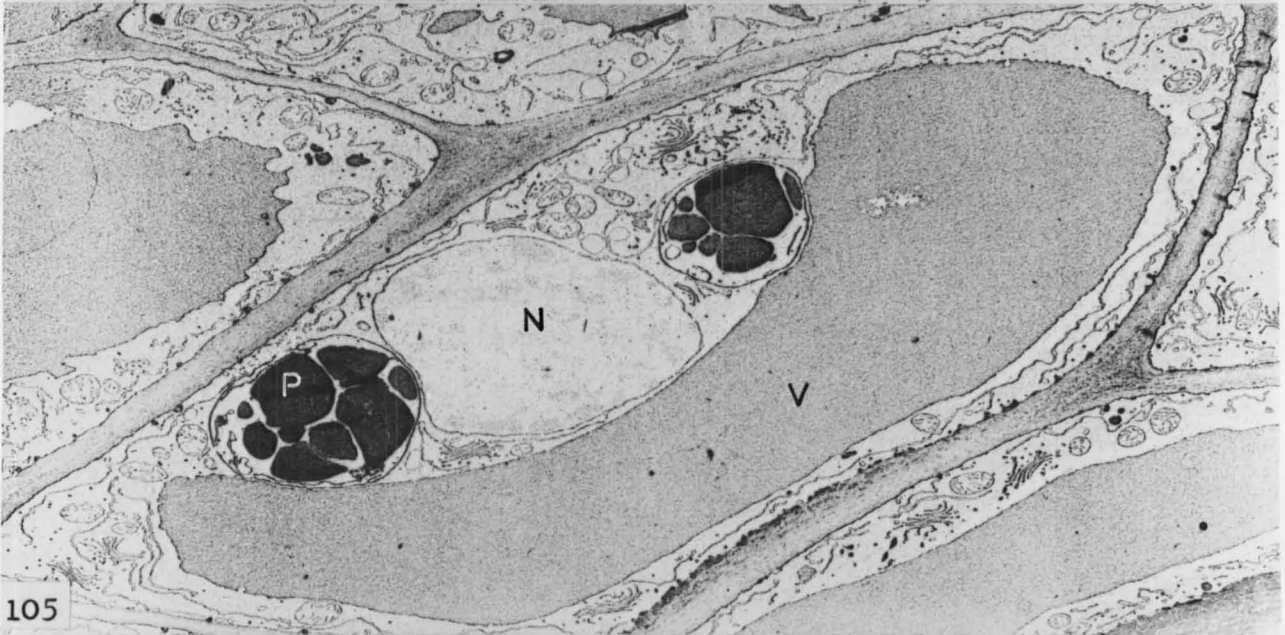
Fig. 107 8 hours. Mid cap. (x 7,000)



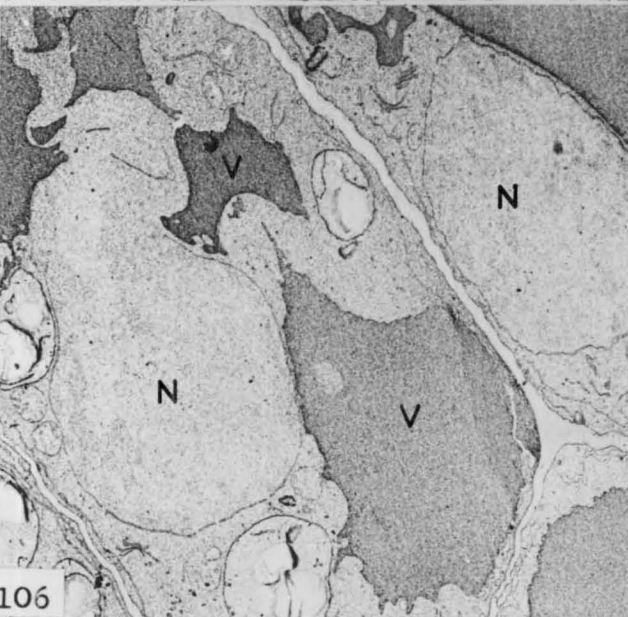
103



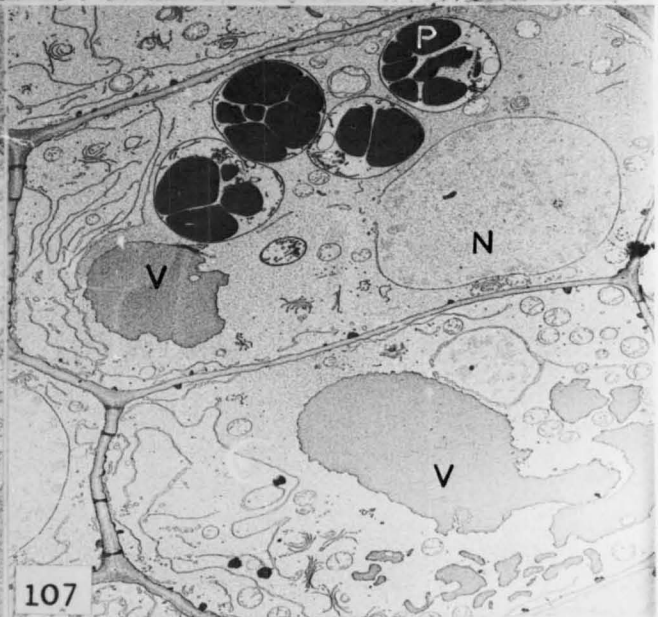
104



105



106



107

Figures 108 & 109 Effect of addition of
Zetterqvist's "balanced" salt solution.

Fig. 108 Aqueous KMnO_4 fixation. Mid root cap cells of
L.nitida. Vacuoles show irregular forms. (x 7,900)

Fig. 109 Differentiating cortical cell in M.citrata fixed in
glutaraldehyde/ OsO_4 . Vacuoles are mainly rounded.
(x 16,600)

Figures 110 & 111. Effect of addition of sucrose in
glutaraldehyde/ OsO_4 fixed M.citrata.

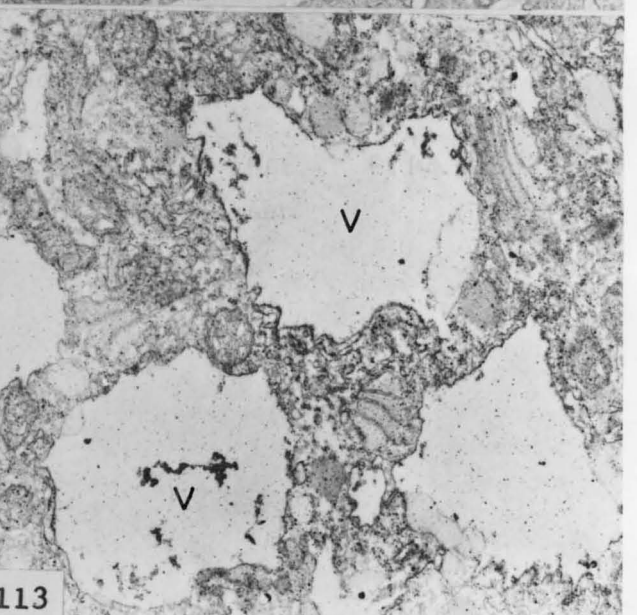
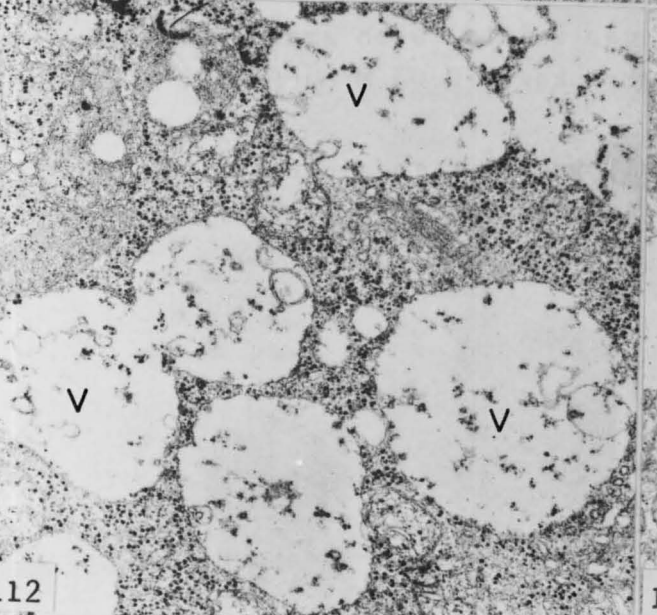
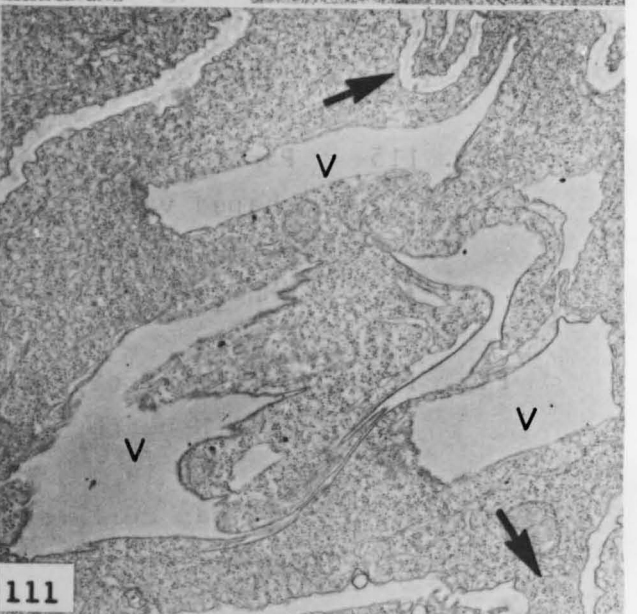
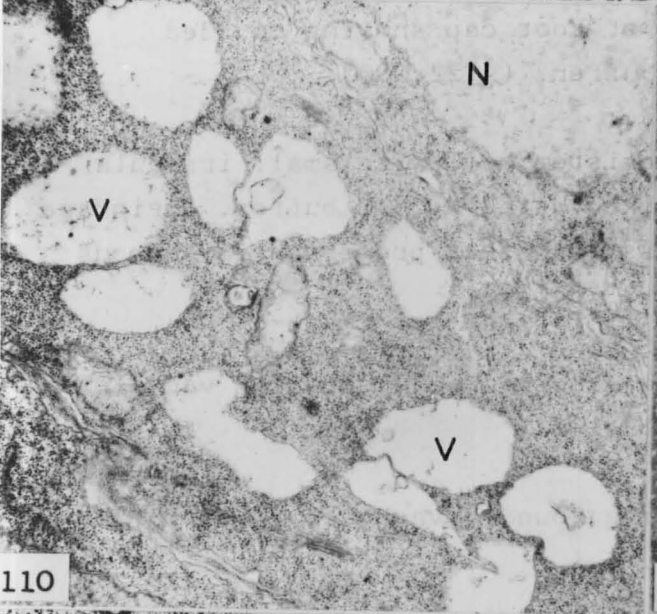
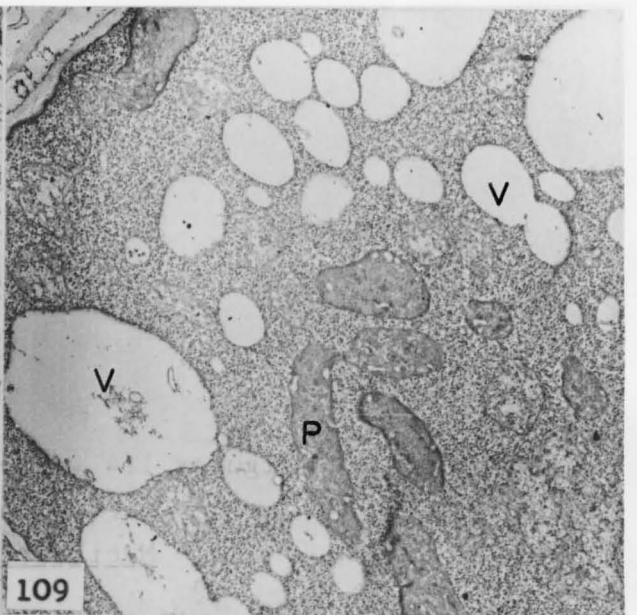
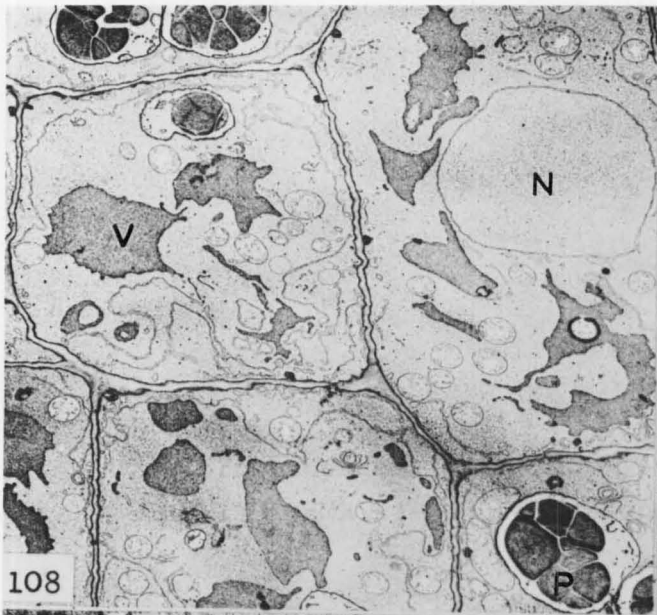
Fig. 110 A group of vacuoles in a procambial cell. Some
vacuoles show good preservation. (x 23,100).

Fig. 111 Root cap cell. The cytoplasm shows reasonable pre-
servation but the vacuoles and outline of the cell
(arrows) exhibit gross distortion due to shrinkage.
(x 20,900).

Figures 112 & 113 Effect of concentration of
phosphate buffer in glutaraldehyde/ OsO_4 .
fixed A.sativa.

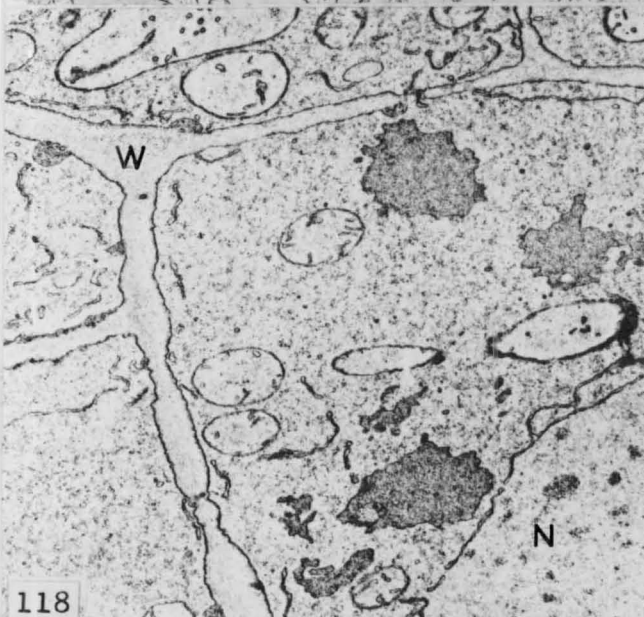
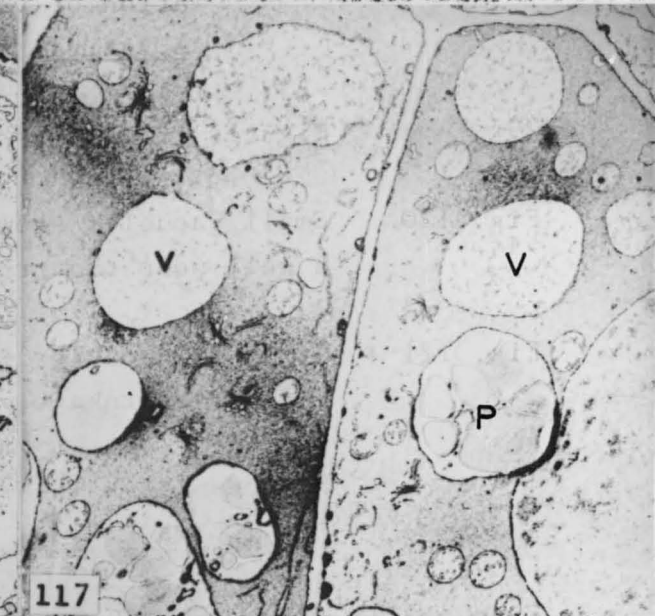
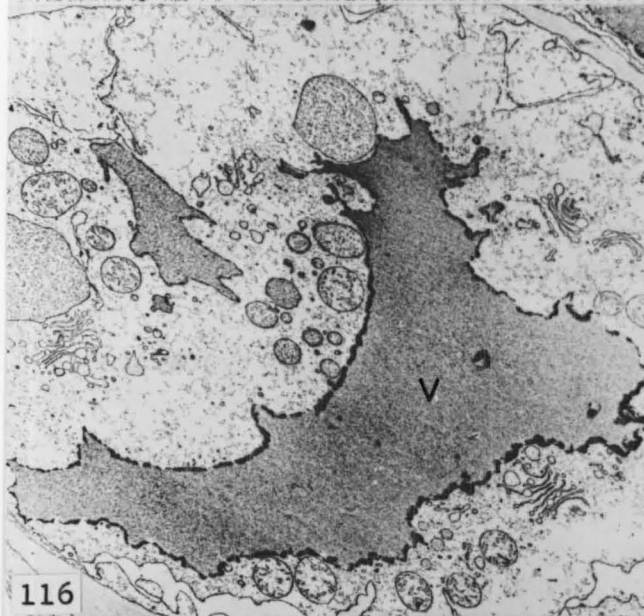
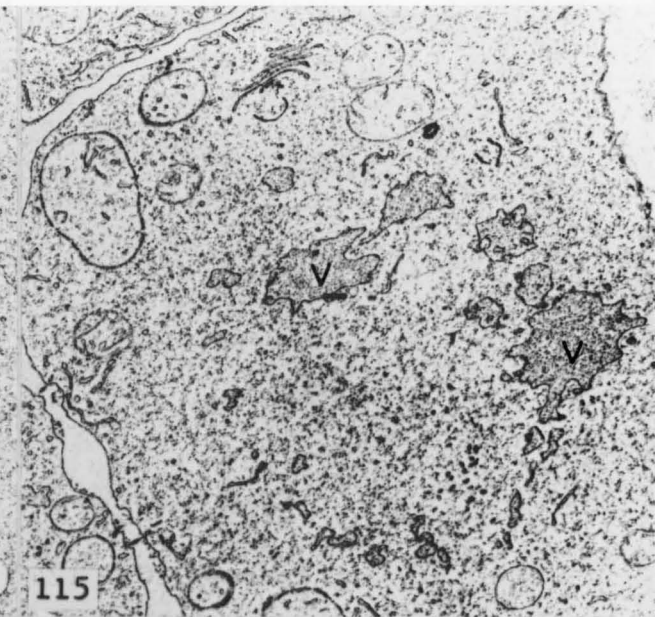
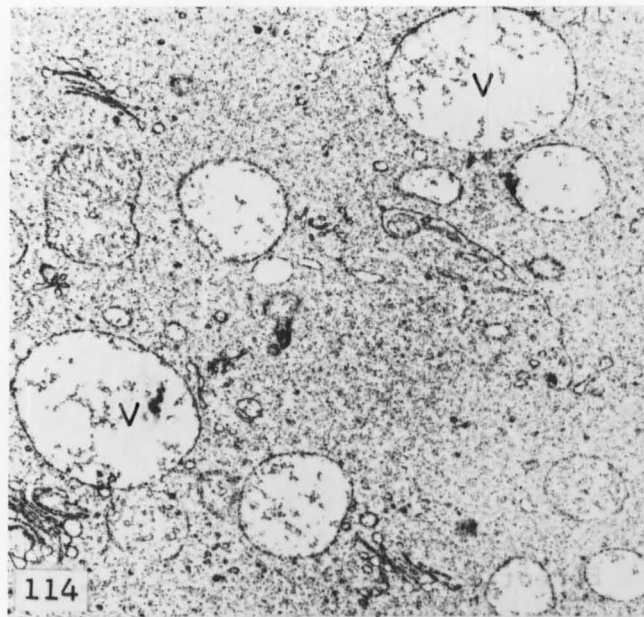
Fig. 112 Mid cap cell showing rounded vacuoles. 0.066 M buffer
(x 14,800)

Fig. 113 Root cap cell showing slightly shrunken vacuoles.
0.016 M buffer (x 26,200)



Figures 114 - 119 Effect of concentration of
veronal acetate buffer in KMnO_4 fixed
M.citrata.

- Fig. 114 Cell near surface of root cap showing rounded vacuoles. 0.28 M buffer. (x 22,000).
- Fig. 115 Portion of a promeristem cell with small irregular vacuoles fixed using 0.28 M buffer. Shrinkage of vacuoles is less than that preserved in aqueous KMnO_4 (Fig. 119) (x 23,100).
- Fig. 116 Outermost cap cell showing badly shrunken vacuoles. 0.14 M buffer (x 12,100).
- Fig. 117 Mid cap cell showing rounded vacuoles. 0.14 M buffer (x 11,000).
- Fig. 118 Portion of a root cap initial with small vacuoles showing moderate shrinkage. 0.05 M buffer (x 23,100).
- Fig. 119 Badly shrunken small vacuoles in a cortical cell near promeristem. Aqueous KMnO_4 (x 31,300)



Figures 120 - 124 Effect of fixation on preservation of vacuoles using osmium tetroxide in A.sativa.

Figures 120 & 121 Aqueous OsO_4 .

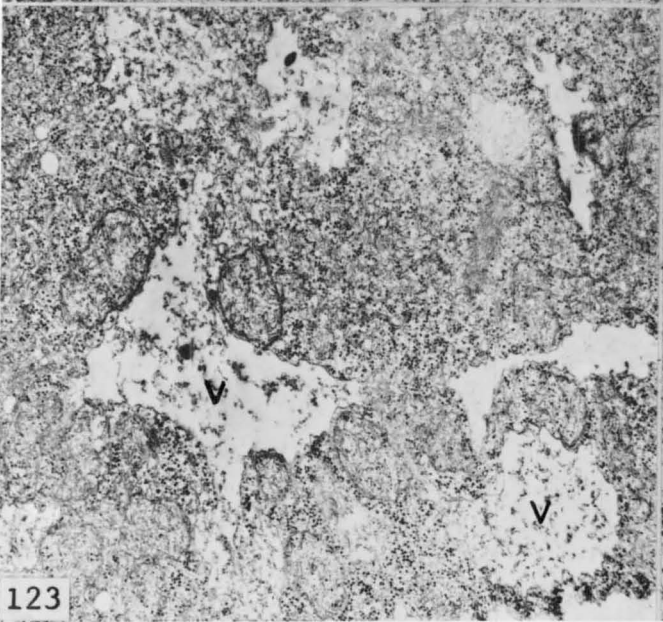
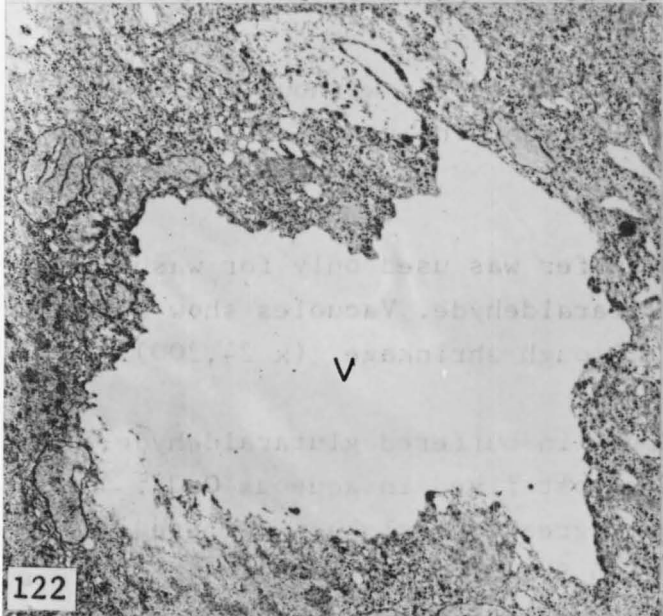
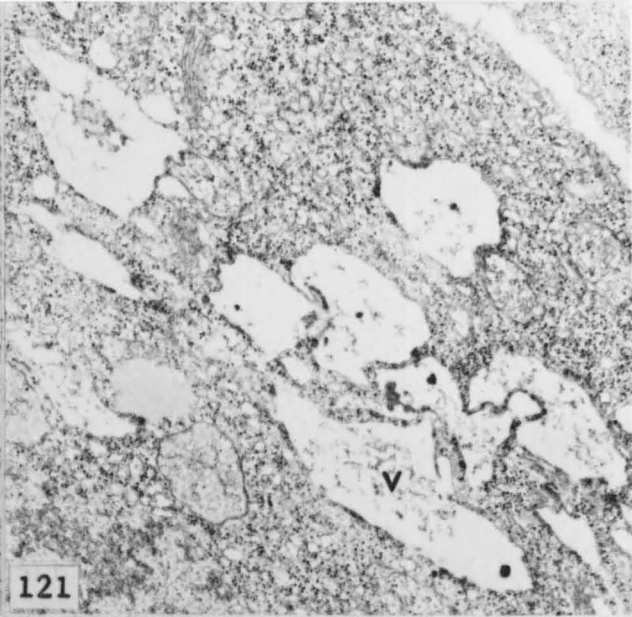
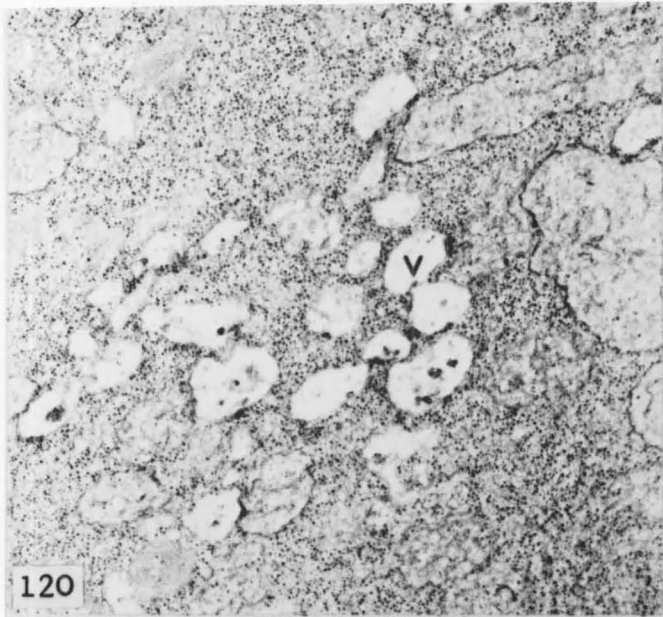
Fig. 120 Small vacuoles showing reasonable preservation in a cell near the promeristem. (x 38,200)

Fig. 121 Group of vacuoles illustrating irregularity caused through shrinkage. (x 28,000)

Figures 122 - 124 Veronal-acetate-HCl buffered OsO_4 .

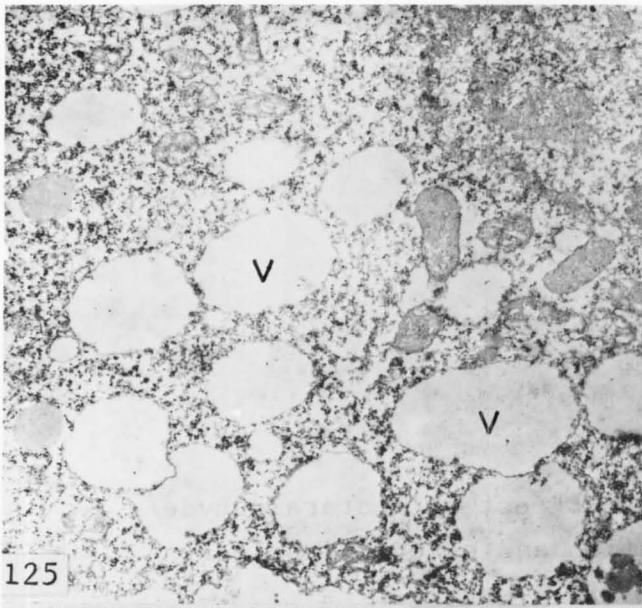
Figs. 122 & 123 Shrunken vacuoles in protoderm cells. (Fig. 122, x 18,700; Fig. 123, ca. x 25,000).

Fig. 124 Protoderm cell showing rounded vacuoles but with some sign of shrinkage. (x 30,000)

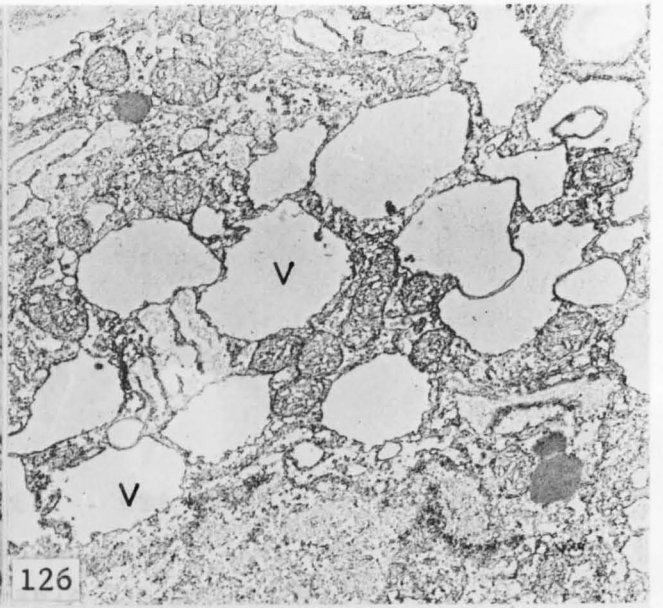


Figures 125 - 128 Effect of buffering at different stages during glutaraldehyde/OsO₄ fixation in A.sativa.

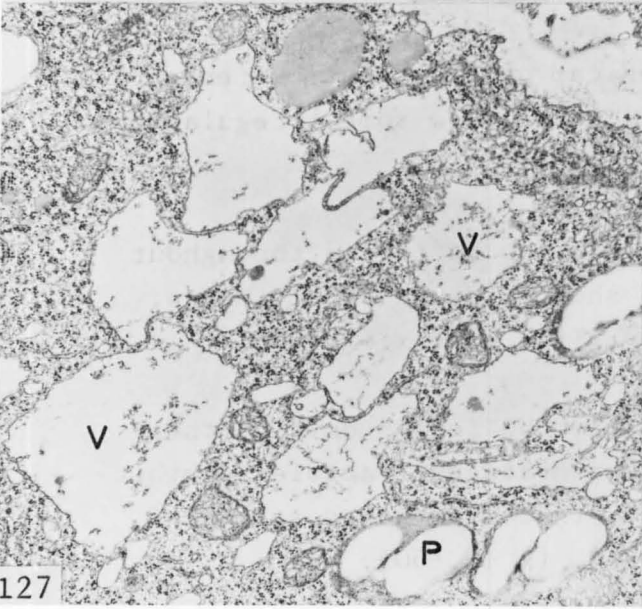
- Fig. 125 Embryonic cortical cells preserved without buffering at any stage during fixation. The vacuoles are mainly rounded. (x 23,100).
- Fig. 126 Outermost cap cell. Buffer was used only for washing after fixation in glutaraldehyde. Vacuoles show some irregularity caused through shrinkage. (x 24,200).
- Fig. 127 Root cap cell preserved in buffered glutaraldehyde, washed in buffer then post fixed in aqueous OsO₄. Vacuoles show slightly greater shrinkage than those in Figs. 125 & 126. (x 22,000).
- Fig. 128 Embryonic root cap cells preserved with buffering only during post fixation in OsO₄. (x 22,000).



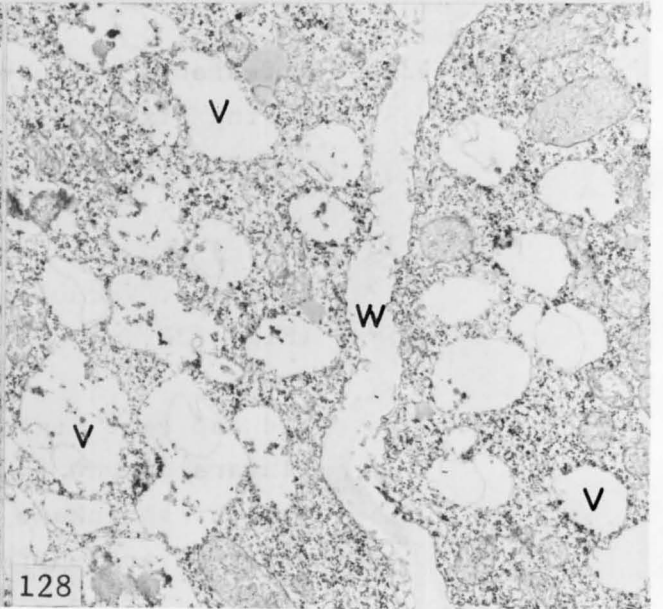
125



126



127



128

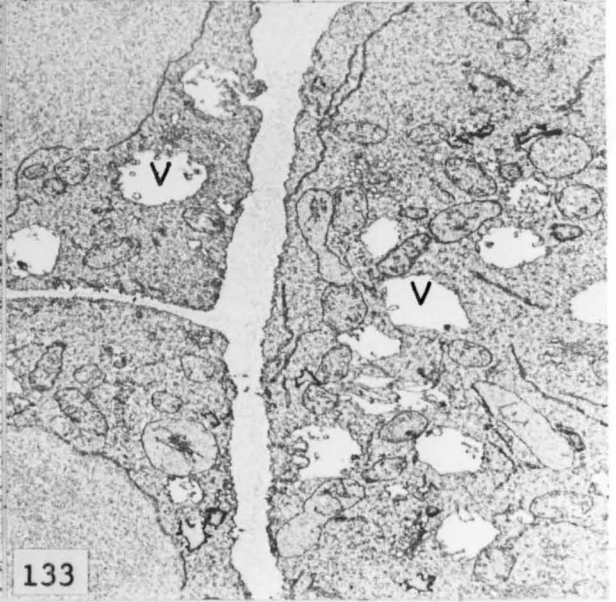
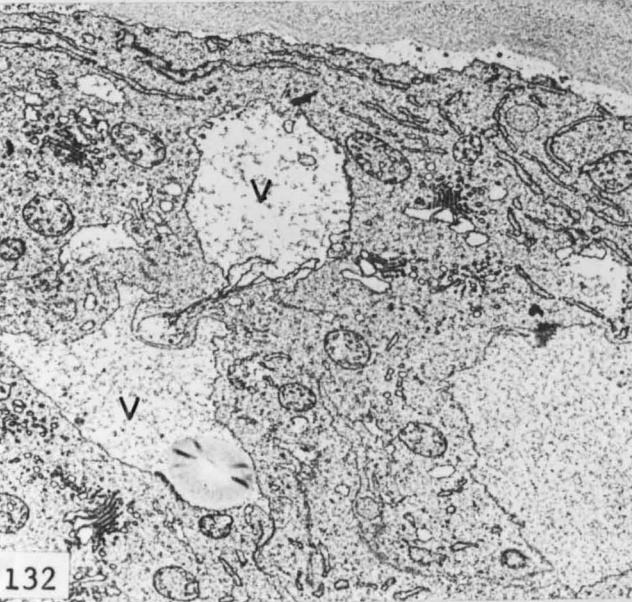
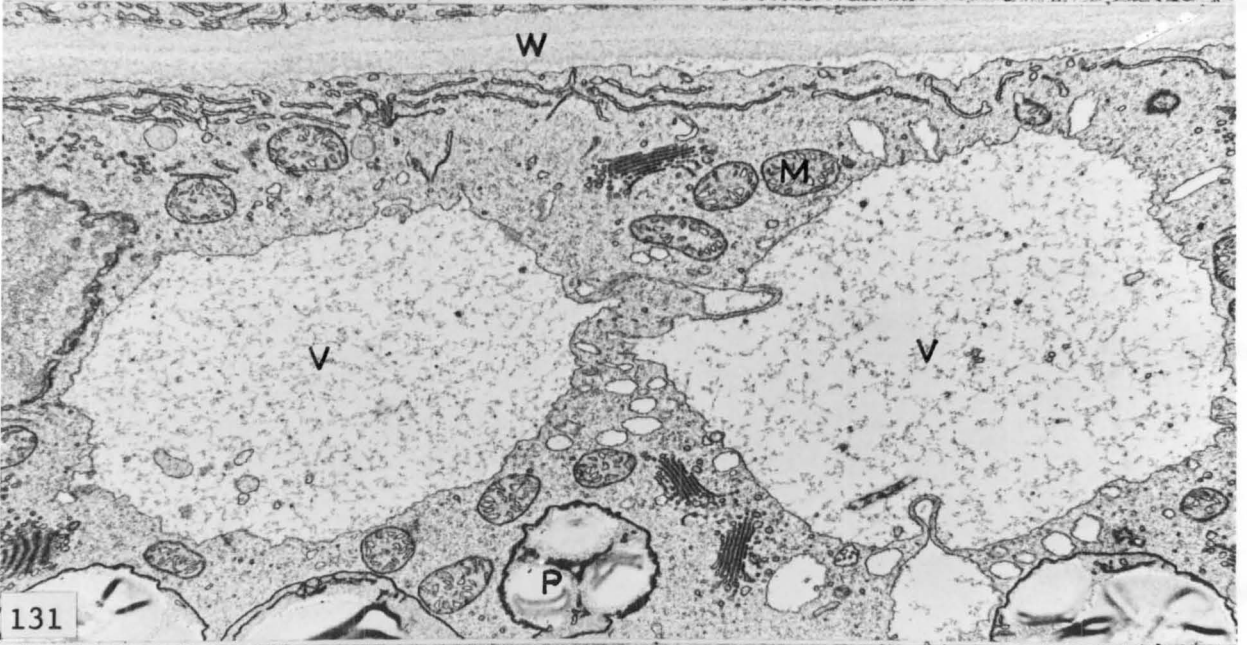
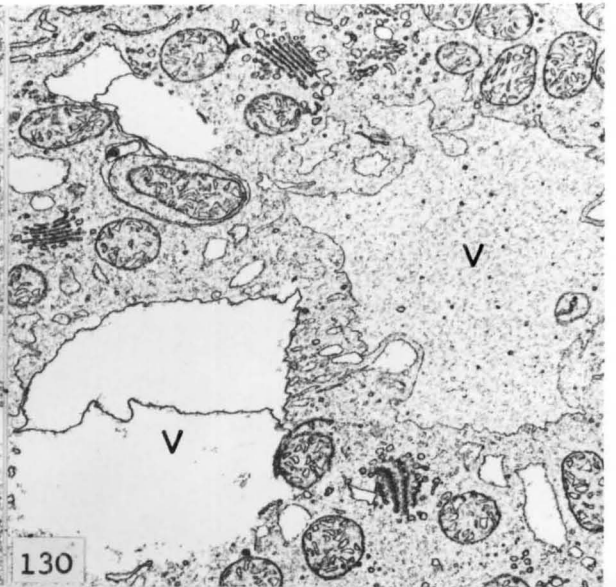
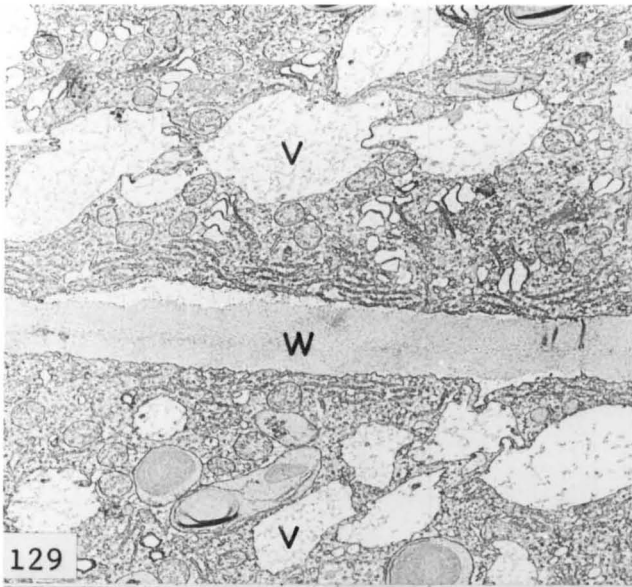
Figures 129 - 133 Effect of glutaraldehyde/
 KMnO₄ fixation and effect of buffering
at different stages in A.sativa.

Fig. 129 Appearance of outer cap cell without buffering
during fixation. Vacuoles show some irregularity
due to shrinkage. (x 13,700)

Figs. 130 & 131 Mid cap cells after buffering throughout
fixation. Vacuoles show evidence of shrinkage.
(Fig. 130, x 18,700; Fig. 131, x 17,400)

Fig. 132 Mid cap cell fixed with buffering only at the
glutaraldehyde and wash stages. Vacuoles exhibit
similar shrinkage to that when other stages of
fixation are buffered. (x 14,800)

Fig. 133 Root cap cells near the promeristem with buffering
only at the KMnO₄ stage. Some shrinkage of vacuoles
is evident. (x 16,500).



6. ULTRASTRUCTURE OF THE TONOPLAST

6. 1 Introduction

The tonoplast is the membrane in plant cells which delimits the vacuole from the groundplasm. The term was originally coined by de Vries, in 1884, for distinct primordia from which he believed vacuoles originated. Since then the name has become restricted to the limiting membrane of the vacuole (see Zirkle, 1937; Voeller, 1964). The tonoplast has been studied intensively in relation to its physical and physiological properties (e.g. Greenham, 1966) and in recent years its structure has been examined under the electron microscope. From early electron microscope observations it was suggested that the tonoplast was a "double membrane" but with improved techniques it has now been shown to comprise a single unit membrane (see Voeller, 1964) about 70 to 100 Å thick. High resolution studies have demonstrated that the tonoplast in chemically fixed cells has a tripartite structure (Grun, 1963) comparable to that of other membranes (Sjöstrand, 1963).

With the introduction of the freeze-etch technique it is now possible to examine the ultrastructural detail of membrane surfaces. Frozen-etched membranes were first studied by Moor and associates in yeast cells (Moor et al., 1964; Moor & Mühlethaler, 1963). Branton and Moor (1964) and Northcote and Lewis (1968) have examined membranes of various organelles in root tips of onion and pea, respectively. The endoplasmic reticulum (Moor, 1967), chloroplast thylakoids (Mühlethaler et al., 1966; Branton & Park, 1967) and the plasmalemma (Stachelin, 1966, 1968b) have also been investigated but so far little work has been devoted specifically to the tonoplast in root tips. Branton (1966) has used the tonoplast to demonstrate where he believes fracturing occurs in frozen-etched membranes. Recently in a study on vacuolation Matile and Moor

(1968) have briefly examined the organization of the tonoplast in root tips of corn.

A feature typical of exposed membrane faces in freeze-etch preparations is the presence of particles. The particles on the tonoplast were first considered from circumstantial evidence to be ribosomes (Moor & Mühlethaler, 1963) but subsequent studies (Branton & Moor, 1964) cast doubt on this idea since the particles were too small. Also, the suggestion is untenable on the grounds that ribosomes have not been observed in association with the tonoplast in osmium fixed material (e.g. Whaley et al., 1960a; Buvat, 1963a). Branton (1966) considered that the particles on the tonoplast, and similar particles found on other membranes, represented structural components of a globular or micellar form lying within the membrane. He thought that these particles could possibly represent lipo-protein associations somewhat analogous to the oxysomes of mitochondria (Branton & Moor, 1964). From studies on chloroplasts, Mühlethaler et al., (1965) believe that the prominent particles on thylakoid membrane surfaces are multi-enzyme complexes. Some workers adopt similar views for particles on other membrane systems (Hess, 1968; Staehelin, 1968b). It has been suggested that the function of such enzyme complexes could be concerned with synthesis of membrane material (Moor & Mühlethaler, 1963) and in the case of the plasmalemma with cell wall synthesis (see Clowes & Juniper, 1968).

Opinion differs as to which part of a membrane is exposed during fracturing in freeze-etching (see Koehler, 1968). Moor and associates (Moor & Mühlethaler, 1963; Jost, 1965; Mühlethaler et al., 1965; Moor, 1966a; Staehelin, 1966) believe fracturing reveals the true outer surfaces of membranes; the particles are regarded as being attached to or slightly embedded in the surface layers. However, Branton and co-workers (Branton, 1966; Bamberger & Park, 1966; Branton & Park, 1967; Branton & Southworth,

1967) claim that fracturing splits the membrane to show its internal organization. The particles are believed by Branton to be embedded within the membrane as a single layer. Recently, from work on artificial and biological membranes, Staehelin (1968a) presents evidence that under certain conditions both surface fracturing and splitting of the membrane may occur. As an alternative to these explanations, Northcote and Lewis (1968) suggest that the image in freeze-etch replicas might represent an imprint of the original membrane on the surrounding material; the membrane itself is fractured away. They conclude that membranes, as visualized in freeze-etch preparations, are best interpreted either as replicas of the true external surfaces or as imprints of these surfaces.

This chapter presents observations on the fine structure of the tonoplast. Since thin sectioning revealed little new information, most observations were based on frozen-etched replicas. These replicas were prepared from both frozen fresh root tips and specimens prefixed with GA during glycerination prior to freeze-fixation. Avena sativa, Triticum vulgare and, to a lesser extent, Zea mays were used for investigations on frozen-etched fresh material. Information on prefixed roots was obtained from five species, with particular reference to Lonicera nitida.

6. 2 Observations

6.2. 1 The tonoplast in ultrathin sections

A tonoplast completely surrounds each vacuole unless it has ruptured during fixation (Figs. 55, 58). In well preserved vacuoles the tonoplast is smooth in outline (Figs. 47, 62, 66) but where fixation causes shrinkage the tonoplast may be irregular and wrinkled in appearance (Figs. 52-60, 70).

The tonoplast cannot always be seen as a distinct membrane, especially in vacuoles where the contents are precipitated as electron-dense material on fixation in KMnO_4 (Fig. 57). Nevertheless, the presence of a tonoplast can be inferred by the sharp separation of vacuolar contents from the groundplasm. In other vacuoles, where the vacuolar sap is less dense after permanganate fixation, the tonoplast is clearly visible as a dark line surrounding the organelle (Figs. 52, 55, 58, 62). On fixation with GA/OsO_4 the tonoplast appears as a single sharp line between the groundplasm and the electron transparent contents of the vacuole (Figs. 47, 68). In poorly preserved GA/OsO_4 material the tonoplast often pulls away from the groundplasm and lies within the vacuole (Fig. 130). A similar displacement of the membrane has been noted by other workers (Jensen, 1965; Gifford & Stewart, 1968).

At most magnifications the tonoplast appears as a single line but with good resolution it can be shown to possess a tripartite structure (Fig. 134) similar to that found by Grun (1963). However, on no occasion has the membrane been found with an asymmetrical tripartite structure. Surface views of the tonoplast are rarely seen in thin sections but when they occur they usually show an undifferentiated membrane. Because thin sectioning yields only limited structural information, one is apt to regard the tonoplast as a comparatively simple membrane. Freeze-etching shows that this is not so.

6.2. 2 Appearance of the tonoplast in frozen etched fresh root tips

A. The tonoplast in cross fracture: Freeze-etching (Figs. 48, 137) supports observations from thin sectioning that the tonoplast is a single unit membrane. However, owing to the granularity caused by freezing and the angle of fracturing it is usually difficult to obtain precise measurements of the thickness of the tonoplast in

frozen-etched material. In most specimens the structure of the membrane is not shown clearly in cross fracture. Generally cross-fractured views reveal less information than chemically fixed and sectioned material of the tonoplast.

According to Branton (1966), tonoplasts and other membranes fractured obliquely reveal a "step" or "ridge" at the base of the membrane which is believed to indicate where the plane of fracture has followed the middle layer of the membrane. Similar steps are noted in the present material especially at the junction between the exposed outer surface of the tonoplast and the adjoining cross fractured groundplasm (Fig. 136).

B. The tonoplast in face view: In addition to cross fractured membranes, freeze-etching provides extensive surface views of the tonoplast (Figs. 135-137). Both outer and inner faces of the membrane may be exposed (Figs. 135, 136, 137). A prominent feature of the exposed faces of the tonoplast after surface fracturing is the appearance of particles (Figs. 135-148). These occur on both sides of the membrane in all the species studies (Figs. 135, 136, 137, 139). While particles are not confined to the vacuolar membrane, the tonoplast can be distinguished from other membranes by the size, density and distribution of its particles. For example, on the endoplasmic reticulum particles are less numerous than on the tonoplast and show only a small difference in density on the two sides of the membrane.

On both sides of the tonoplast the particles are globular (Figs. 139-148). The particles vary little in size (Figs. 139, 143, 144) but those on the inner face appear to be slightly smaller than those on the outside of the tonoplast (Fig. 139). The dimensions of the particles are about $90 - 150 \text{ \AA}$ in diameter similar to those measured

by Matile and Wiemken (1967) in yeast cells and Matile and Moor (1968) in corn root tips.

The density of particles on the two exposed faces of the tonoplast is strikingly different in all the species studied (Figs. 135-144). To determine quantitatively the difference in density per unit area between the two surfaces an analysis was made using Avena and Triticum. The results are given in Figure 138 and the statistical analysis in Table 7. In both species the density of particles on the two surfaces is significantly different with a greater density on the inner side of the tonoplast; the ratio is approximately 5:3. The analyses indicate that the inner faces of the tonoplasts in Avena and Triticum have comparable densities of particles but that a difference may exist for the outer faces. Although the number of particles on the outer surfaces are statistically different in the two species, further work is required to determine whether this difference is real, due to sampling or due to a greater loss of particles during fracturing in Avena.

To determine whether the number of particles changed with development of the vacuole, the density of particles was analysed on vacuoles of different size. In Avena no obvious difference was found between small and large vacuoles for either face of the tonoplast (Figs. 145-148). This applied also to Triticum except that the inside of one large vacuole showed fewer particles than the inner faces of other vacuoles of similar size (see 5th histogram in Fig. 138).

At magnifications of about x50,000, both faces of the tonoplast appear uniformly covered with particles (Figs. 140, 141). But when the tonoplast is studied at higher magnifications the particles are found to be unevenly distributed and interspersed with bare areas

(Figs. 143, 144). This is more apparent on the outer face than on the inner face of the tonoplast. On the outer face the particles sometimes occur in small indefinite clusters (Figs. 139, 143). In many instances, small depressions in the membrane (Fig. 142) show that some particles are dislodged during fracturing. However, not all the bare areas on the outer face of the tonoplast are due to this loss of particles; they appear to be a natural feature of the membrane. On the inner face of the tonoplast particles are also dislodged during fracturing but apparently to a lesser extent.

6.2 3 Appearance of the tonoplast in prefixed root tips.

- A. Tonoplast in cross fracture: Roots prefixed in GA in 30% glycerol prior to freeze-fixation show tonoplasts similar in cross fracture to those of frozen fresh roots.
- B. Tonoplast in face view: The appearance of the tonoplast in prefixed material is usually distinctly different from that in fresh roots (Figs. 150-160). Instead of globular particles, exposed faces often display platelet-like structures or plaques (Stahelin, 1968a). These occur on both sides of the tonoplast (Figs. 151-153). Although plaques are characteristic of prefixed material their occurrence is variable. In Lonicera some trials revealed plaques almost exclusively (Figs. 150-153) while others showed mainly particles (Fig. 149) comparable to those found on tonoplasts in frozen-etched fresh roots. Similar variations were noted in the other species examined, but as in Lonicera plaques were more common than particles.

Particles and plaques were rarely seen together on exposed faces of tonoplasts (Fig. 154). Where this did occur the particles were found only on top of the plaques (insert, Fig. 154). Tonoplasts displaying particles rather than plaques showed considerable variation

in particle density. The density ranged from that shown by frozen-etched fresh roots to one where particles were almost absent.

The plaques occur as slightly raised structures scattered over the exposed faces of the tonoplast (Fig. 152). They assume various forms but are mainly round to oblong with an angular outline. The size of plaques, in general, is similar on a given tonoplast (Figs. 156-158).

The majority of plaques have a smooth surface but occasionally the surfaces are finely granular. The areas between the plaques are more granular (Figs. 152, 156b, 158b).

Counts made on Lonicera showed that the density of plaques on the inner and outer faces of the tonoplast did not differ significantly (Fig. 161; Table 8). The density of plaques per unit area is considerably lower than that of particles on both faces of the tonoplast (Fig. 162; of Figs. 156c and d). Plaque density in different vacuoles (Fig. 161) varied more than did particle density in the frozen-etched fresh root tips (Fig. 138). However, between vacuoles, there was a considerable variation in the density of plaques within a certain range (Fig. 161). This is in marked contrast to the distribution of particles in frozen-etched fresh root tips (Fig. 138). These results from Lonicera are supported by observations on Avena.

Among vacuoles of different size and stage of development little difference was detected in the density of plaques (Figs. 156a-c; 157a-d, 158a-b). The density of plaques on the inner faces of the tonoplasts in Lonicera and Avena was similar in both large and small vacuoles, except that the outer face of one large vacuole in Lonicera showed about a third more plaques (but these are smaller) per unit area than did small vacuoles (Fig. 156c). This could indicate a change in

the structure of the membrane during development of the vacuole. Owing to the uniformity of freezing in prefixed material and its effect on fracturing, such views of large vacuoles are rarely obtained for comparison; large vacuoles are usually cross fractured.

Whereas Lonicera and Avena showed prominent plaques on the tonoplast, in Triticum plaques were less apparent. Figures 155 and 159 show that only a few small indistinct plaques are recognisable on the exposed inner face of the tonoplast. Instead, the membrane has a finely granular appearance similar to the areas between plaques on other tonoplasts.

6.2. 4 Differentiation of the tonoplast

The tonoplast is a dynamic membrane. As the vacuole enlarges the surface area of the tonoplast increases, yet at the same time the membrane maintains a constant thickness as shown by thin sectioning. The tonoplast is involved in the fusion of vacuoles and in the incorporation of cytoplasmic material into the organelle (see Chapter 7). Where incorporation of cytoplasmic material occurs, to form vacuolar inclusion bodies, the tonoplast shows local differentiation. In thin sections and ~~cross~~-fractured views, the organization of the membrane in such areas cannot be distinguished from the rest of the tonoplast (Figs. 193, 291), but surface fracturing reveals this local differentiation of the membrane (Figs. 163-174).

The inclusion bodies develop from invaginations of the tonoplast. The invaginations are most common on small vacuoles (Figs. 163-165) but they occasionally occur on larger vacuoles (Fig. 166). On exposed portions of small vacuoles the tonoplast may show only one differentiated area (Figs. 163-165) but on larger vacuoles several areas may occur at different stages of development (Fig. 166).

The differentiated areas develop as circular regions on the tonoplast

(Fig. 163). They first appear as small local flattenings of the curved surface of the tonoplast (Fig. 163) and as development proceeds they deepen (Figs. 164-165) forming a diverticulum (see Fig. 188). The invaginations eventually become pinched off as vesicles and come to lie free within the vacuole (see Chapter 7). The differentiated areas vary in size depending on the stage of development of the invagination and the particular type of inclusion body being formed.

A feature of differentiated areas is a local aggregation of particles. On the outer exposed face of the tonoplast, the areas are marked by a greater density of particles than on the rest of the tonoplast (Figs. 163-170). Even before the tonoplast shows signs of flattening, the site of a future invagination is marked by an aggregation of particles (Figs. 163, 167). As differentiation proceeds the aggregations become defined as circular areas which enlarge to a certain stage. The tonoplast then becomes flattened and begins to grow into the vacuole (Figs. 164, 165). A selection of stages in the differentiation of the outer surface of the tonoplast is shown in Figures 167 to 170.

On sites of developing inclusion bodies the density of particles is about twice that of the rest of the tonoplast. This becomes apparent very early (Fig. 168) and remains throughout subsequent stages of early development (Figs. 163-165). It has not been possible to determine the density of particles at stages beyond that shown in Figure 165, since once the invaginations deepen the fracture plane no longer follows the surface of the depressions but cross-fractures them. Similarly, on the inner side of the tonoplast, once the invagination grows beyond a certain point it is broken off from the tonoplast as the vacuolar contents are plucked out during fracturing (Fig. 173, 174). On the inner face of the tonoplast, the density of particles on the invagination is only slightly greater than

the density on the rest of the tonoplast (Fig. 172).

The size of particles on sites of developing invaginations is similar to that noted for particles on the rest of the tonoplast. This applies to both the outer and inner faces of the membrane.

6.3 Discussion

The advantages of freeze-etching in studying the surface detail of membranes are now becoming well established (see Koehler, 1968). A variety of animal membranes (Moor et al., 1964; Moor, 1966a & b; Branton, 1967; Deamer & Branton, 1967; Rayns et al., 1967; Friederici, 1968a & b; Leak, 1968b), plant membranes (Moor & Mühlethaler, 1963; Branton & Moor, 1964; Branton & Park, 1967; Staehelin, 1968a) and artificial membranes (Branton, 1966; Moor, 1966a; Staehelin, 1968a) have been investigated but to date comparatively little work has been done on the tonoplast. Electron micrographs of frozen-etched tonoplasts are illustrated in a number of papers (e.g. Moor, 1964; Staehelin, 1966; Matile & Wiemken, 1967; Sassen et al., 1967; Hess, 1968) but most workers mention the membrane only briefly or not at all. At the start of the present work two papers were available which contained brief observations on the tonoplast in root tips (Branton & Moor, 1964; Branton, 1966). Since then other papers have appeared (Matile, 1968; Matile & Moor, 1968; Northcote & Lewis, 1968).

6.3. 1 Particles on the tonoplast in frozen fresh roots

The characteristic feature of the tonoplast in frozen fresh roots is the presence of particles on both exposed faces of the membrane. These particles are similar to those shown on tonoplasts by other workers (Moor & Mühlethaler, 1963; Branton & Moor, 1964; Moor, 1965; Branton, 1966; Sassen et al., 1967; Hess, 1968; Matile, 1968; Matile & Moor, 1968; Northcote & Lewis, 1968) and possibly represent enzyme complexes. The function of such enzyme complexes could be concerned with the synthesis of membrane material (Moor & Mühlethaler, 1963), the passage of substances into

and out of the vacuole and/or enzyme modification within the membrane.

6.3. 2 Size of the particles

The size of the majority of particles on the tonoplast falls within the range 90-150 Å. The particles have been measured on the vacuoles of a variety of organisms (Moor & Mühlethaler, 1963; Branton & Moor, 1964; Staehelin, 1966; Matile & Weimken, 1967; Hess, 1968; Matile & Moor, 1968). These observations suggest that the particles are definite entities and that there is a minimum and maximum size of development.

There are several explanations for the variation in size of the particles. As fracturing occasionally removes particles to leave small depressions in the membrane surface (Fig. 142), it is equally possible that it may partly break some particles so that they appear smaller than normal. On the other hand, this range in size of particles may be an expression of their different states of activity. Staehelin (1966) suggested that the particles on the plasmalemma of Chlorella passed through the membrane and that their size varied in accordance with the extent of penetration. Although this explanation may be applicable to the plasmalemma it seems a less likely explanation for the tonoplast. The

strikingly different density of particles on the two sides of the tonoplast suggests that the particles are very much a structural part of the membrane. Another alternative is that the various sizes of particles represent stages in their development or degradation. This is what might be expected if the particles are integrated dynamic components of membranes.

6.3. 3 Distribution of particles on the two faces of the membrane

The feature which has been shown to distinguish the tonoplast from other membranes (e.g. the endoplasmic reticulum and plasmalemma) is the difference in density of particles on the two sides of the membrane. Until recently this feature had received little attention. In the first study of frozen-etched root tips (Branton & Moor, 1964) no difference was

reported between the two sides of the tonoplast. In a subsequent paper Branton (1966) did not mention this feature although it is evident in one of his micrographs. Staehelin (1966) shows a similar condition in the tonoplast of Chlorella. In pea root tips Northcote and Lewis (1968) illustrate the inner surface of the tonoplast with a greater density of particles than the outer surface but without comment. Since the present study was completed the feature has been noted in a general way by Matile (1968) and more specifically by Matile and Moor (1968) in root tips of Zea seedlings.

Matile and Moor observed that the inner face was densely covered by globular particles interspersed with small uncovered areas; on the outer face particles were less numerous. In addition they noted many tiny holes on the outer face of the tonoplast which suggested that most of the particles were detached through fracturing. However, in isolated vacuoles the outer face was found to closely resemble the inner face and no holes were present. Matile and Moor therefore concluded that the plane of fracturing was greatly influenced by the surrounding medium (in situ by groundplasm and in the isolated state by 30% glycerol).

While the above workers show a different density of particles on the two sides of the tonoplast the difference is less striking than that found here. Matile and Moor (1968) used prefixed root tips rather than specimens grown in glycerol and this may have influenced the numbers of particles. The results obtained in the present work more closely resemble those shown by Branton (1966) and Northcote and Lewis (1968) who used frozen-etched living roots. Frozen-etched fresh root tips undoubtedly give more reliable information than prefixed specimens where the tonoplast has been modified by chemical treatment. If fracturing removes particles (Matile & Moor, 1968), one would expect from mechanical considerations that more particles

would be brushed off a convex surface than a concave one. This does not seem to happen; on both sides of the tonoplast hollows where particles have been removed occur with about equal frequency. Also little difference is noted among vacuoles of different curvature. The particle densities on the two faces of the tonoplast are therefore regarded as reflecting real differences in membrane organization.

What does the difference in density of particles between the two faces of the tonoplast mean in terms of membrane organization? If results from freeze-etching are valid, as we have reason to believe (Moor & Mühlethaler, 1963; Moor, 1964, 1966b; Staehelin, 1968a & b), observations from the present study show that the tonoplast is distinct from other membranes of the cell. The structure of a membrane is undoubtedly related to its function. This concept is now becoming more widely appreciated (e.g. Cunningham & Crane, 1966; Glauert, 1968; Hess, 1968; Staehelin, 1968a & b) and is replacing earlier ideas of a universal organization for membranes (e.g. Davson & Danielli, 1952; Robertson, 1959). The present results clearly indicate that the tonoplast has two distinct sides and therefore cannot be regarded as a simple membrane. An asymmetrical organization of the tonoplast is evident also from sectioned material (Grun, 1963). The different density of particles could reflect different physiological activities at the two faces of the membrane and/or differences in the nature of the surrounding media (i.e. groundplasm on the outside and vacuolar sap on the inside).

6.3. 4 Grouping of particles on the tonoplast

It has already been stated that particles occur over the entire face of the tonoplast but that their distribution is seldom uniform, especially on the outer face; some of the bare areas are caused by dislodgement of particles but most appear to be natural. Areas devoid of particles are

more evident in illustrations in some reports than in the present material (Moor & Mühlethaler, 1963; Moor, 1964, 1965; Matile & Weimken, 1967; Matile, 1968; Matile & Moor, 1968). Results from this study are more similar to those shown by Branton and Moor, (1964) and Northcote and Lewis (1968). In most of these studies, as in the present work, there is little difference in the grouping of particles on the outer faces of different vacuoles. In contrast, Hess (1968) found that particle groupings varied from one region of the cell to the other although particle size remained relatively constant.

If the particles are an integral part of the membrane, we might expect their distribution and abundance to indicate different states of activity within the membrane. Similarly, variations in particle size and concentration on different membranes might reflect different functions of particular membrane systems (Hess, 1968). From observations on yeast cells, Moor and Mühlethaler (1963) concluded that the membranes grew only in the particle covered regions. It was assumed that there was a functional relationship between the particles and the production of membrane material. On this basis, different concentrations of particles might be expected to occur on the tonoplast depending on its state of growth. More particles might be anticipated on young and enlarging vacuoles than on fully differentiated ones. While there is some evidence from the present study supporting this (Figs. 147, 148) most vacuoles show little variation irrespective of size. (Figs. 145, 146). However, this does not necessarily invalidate the argument as most vacuoles in the root tips examined were probably actively growing so that little variation in density would be expected. The problem requires further study and might be approached by freeze-etching progressively older segments of root tissue.

The particles on a given tonoplast are usually uniformly distributed. If particles are involved in, or reflect, membrane growth this suggests that vacuoles in root tips grow mainly throughout their extend rather than in the local areas.

6.3. 5 Differentiation of the tonoplast with development of vacuolar inclusion bodies

While little difference is detected in particle distribution during general growth of the tonoplast, local differences are clearly apparent on formation of vacuolar inclusion bodies. The high concentration of particles in regions where these arise supports Moor and Mühlethaler's (1963) suggestion that the particles are directly involved in, or indicate membrane growth. The invagination of the tonoplast is an active rather than a passive process; therefore a greater synthesis of membrane material would be expected in these areas than in the rest of the tonoplast. Whether the particles are directly concerned with the synthesis of membrane material cannot be answered until more is known about their composition and their structural relationship with the membrane. However, the concentration of particles at points of local growth clearly establishes a functional relationship with membrane activity.

It seems unlikely that the aggregation of particles where inclusion bodies develop is an artifact. While fewer particles might be plucked from concave than from convex surfaces, this could not explain the marked difference in particle density between the sites of inclusion body formation and the rest of the tonoplast. The aggregation of particles becomes apparent before the membrane flattens and is well developed when only slight depressions have formed (Figs. 163, 170).

It has been shown that the difference between the density of particles on the invagination and on the rest of the membrane is less striking for the inner face of the tonoplast than for the outer face. What possible

explanations are there for this? On the inner side of the membrane particles are normally closely packed. Therefore in areas of localized growth only a small increase in the number of particles could be accommodated. Although on the inner face of the tonoplast invaginations provide a convex rather than a concave surface, this too would not greatly increase the space available for particles. In contrast, particles are widely distributed on the outer face of the tonoplast before invaginations develop. Another possible explanation is that the particles on the inner face of the tonoplast are less concerned with the synthesis of membrane material than those on the outer face and therefore would increase less in number.

6.3. 6 Interpretation of exposed faces of the tonoplast in frozen-etched fresh root tips

From examination of several hundred vacuoles it is concluded that fracturing in fresh root tips normally exposes the true surfaces of the tonoplast. Both the outer and inner faces are revealed. The following argument is presented in support of this conclusion.

If particles lie within the membrane as a single layer (Branton, 1966) and if fracturing splits the tonoplast one might anticipate a random apportioning of particles to the two halves of the membrane. Instead, the exposed faces of the tonoplast have distinctly different particle densities. For Branton's concept to hold, there would need to be a selective adherence of particles to particular sides of the membrane during fracturing. Furthermore, if particles remain attached to one half of the membrane corresponding depressions should occur on the other. However, the proportion of bare areas and depressions on the inner face does not account for the density of particles on the outer face.

Thus, while it is difficult to reconcile observations from the present study with Branton's (1966) original model, it might be possible to

reconcile them with a model in which the particles are in two distinct layers which separate on fracturing. If this was the model, the bonds between particles within a given layer would need to be stronger than those between layers. Furthermore, one layer would need to contain a greater density of particles than the other to explain the observed facts. Such an unequal density of particles in the two layers might help to explain the asymmetrical organization of the tonoplast seen in thin sections (Grun, 1963).

Although this hypothesis could explain the constant differences between the two exposed faces of the tonoplast there is an objection to its acceptance. The particles are too large to ^{be} accommodated within the tonoplast if we accept the dimensions of the membrane stated by Grun (1963) and others and seen in cross-fractured vacuoles (Fig. 48). It would even be impossible for a single layer of particles 100 \AA in diameter to fit into a tonoplast 75 \AA in thickness. The most asymmetrical tonoplasts measured by Grun did not exceed a total thickness of 300 \AA . Even then it would be difficult to accommodate two layers of particles as a constant feature as one layer of the tripartite structure seldom exceeds 25 \AA in thickness. This argument assumes that the layers of particles correspond in position to the outer dark lines of the tripartite membrane seen in chemically fixed tissue.

Branton (1966) uses as ~~evidence~~ evidence in support of his interpretation work on prolonged etching of yeast vacuoles. After normal etching a ridge is present at the base of many exposed membranes but when the contents of the vacuole are sublimed during prolonged etching the face of this ridge becomes exposed. Branton regards the exposed face of the ridge as the "true membrane surface" of the tonoplast. However, the ridge might also be interpreted as a phase boundary, which has been enhanced by freezing and etching, between the membrane and the surrounding medium. With

prolonged etching, the deposition of non-etchable vacuolar contents against the true face of the membrane might have produced the "surface" of the ridge.

The suggestion of Northcote and Lewis (1968) also warrants discussion. In view of results obtained from the present study it does not satisfactorily explain the observed facts. In freeze-etch material, one would have to regard the image of scooped-out vacuoles as the imprint of the outer surface of the tonoplast and the image of convex surfaces as the imprint of the inner surface of the tonoplast. While Northcote and Lewis's suggestion is not improbable it presents certain difficulties. It implies that the membrane is always stripped away because exposed surfaces show only one view. If the membrane sometimes remained, positives and negatives of a given surface should occur; but this does not happen. Northcote and Lewis's idea implies also that the particles observed would correspond to hollows in the original membrane. On this basis depressions would be a structural feature of membranes but there is no evidence to support this. Alternatively, the particles might be real and attached to the membrane surface but left adhering to the surrounding medium after the membrane had been torn away. However this idea provides unnecessary complications. If the particles are real structures closely associated with membrane organization one would expect them to be strongly bonded to the membrane. Weak bonding forces have been suggested to explain the absence of ribosomes on freeze-etch ER membranes (Staehelin, 1968a) but it seems unlikely that this applies to the particles on the tonoplast. Observations show that very few particles are in fact detached from the tonoplast in frozen-etched fresh cells (Fig. 142).

From the above discussion, it is concluded that fracturing in frozen-etched fresh cells follows the real surfaces of the tonoplast. Either the surface bordering the groundplasm or that abutting the vacuolar contents

is exposed. Further evidence to support this interpretation comes from observations on partly torn portions of the tonoplast which have been folded back on to the inner surface of the membrane. The folded back portion reveals particles identical to those seen on the outer surface of the tonoplast (Fig. 196). If the particles were within the membrane, as Branton (1966) suggests, and not on the surface, the outer exposed surface of the tonoplast might be expected to be bare when folded back on itself.

6.3.7 Interpretation of the tonoplast in prefixed material:

Interpretation of the tonoplast in prefixed roots is complicated because the membrane presents a variety of appearances:

- (a) surfaces bearing particles as in frozen fresh cells
- (b) surfaces showing plaques but no particles
- (c) surfaces showing neither plaques nor particles
- (d) intermediate conditions

In prefixed root tips, other workers have described only particles on the tonoplast (Matile, 1968; Matile & Moor, 1968). However, it is possible that results may be influenced by specimen preparation; in Matile's studies prefixation was followed by glycerination whereas in the present work these steps were combined.

Besides prefixed material, plaques are occasionally found on tonoplasts of Lonicera and Mentha (Figs. 21, 43) placed in 20% glycerol overnight and on membranes of fungal contaminants grown in 15% and 20% glycerol (Fig. 44). Plaques on tonoplasts have also been reported by Staehelin (1968a) who found them in the alga Cyanidium grown in glucose solution. Similar plaques are described on the lamellae in the myelin sheath of mouse nerve (Moor, 1966a) and on artificial and biological membranes besides the tonoplast (Staehelin, 1968a). Moor (1966a) thought that the plaques were attached to the outer surface of the membrane. Staehelin (1968a) however, has

demonstrated that the plaques are part of the membrane. The plaques are exposed by fracturing both within and along the surfaces of the membrane. Stashelin regards the plaques as "island-like remnants of partly broken away upper-lying halves of the bilayers" of the membrane.

Stashelin's hypothesis satisfactorily explains the situation found in tonoplasts and in other membranes of the present material. Thus tonoplasts bearing plaques represent a modified condition in which fracture planes within the membrane are altered by pretreatment. Possibly GA replaces the original bonds between molecules; it is known to cross-link proteins (Sabatini et al., 1963). As a result portions of the membrane may not be held so strongly and may break away during fracturing. Other conditions of pretreatment and subsequent freezing undoubtedly also influence the fracturing properties of the membrane. Matile and Moor (1968) for example did not find plaques in prefixed root tips of Zea. It therefore seems that GA and glycerol used simultaneously cause a greater alteration in fracture planes within membranes than when glycerination follows prefixation.

Although the presence of glycerol during prefixation may explain some differences between results obtained by Matile and Moor (1968) and in the present study, glycerol cannot be the only factor involved. The variation in appearance of the tonoplasts prepared with the same prefixation schedule suggest that other conditions are important. Some tonoplasts, for example, retain a normal complement of particles but others show variously depleted populations (Figs. 149, 156d; see also micrographs of Matile & Moor 1968) until in the extreme condition almost no particles are visible. At this stage fracturing seems to follow the surface of the tonoplast with the outer portion of the membrane remaining intact. Intermediate conditions showing particles, smooth exposed outer surfaces, and areas bearing plaques are found occasionally (Fig. 160). Once fracturing starts to pluck away the outer portion of the membrane, it (with few intermediate stages)

proceeds to the condition illustrated (Figs. 150-153). Sometimes the properties of the membrane are so altered that most of the outer layer is stripped away leaving only occasional traces of plaques (Figs. 155, 159). The various appearances of the tonoplast, within and between specimens, suggest that ⁿmany factors are involved in altering the fracture plane within frozen-etched membranes. The plane of fracture is probably influenced by the chemical and physical treatment of the tissue and by the physiological state of organelles and specimens.

The variation in results shows that while fracturing is somewhat random it is confined within certain limits. The similar densities of plaques on both sides of the tonoplast suggest that the inner and outer faces are equally affected. This emphasizes that the fracture plane is not greatly influenced by the curvature of the vacuole; the organization of the membrane instead determines this.

If changes occur in membrane organization, alterations in fracturing might be expected. Staehelin (1968a) noted that the size and distribution of plaques altered in varying polar lipid mixtures. Similarly, if the membranes of an organelle change during development, an alteration in the pattern of fracturing might be anticipated. This might explain why different plaque densities occur on some large vacuoles compared with small vacuoles (Figs. 165b & c). Some large vacuoles in frozen-etched living cells have a slightly lower density of particles on the inner surface than do small vacuoles; this supports the notion that a slight change may have occurred in the organization of the tonoplast during differentiation.

A feature not satisfactorily explained is that plaques are distributed more or less regularly on the tonoplast. If fracturing is a random process and if prefixation affects the tonoplast equally, one might expect a variety of arrangements of fractured and unfractured regions. However,

the regular pattern found suggests that fracturing is not completely random but reflects some internal organization of the membrane. Where plaques are present, bonding within the membrane may be strong. Possibly prefixation induces an aggregation of bonds at sites more or less evenly distributed throughout the membrane. On this hypothesis the size of the plaques might also reflect the concentration of bonds at any one site.

6. 4 Summary

The tonoplast has been studied with particular reference to its appearance in freeze-etch replicas. Root tips grown in 20% glycerol and those prefixed in GA/glycerol prior to freeze-fixation were used. In frozen-etched fresh cells the tonoplast is covered with globular particles; on the inner face the particles are about two thirds more numerous than on the outer face. On both exposed faces the particles are uniformly distributed, except where the tonoplast invaginates to form vacuolar inclusion bodies. At these sites, especially on the outer face, the particles are more concentrated than on the rest of the tonoplast. This aggregation of particles is considered to reflect high membrane activity where invaginations develop compared with that of the rest of the tonoplast.

In prefixed material exposed faces of the tonoplast showed either particles, particles on top of plaques (rare), plaques, or smooth surfaces devoid of both particles and plaques. Membranes showing plaques only occurred most commonly; the plaques were of similar frequency on both sides of the membrane.

In frozen-etched fresh cells it is concluded that fracturing normally follows the true surfaces of the tonoplast. The particles are therefore believed to be attached to and slightly embedded in these surfaces.

When specimens are prefixed the fracture plane is modified; occasionally

it follows the surface but usually it strips away the particles and plucks out portions of the outer layer of the membrane leaving only remnants of it in the form of plaques. In extreme conditions, the outer layer of the tonoplast is completely torn away exposing the inner organization of the membrane.

Fig. 134 High resolution of the tonoplast in a root cap cell of Zea mays showing the tripartite organization of the membrane (arrows) (x 82,500).

Figures 135 - 137 General appearance of fractured faces of the tonoplast in frozen-etched fresh root tips.

Fig. 135 Two adjacent vacuoles showing outer (left) and inner (right) exposed surfaces of the tonoplast in Avena sativa. The greater density of particles on the inner side compared with the outer is evident. Observe the similar density of particles on outer surfaces on portions of other vacuoles shown (arrows). (x 71,000).

Fig. 136 Surfaces of the tonoplast in another specimen of A. sativa. An outer surface is exhibited by the middle elongated vacuole; the two adjacent vacuoles show inner surface views. The density of particles on the inner surface of both tonoplasts is similar. Arrows indicate the presence of a "ridge" between the groundplasm and the surface of the vacuole. (x 69,000).

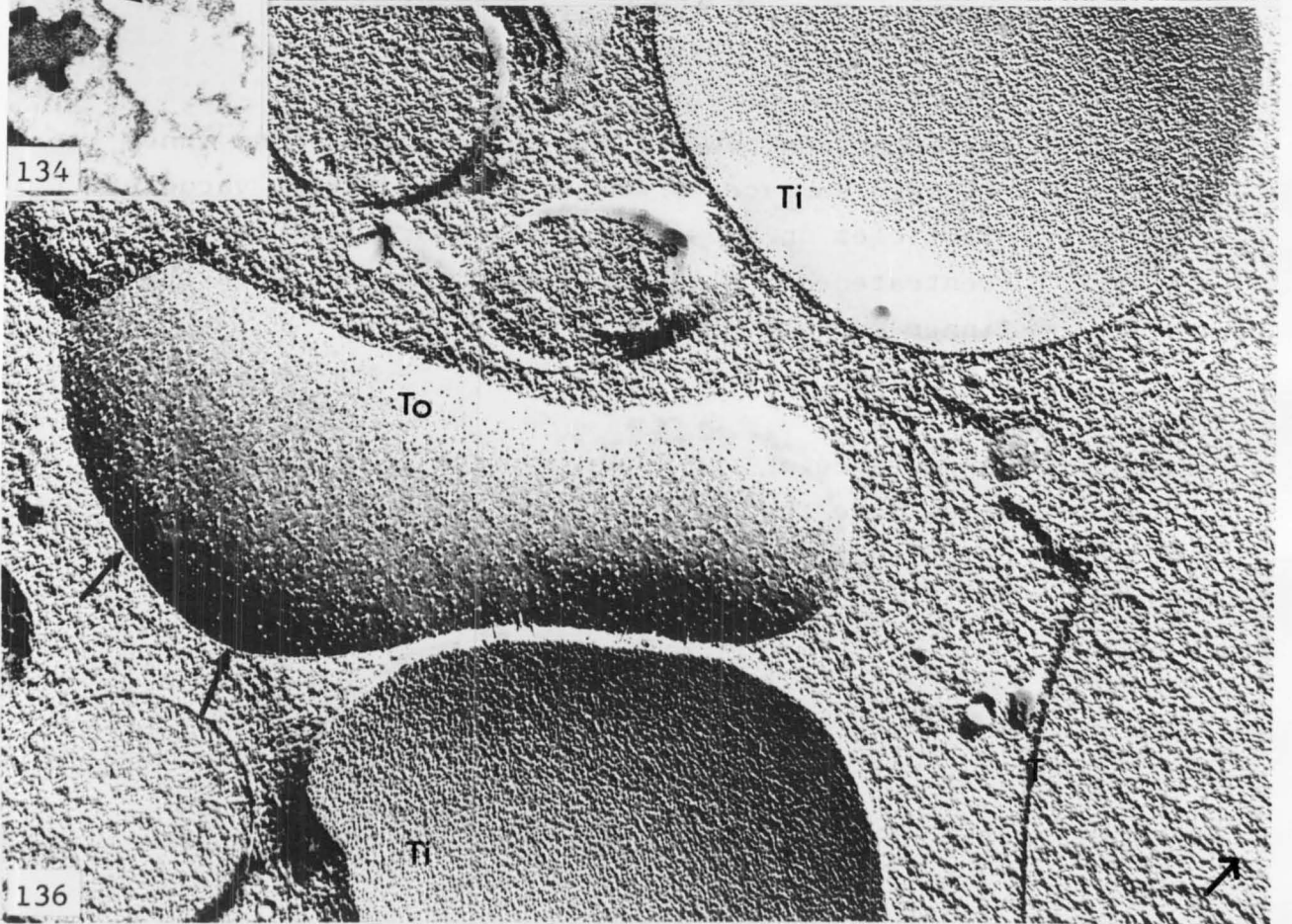
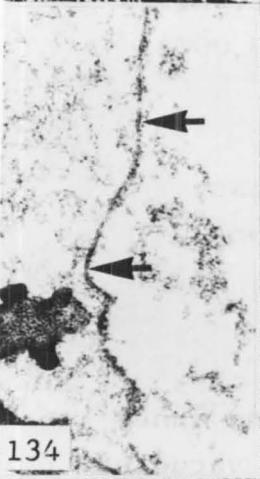
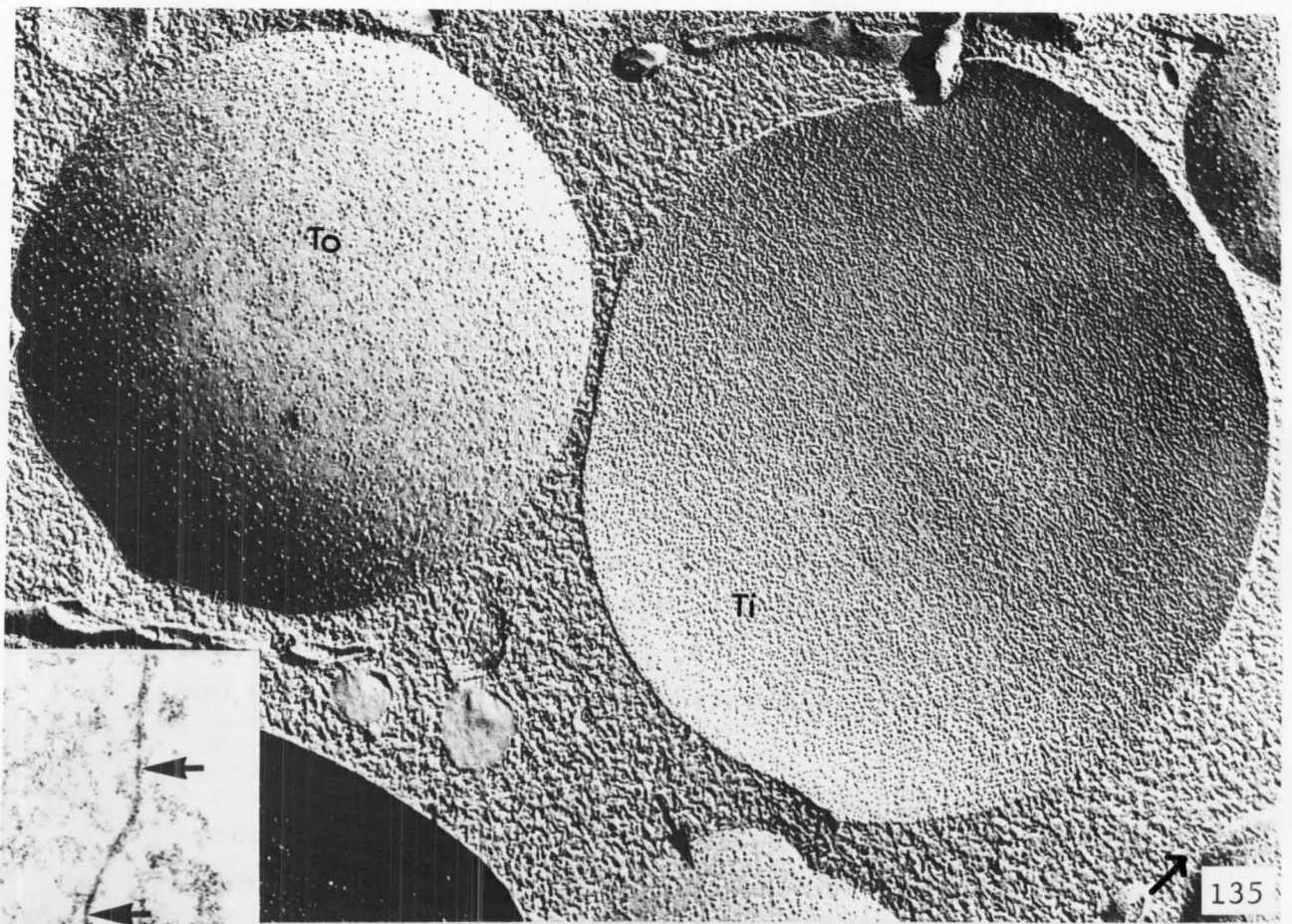
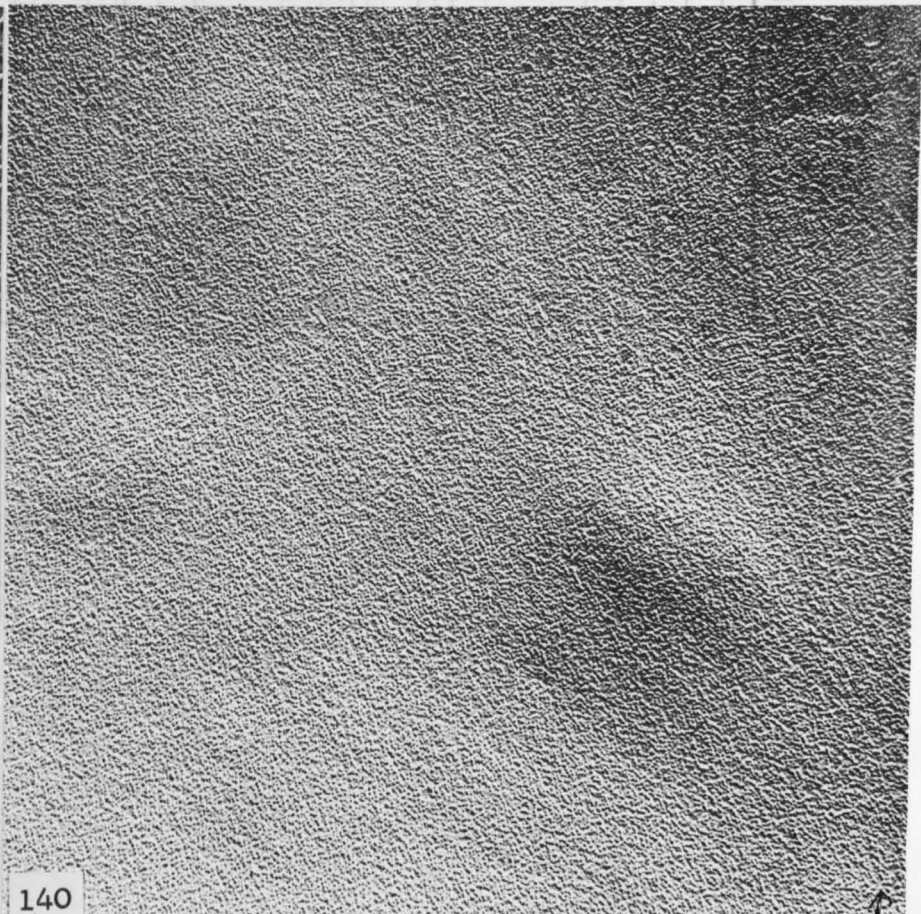
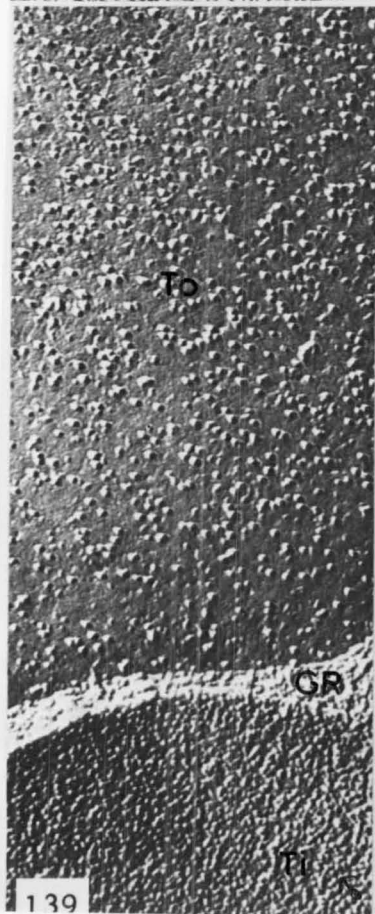
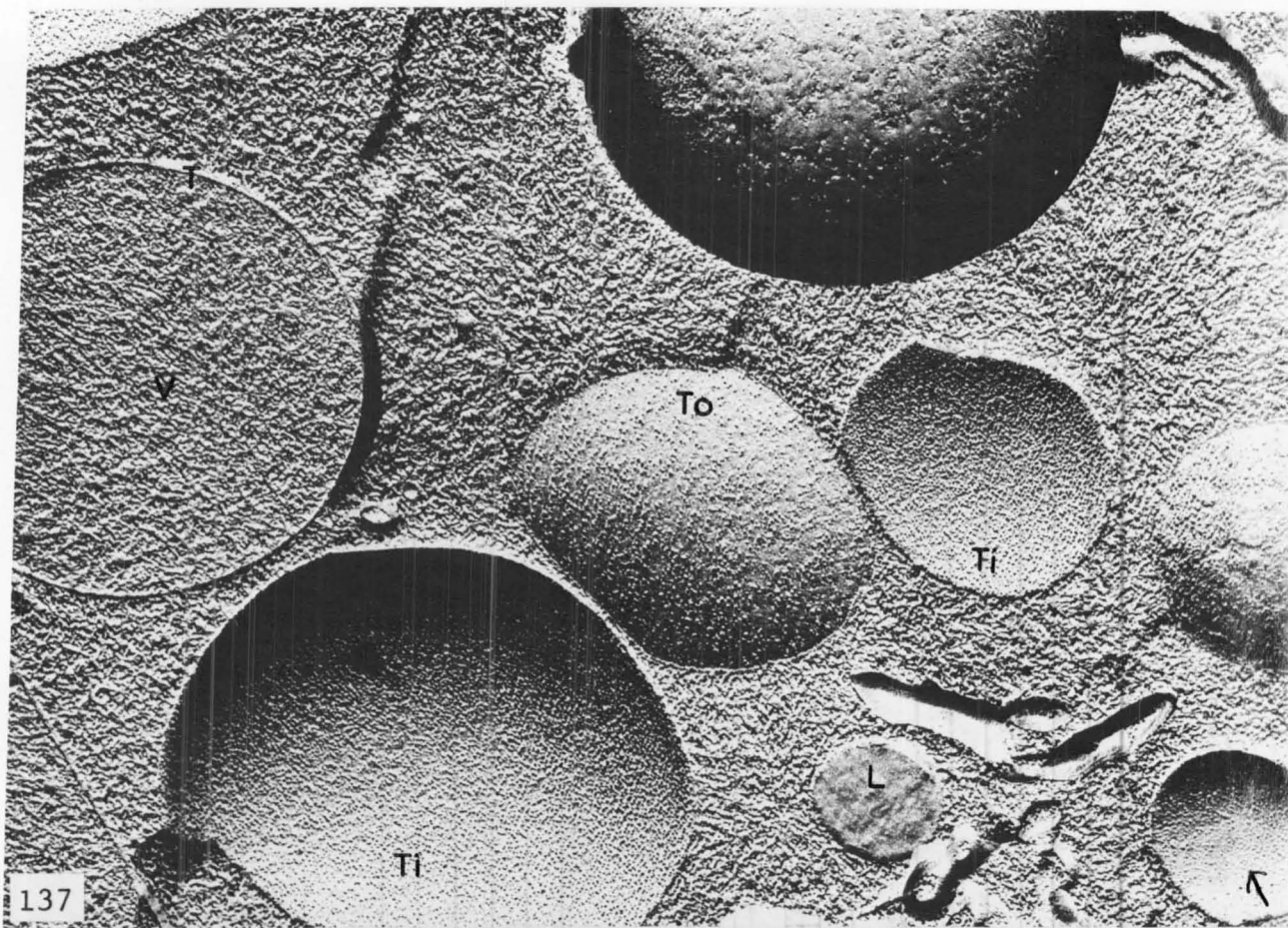


Fig. 137 - Vacuoles showing outer and inner exposed faces of the tonoplast in Triticum vulgare. The density of particles on these surfaces is similar to that shown in Figs. 135 and 136. The cross-fractured vacuole (left) is bounded by a single unit membrane (T). The organelle at top right is probably a plastid in surface view. (x 69,000).

Figures 139 - 148 Details of the tonoplast surface in frozen-etched fresh root tips of A. sativa.

Fig. 139 A comparison between the outer (upper) and inner (lower) surfaces of two closely adjacent vacuoles. Particles on the outer surface are less concentrated and slightly larger than those on the inner surface. (x 103,000).

Fig. 140 Appearance of the inner surface of a large vacuole. Note the close packing and rather uniform distribution of particles. (x 56,000).



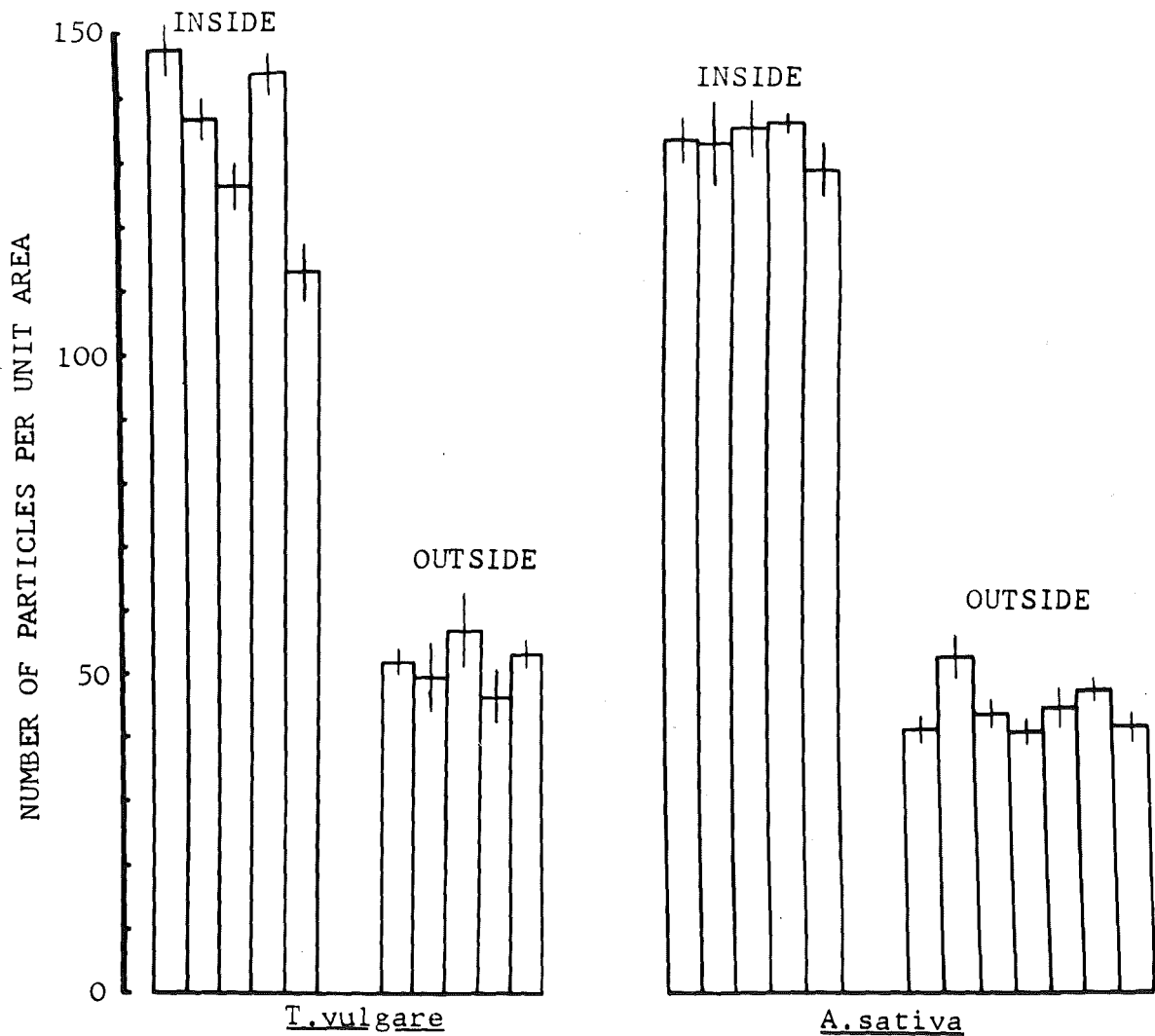


Fig. 138 Density of particles on outer and inner surfaces of the tonoplast in frozen-etched fresh root tips. Each bar in the histogram expresses the mean of quadrats sampled for one vacuole. Vacuoles of various sizes were sampled. The vertical lines indicate \pm one standard error of the mean for each vacuole.

TABLE 7
ANALYSIS OF PARTICLE DENSITY ON TONOPLASTS IN FROZEN-ETCHED
FRESH ROOT TIPS

| Species | Surface of tonoplast | Mean density of particles | t test |
|-------------------------|----------------------|---------------------------|-----------|
| <u>Avena sativa</u> | outside | 44.3 | t = 39.9 |
| | inside | 133.1 | P ≪ 0.001 |
| <u>Triticum vulgare</u> | outside | 51.3 | t = 42.8 |
| | inside | 131.9 | P ≪ 0.001 |

Outer surface of T.vulgare versus A.sativa

t = 2.85
P < 0.02

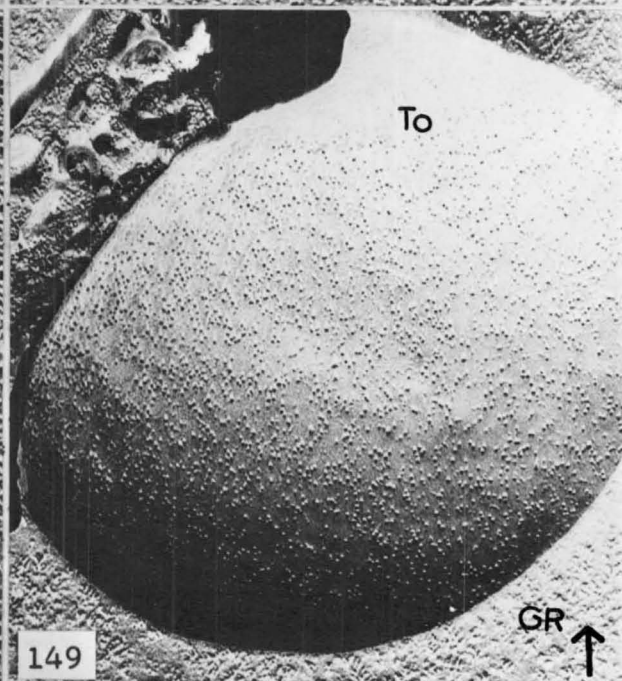
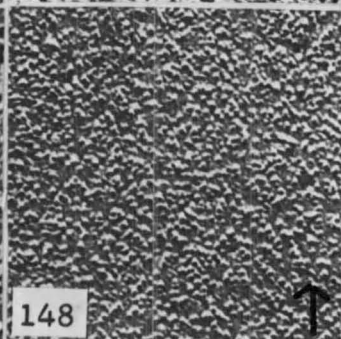
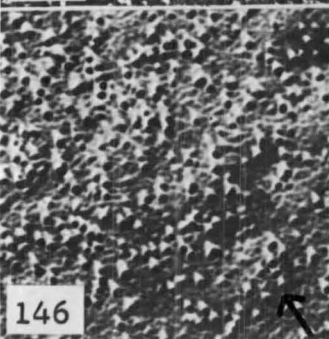
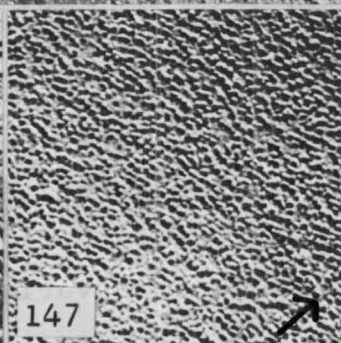
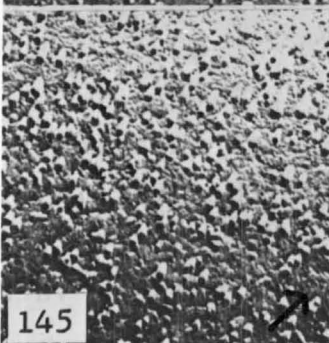
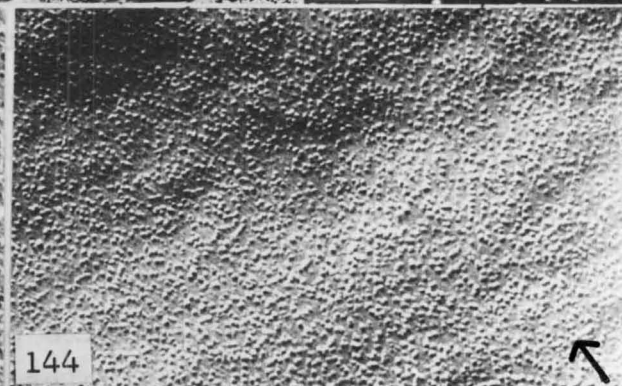
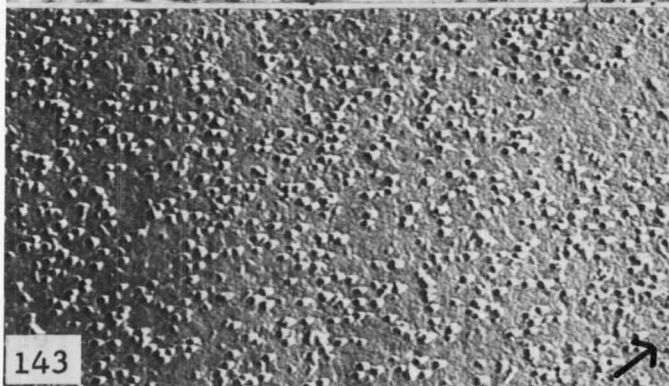
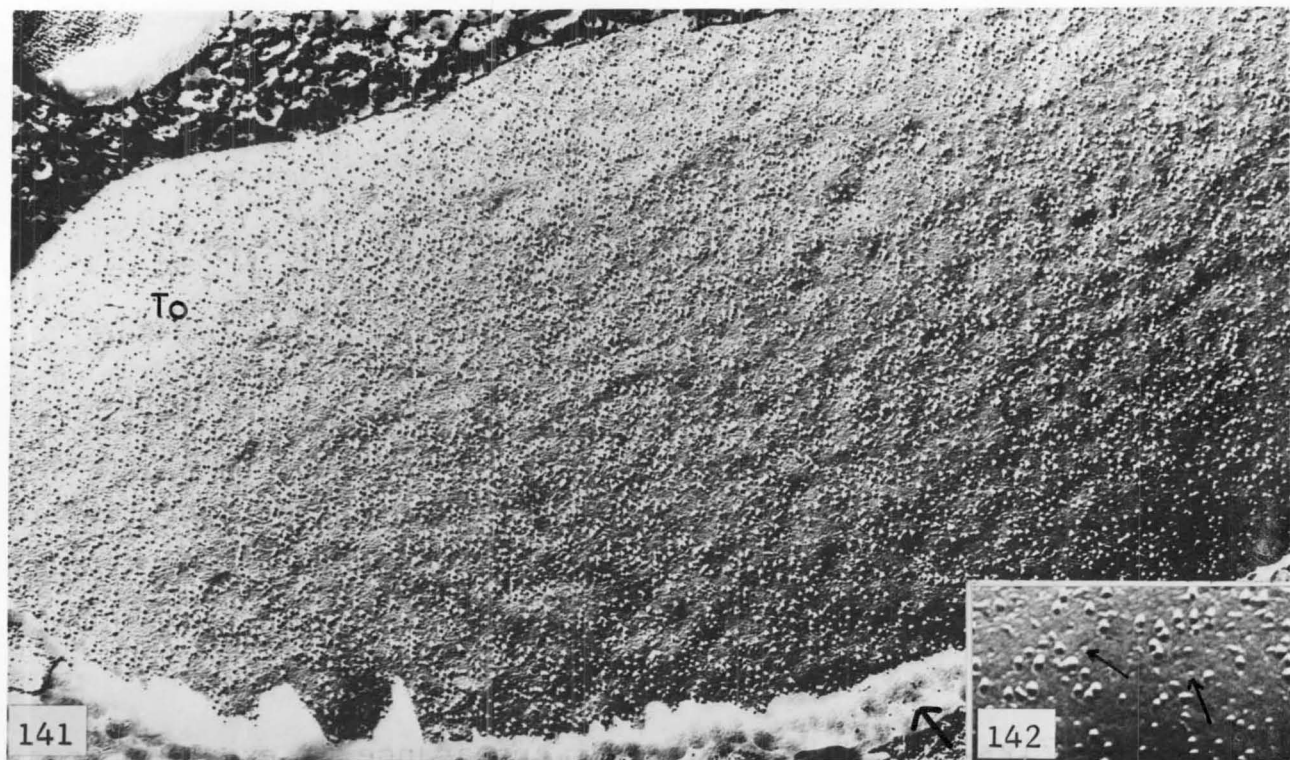
Inner surface of T.vulgare versus A.sativa

t = 0.20
P > 0.01

TABLE 8
ANALYSIS OF PLAQUE DENSITY ON OUTER AND INNER SURFACES OF
TONOPLAST IN PREFIXED ROOT TIPS OF LONICERA NITIDA

| Surface of tonoplast | Mean density of plaques | t test |
|----------------------|-------------------------|----------------------|
| outer | 19.6 | t = 0.93 P > 0.10 |
| inner | 17.7 | |

- Fig. 141 The outer surface of a large vacuole showing particles distributed over the entire exposed surface. The slightly dimpled appearance of the tonoplast is due to freezing. (x 64,000).
- Fig. 142 Portion of the outer surface of a small vacuole. Particles are attached to the surface and small depressions occur where others have been dislodged (arrows). (x 132,000).
- Fig. 143 High magnification of the outer surface of the tonoplast of a medium sized vacuole. Areas devoid of particles are irregularly distributed. (x 110,000).
- Fig. 144 The inner surface of the tonoplast of a medium sized vacuole at high magnification. The particles are more closely packed than those on the outer surface. (x 150,000).
- Figs. 145 & 146 Density of particles on outer surfaces of a small and a large vacuole respectively. The comparison suggests little change in density on enlargement of the vacuole. (both x 115,000).
- Figs. 147 & 148 A comparison between density of particles on inner surfaces of tonoplast in a small (Fig. 147) and enlarged vacuole (Fig. 148). The larger vacuole shows a slightly lower density of particles suggesting a change in the inner surface of the tonoplast on differentiation of the vacuole. (both x 115,000).
- Fig. 149 The outer surface of the tonoplast in a prefixed specimen of Lonicera nitida. Particles are present but compared with frozen-etched fresh cells (c.f. Fig. 141) areas devoid of particles are larger. (x 45,000).

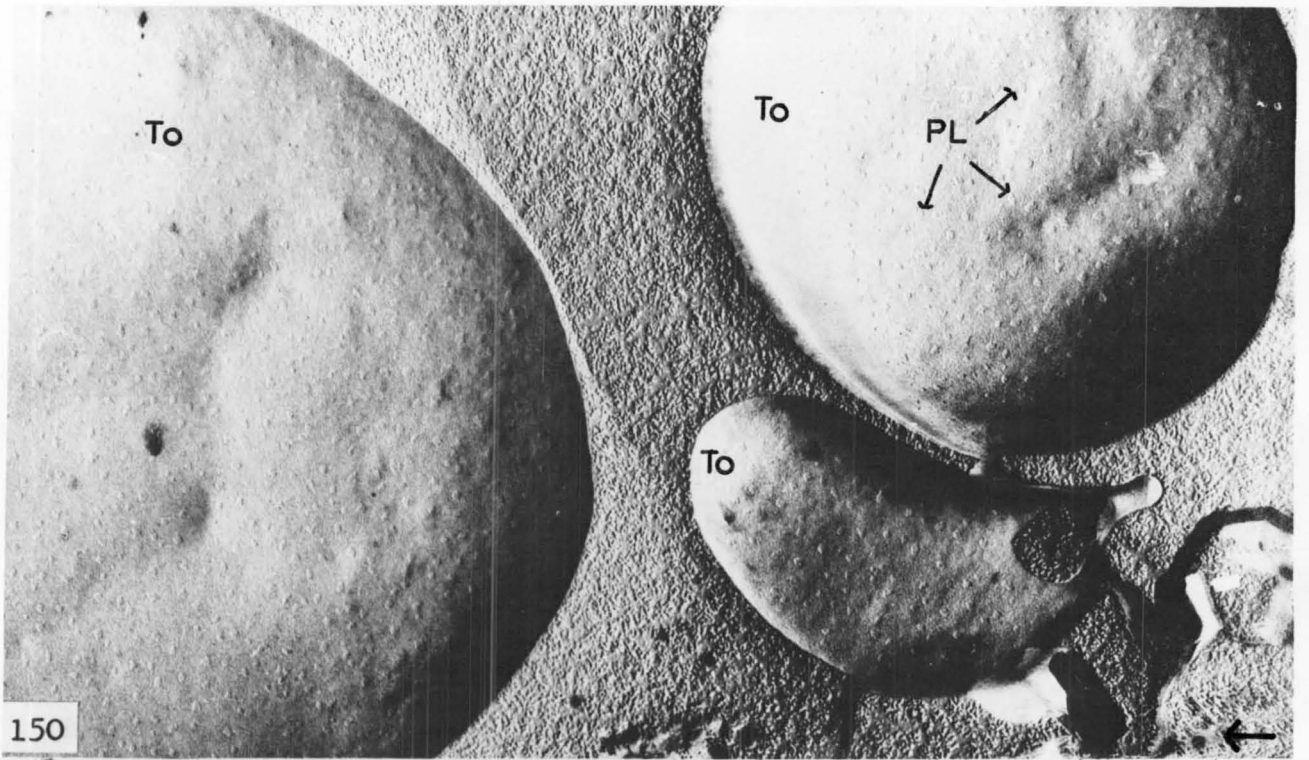


Figures 150 - 160 Fractured faces of the tonoplast in frozen-etched root tips prefixed in glutaraldehyde in 30% glycerol prior to freezing.

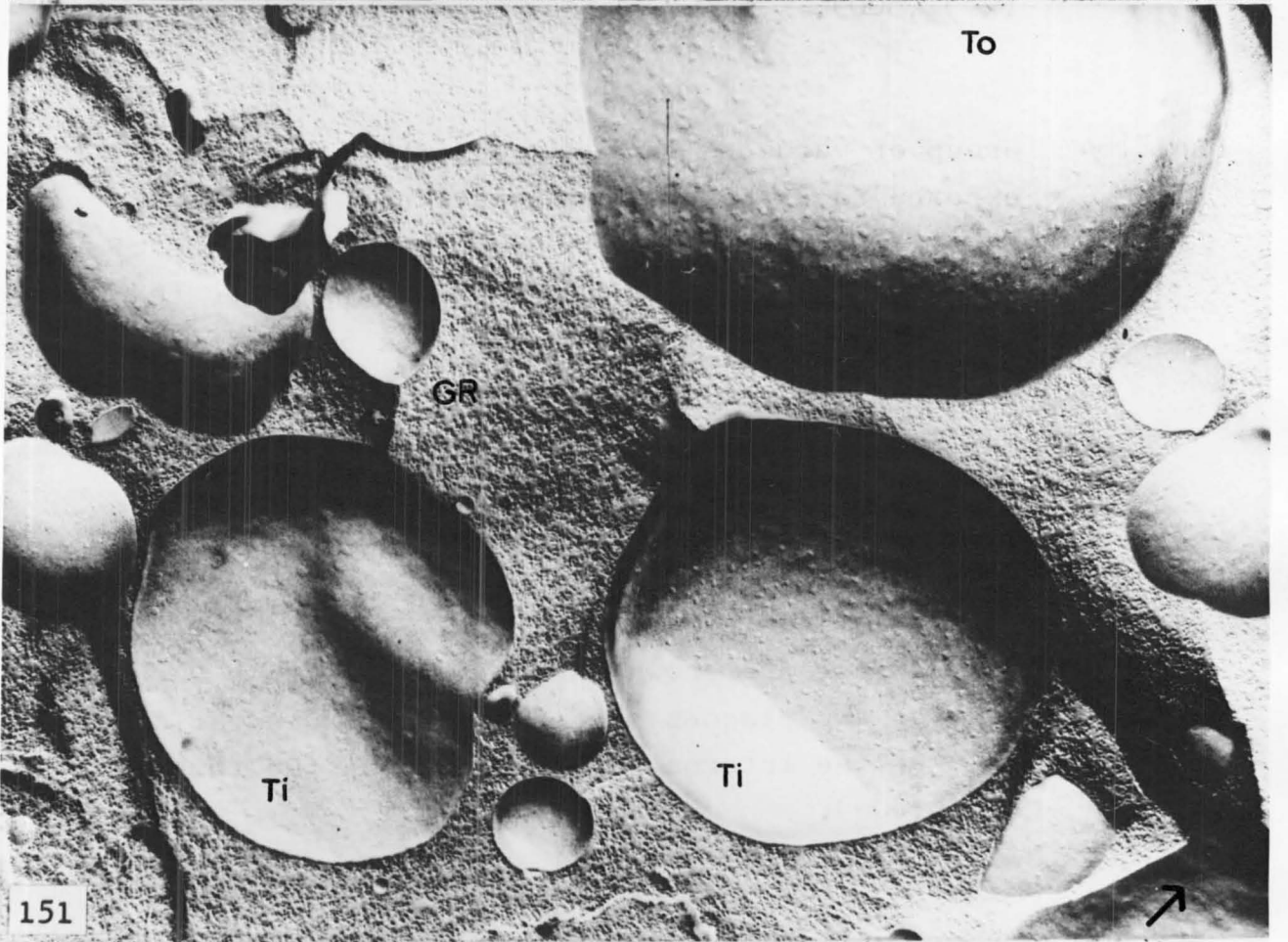
Figs. 150 & 151 General appearance of exposed faces of the tonoplast in L.nitida showing presence of plaques rather than particles. The plaques are mainly uniformly distributed.

Fig. 150 The outer faces of a group of vacuoles. The range in size of plaques is similar on the different vacuoles. (x 45,000).

Fig. 151 A group of vacuoles showing outer and inner exposed faces of the tonoplast. The distribution of plaques is similar on both faces but the size of plaques shows some variation between vacuoles. (x 45,000).

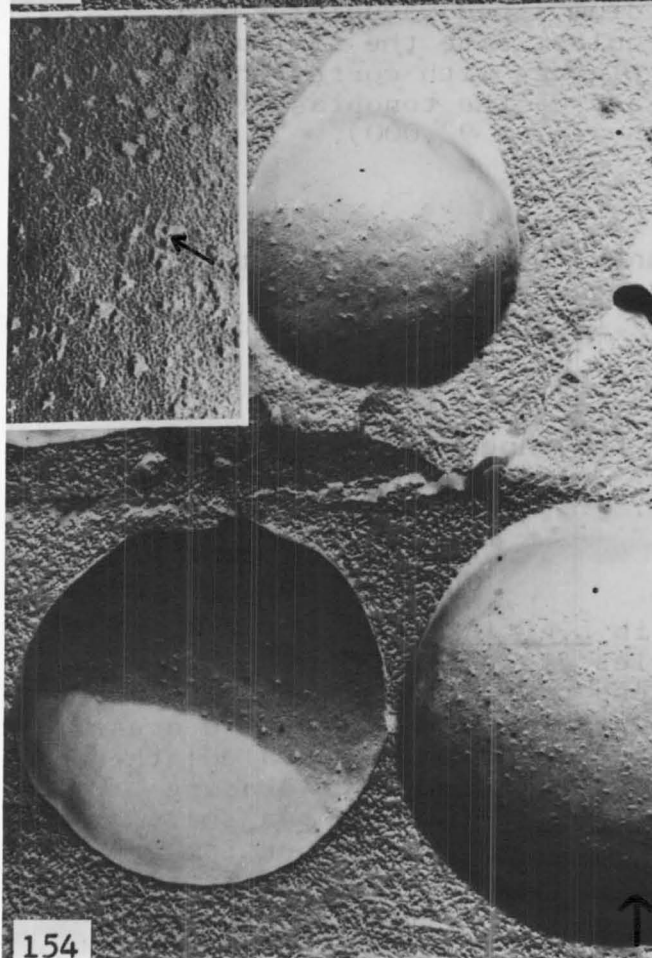
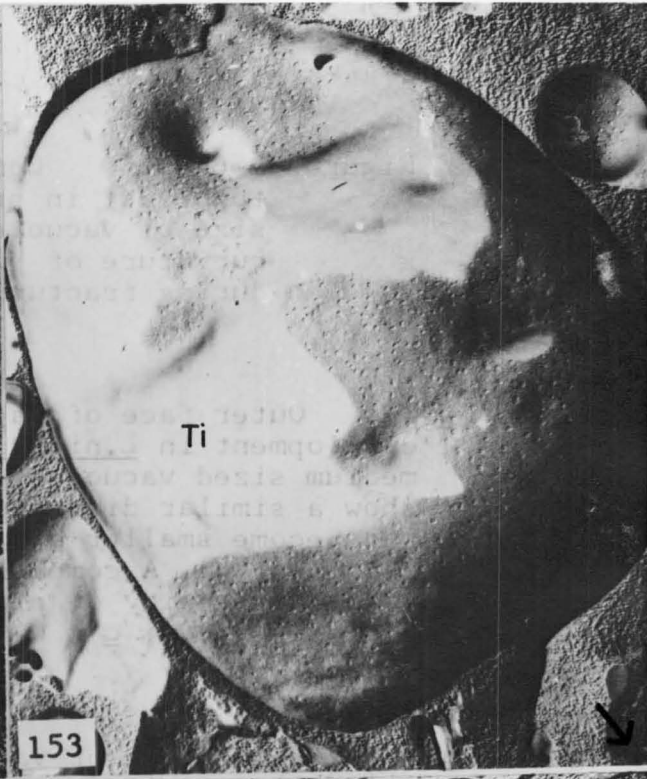
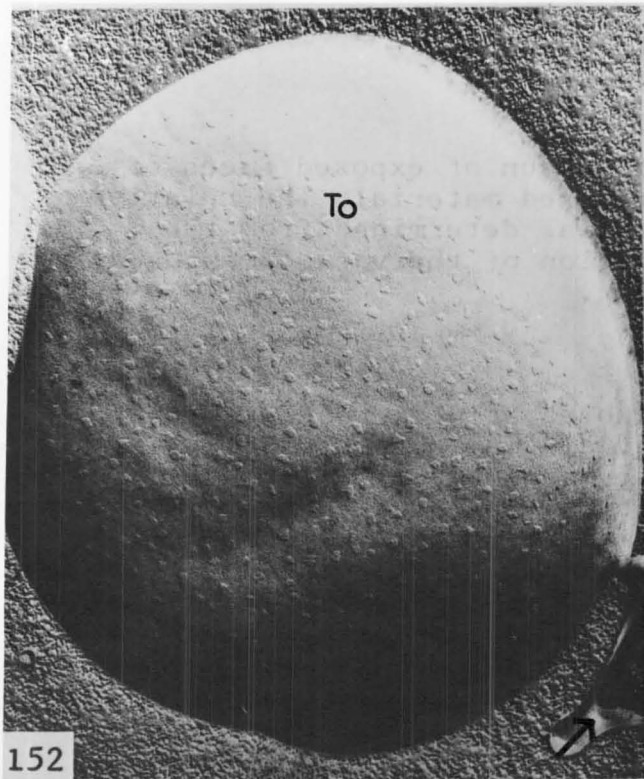


150



151

- Fig. 152 Details of the outer face of a vacuole in L.nitida. The plaques are slightly raised above the rest of the exposed face of the membrane and are angular in outline. The exposed face of the tonoplast between the plaques is finely granular (x 91,000).
- Fig. 153 Plaques on the inner face of a vacuole in L.nitida (x 37,500).
- Fig. 154 Group of vacuoles in T.vulgare showing outer and inner exposed faces of the tonoplast. Both plaques and particles are present on some faces. The particles are attached to the plaques (arrows) rather than to areas between them (see insert) (x 57,000; insert x 123,700).
- Fig. 155 Inner face of a vacuole in T.vulgare showing a fine granular appearance similar to areas between plaques on other vacuoles (c.f. Figs. 150 & 151). Details of the surface are shown in Fig. 159. In this vacuole most of the plaques have been removed revealing much of the internal organization of the tonoplast. (x 36,500).



Figures 156 - 160 Comparison of exposed faces of tonoplast in prefixed material. The relative size of vacuoles was determined from the curvature of portion of the vacuole exposed during fracturing.

Fig. 156 a - d Outer face of vacuoles at different stages of development in L.nitida. a, small vacuole; b & d medium sized vacuoles; c large vacuole. The plaques show a similar distribution at the various stages but become smaller and more numerous in some large vacuoles (c). A comparison of a - c with d shows the relative density of plaques to particles. (a x 75,000; b & c x 92,000; c x 64,000).

Fig. 157 a - d Inner face of vacuoles in L.nitida as above. a, small vacuole; b, medium sized vacuole; c & d two different large vacuoles. Note the similarity in size and density of plaques with corresponding stages from the outer face of the tonoplast (Fig. a - c). (a, x 103,000; b - d, x 92,000).

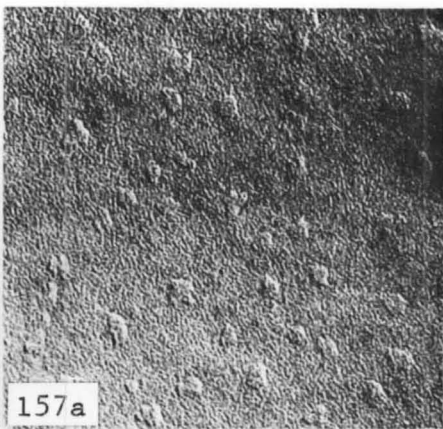
Fig. 158 a & b Outer (a) and inner (b) faces of the tonoplast in A.sativa. The size and density of plaques is similar to that shown by L.nitida. (both x 92,000).

Fig. 159 Higher magnification of the inner face of the tonoplast shown in Fig. 155. Only remnants of plaques are visible. (x 91,000).

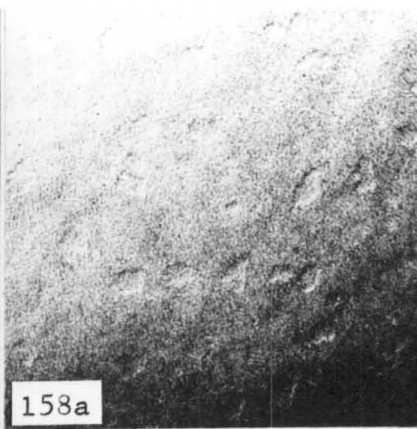
Fig. 160 Portion of a tonoplast in L.nitida showing irregular fracturing. While particles are retained over much of the surface, areas occur where particles have been removed exposing various levels within the membrane. In areas A the outer portion of the membrane is revealed; there is no ridge present separating the region bearing particles from the smooth areas. At site B a small "ridge" occurs indicating that the outer portion of the membrane has fractured away revealing an internal region of the membrane. Near C the outer portion of the membrane has been incompletely removed leaving remnants in the form of indistinct plaques. (x 69,500).



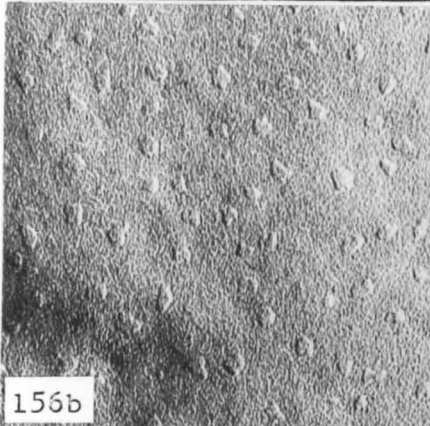
156a



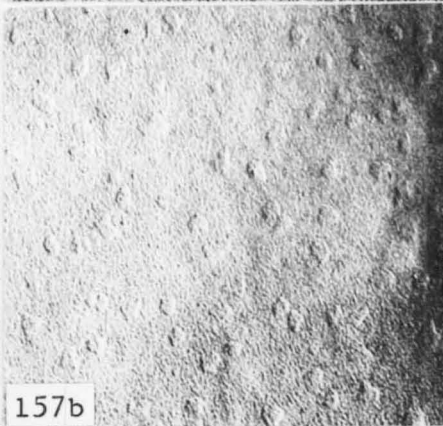
157a



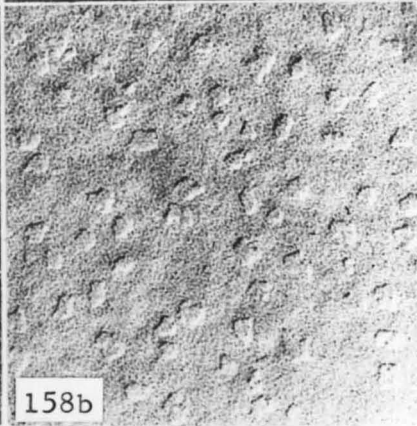
158a



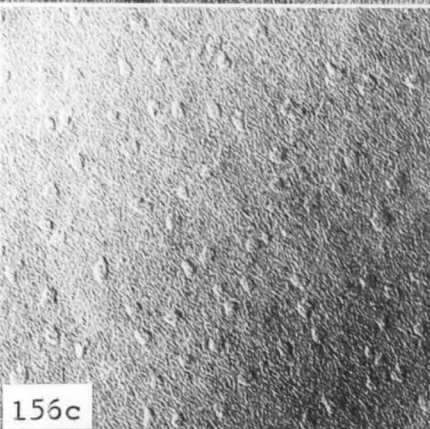
156b



157b



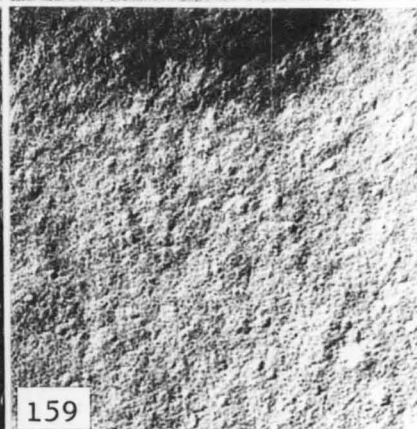
158b



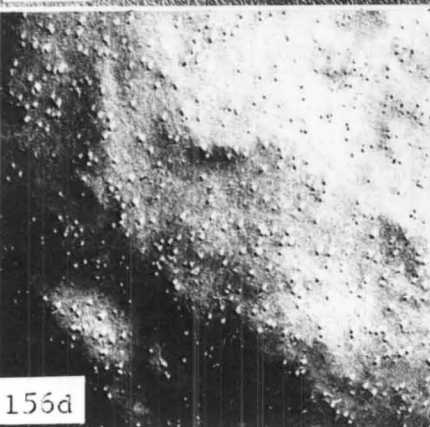
156c



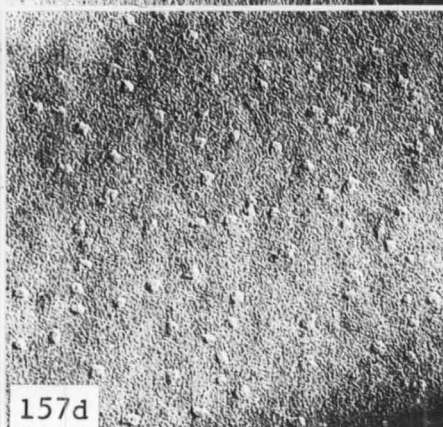
157c



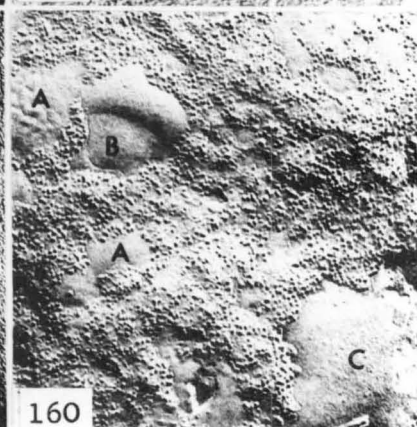
159



156d



157d



160

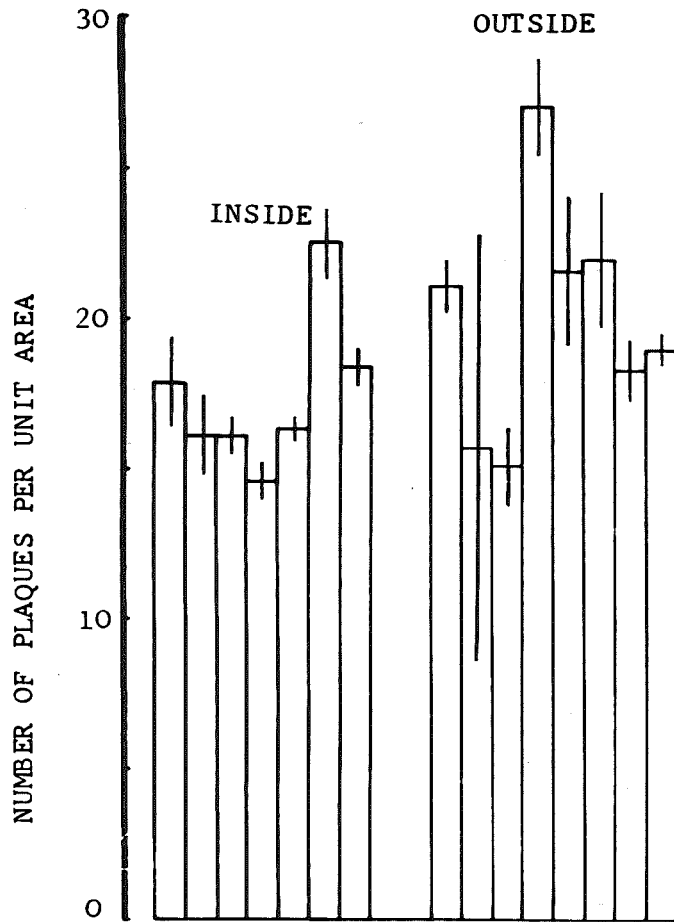


Fig. 161 Density of plaques on inside and outside of tonoplast in prefixed root tips of Lonicera nitida. Each bar in the histogram expresses the mean of quadrats sampled for one vacuole. The vertical lines indicate \pm one standard error of the mean for each vacuole.

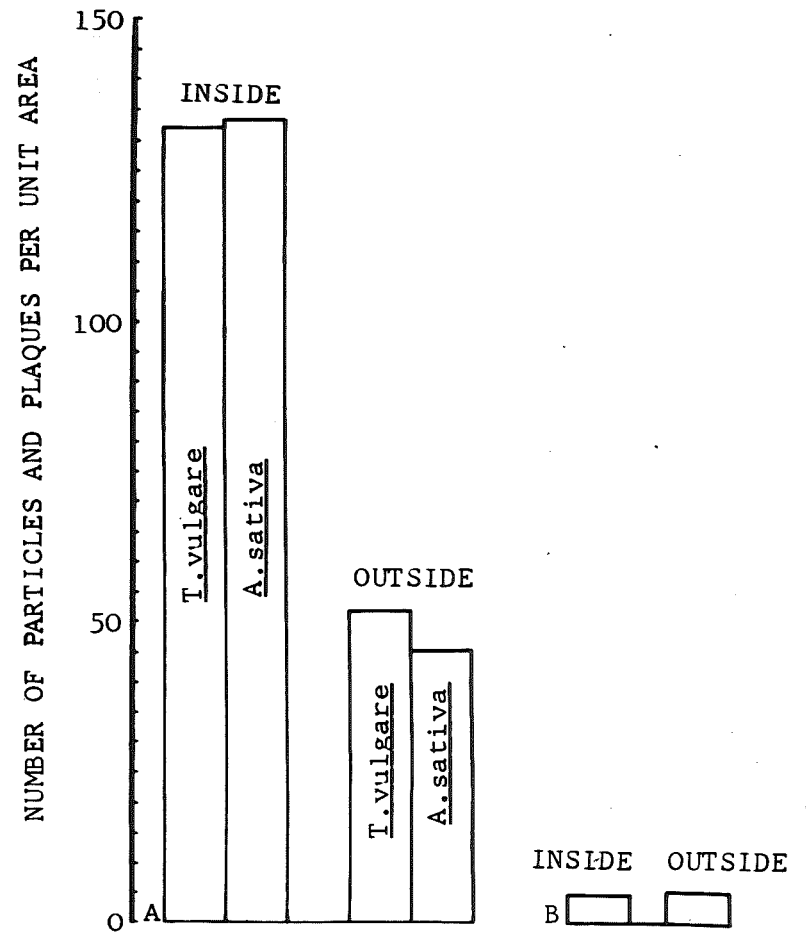


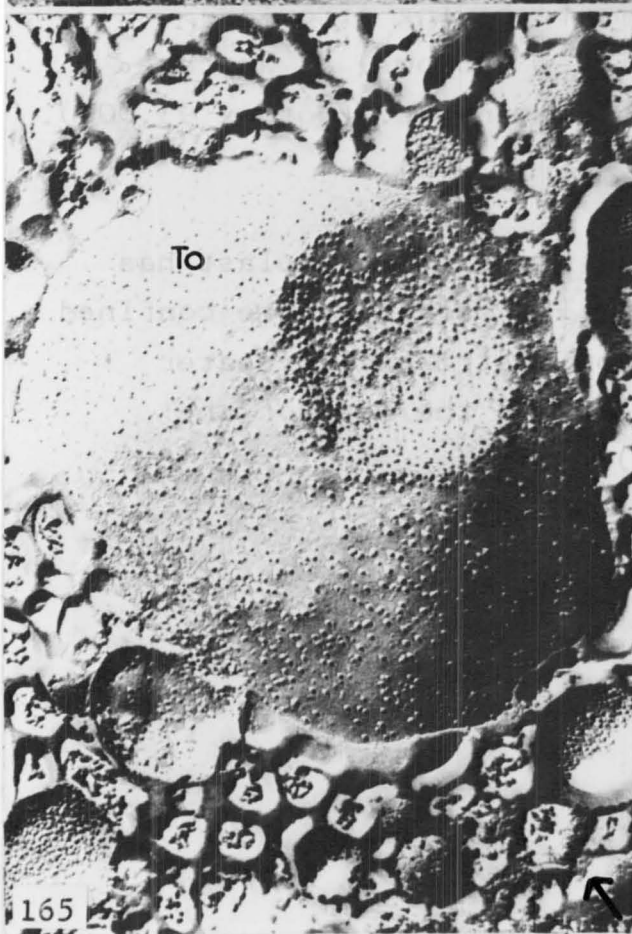
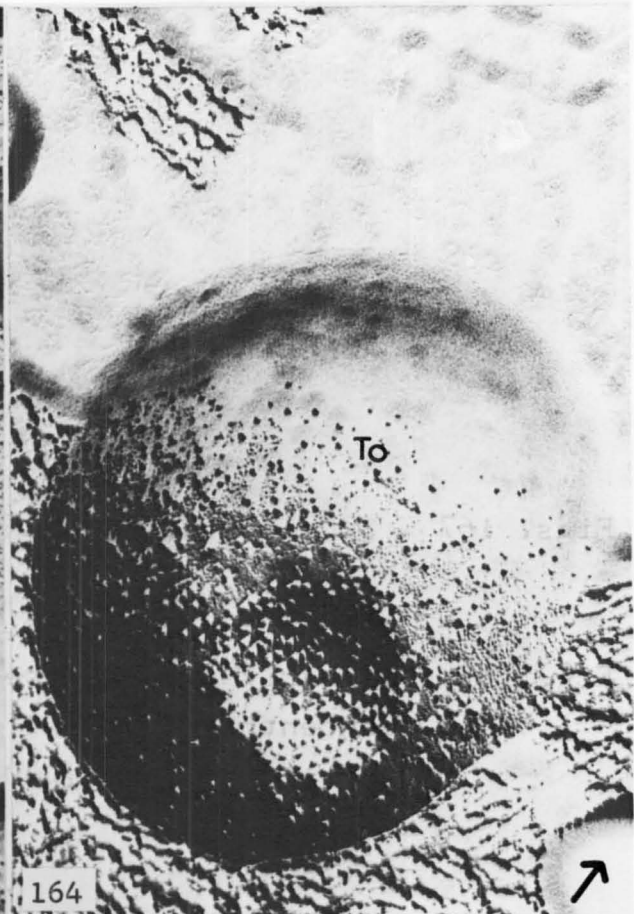
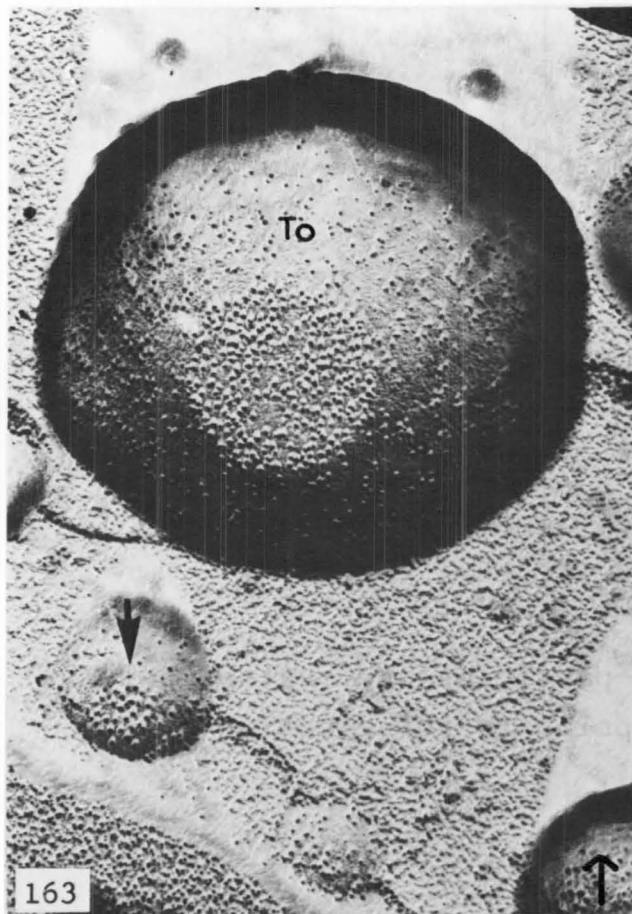
Fig. 162 Comparison of particle and plaque density on inner and outer surface of tonoplast. Results based on means from Figs. 138 & 161. A, frozen-etched fresh roots. B, prefixed root tips of L. nitida.

Figures 163 - 170 Differentiation of the outer surface of the tonoplast in frozen-etched fresh root tips on formation of vacuolar inclusion bodies.

Fig. 163 Two small vacuoles in T.vulgare showing concentration of particles where invaginations develop on formation of inclusion bodies. Particles are aggregated on the smaller vacuole (arrow) but the tonoplast is still rounded at this point. A later stage is shown in the upper vacuole where the tonoplast is flattened where particles are concentrated. The accumulation of particles is confined to circular areas where invaginations develop. (x 101,000).

Figs. 164 & 165 Invaginations of the tonoplast in A.sativa on further differentiation of inclusion bodies. The density of particles is greater in the depression than on the rest of the tonoplast. (Fig. 164, x 94,000; Fig. 165, x 84,500).

Fig. 166 An enlarging vacuole in T.vulgare showing several differentiated areas (arrows) marking sites of developing inclusion bodies on the outer surface of the tonoplast. Fragments of adjoining organelles are attached to the tonoplast. (x 28,500).

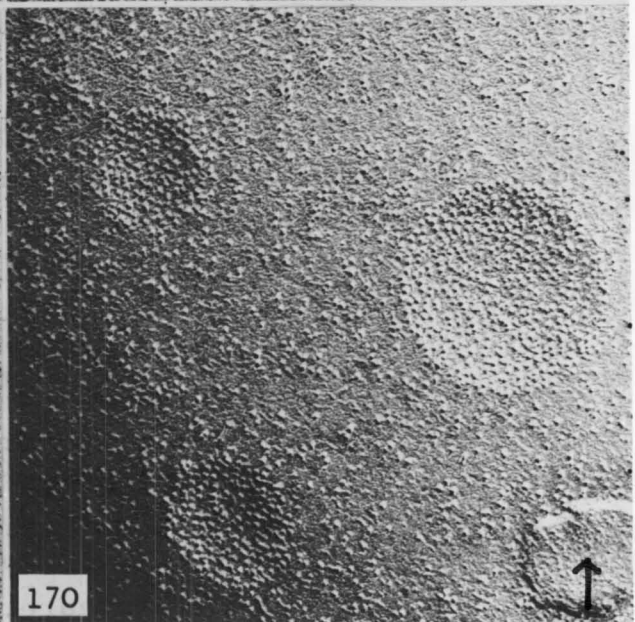
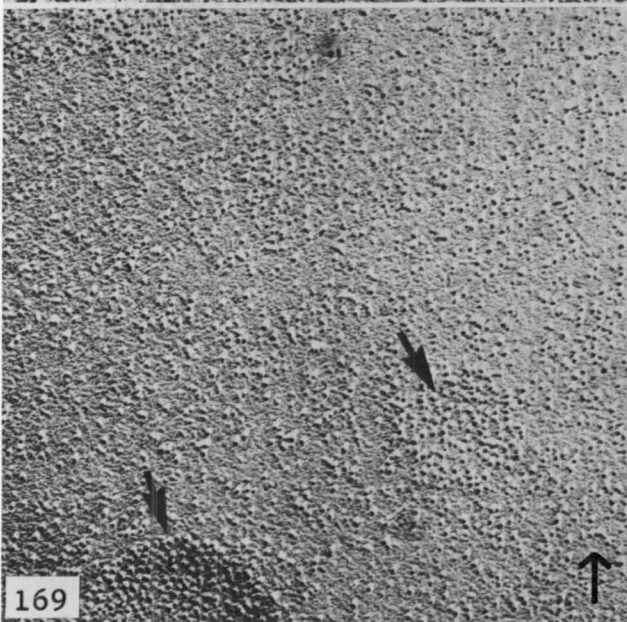
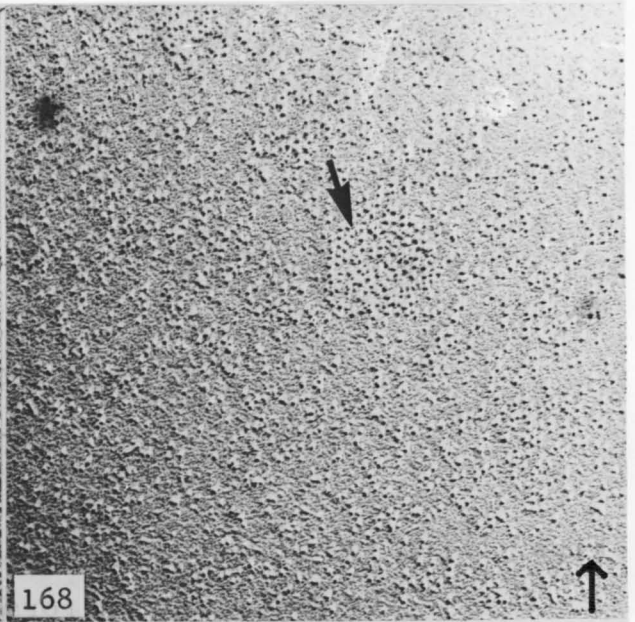
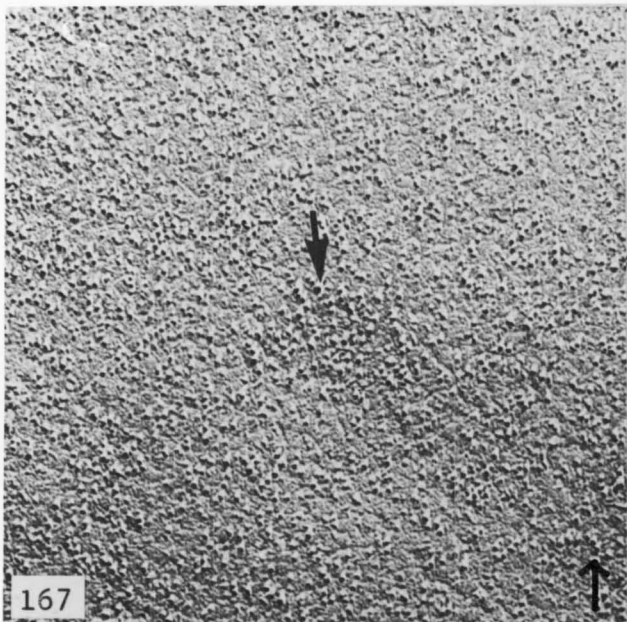


Figs. 167 - 170 Details of the tonoplast shown in Fig. 166.

Fig. 167 An early stage where particles are beginning to concentrate (arrow) prior to invagination of the tonoplast. (x 96,000).

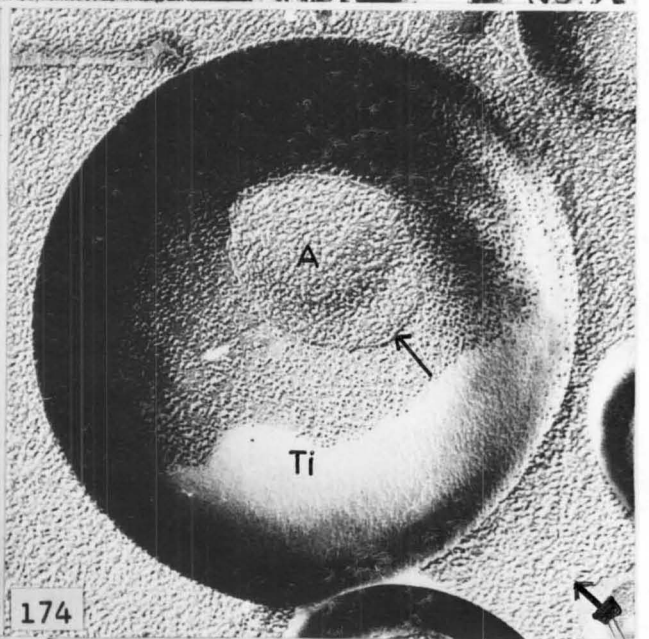
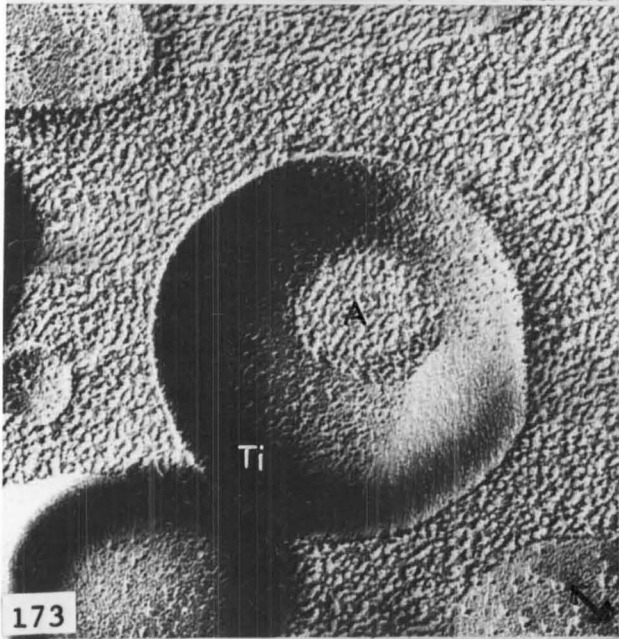
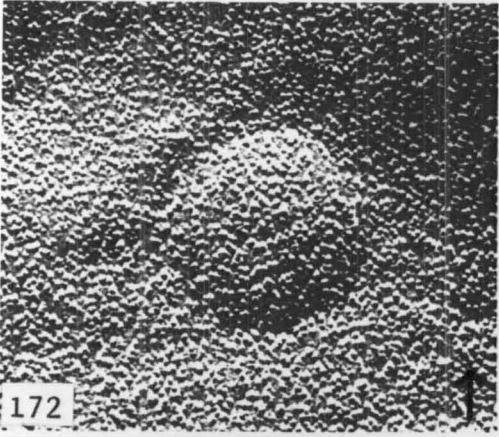
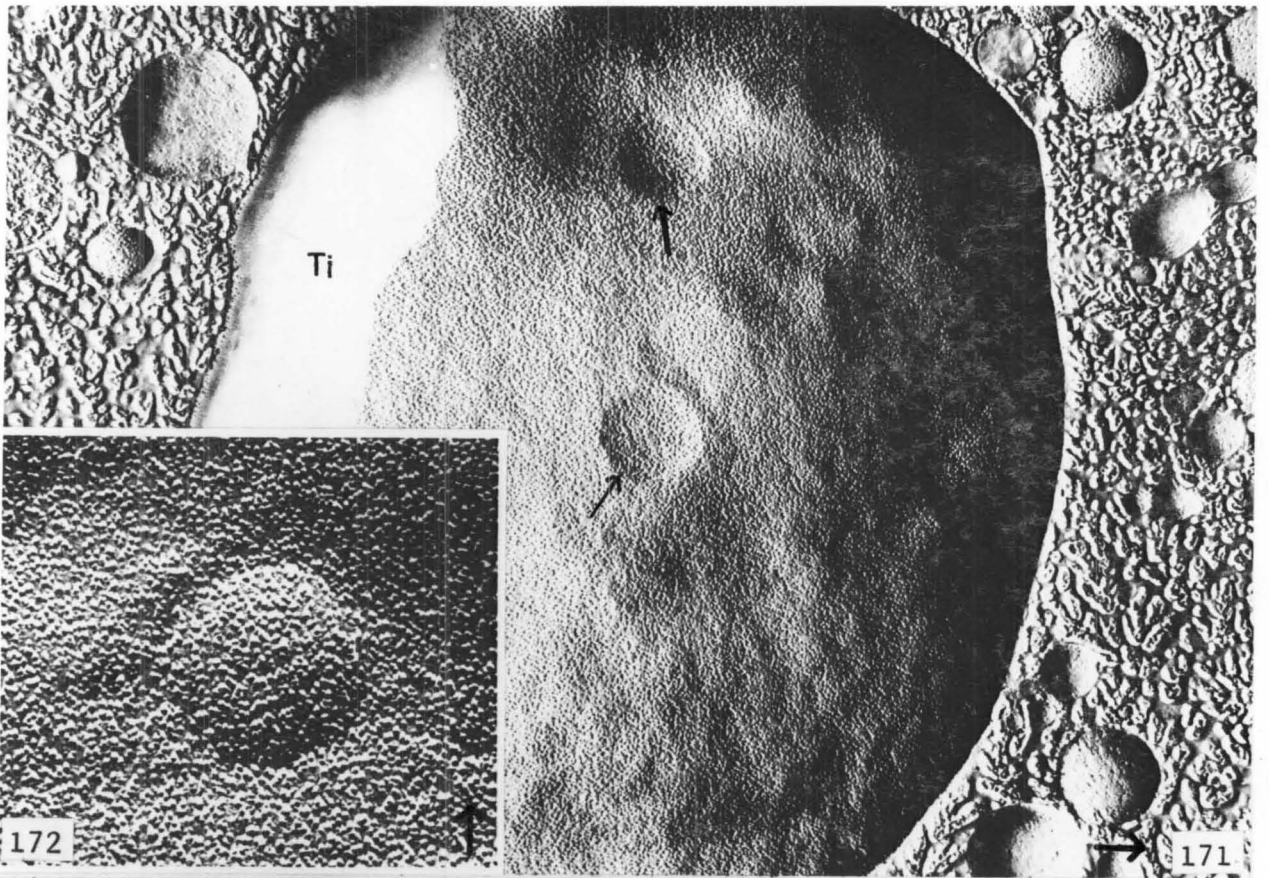
Figs. 168 & 169 Later development showing circular aggregations of particles (arrows) at a stage before the tonoplast invaginates. (both x 93,000).

Fig. 170 Subsequent development where the tonoplast has started to invaginate. The particles are confined to the hollows and show a strikingly greater concentration compared with the rest of the tonoplast. (x 93,000).



Figs. 171 & 172 Differentiation of the inner surface of the tonoplast on formation of vacuolar inclusion bodies. Fig. 171 shows two invaginations of the tonoplast (arrows). The larger size and regular shape of these distinguishes them from the dimples on the rest of the tonoplast caused during specimen preparation. Fig. 172 is a higher magnification of the middle invagination of Fig. 171 and shows a slightly higher concentration of particles than on the rest of the membrane. (Fig. 171 x 90,500; Fig. 172 x 172,000).

Fig. 173 & 174 Inner surface views of tonoplasts in small vacuoles of T.vulgare. The round areas (A) represent groundplasm where putative tonoplast invaginations have been cross-fractured. The cross-fractured tonoplast is clearly visible surrounding the area in Fig. 174 (arrow). (Fig. 173 x 138,000; Fig. 174 x 92,000)



7. ULTRASTRUCTURAL OBSERVATIONS ON VACUOLAR INCLUSIONS

7. 1 Introduction

In addition to water, dissolved substances and colloidal material, vacuoles of plant cells often contain crystalloid objects and various cytoplasmic inclusions. Vacuolar inclusions were studied early by light microscopists (see Zirkle, 1937; Guilliermond, 1941; Voeller, 1964); only recently have they been examined at the ultrastructural level. Several papers on fine structure illustrate inclusions within vacuoles (e.g. Esau & Cronshaw, 1968; Johnson & Porter, 1968) or briefly describe the structures (e.g. Sitte, 1958; Moor & Mühlethaler, 1963; Nougarede, 1963a; Diers, 1965; Jensen, 1965; Staehelin, 1966; Maniruzzaman et al., 1967; Villiers, 1967; Ducreux, 1968; Srivastava & Paulson, 1968; Woodcock & Bell, 1968). A few detailed investigations have also been made.

Vacuolar inclusions have been studied histochemically and with the electron microscope in root meristem cells of Fagopyrum (Hršel, 1961a & b, 1965a) and in the embryo sac of Lilium candidum and embryonic leaves of Triticum vulgare (Hršel & Juráková, 1964). In vacuoles of Lilium and Triticum the inclusions contained cytoplasm, small vesicles, mitochondria and proplastids. Some of the inclusions were attached to the tonoplast by a narrow stalk. Poux (1963a) described intra-vacuolar structures in meristematic cells of the shoot apex and young leaves of Triticum. She found that the intra-vacuolar structures were usually infrequent and appeared as osmiophilic masses but when isolated organs were kept in glucose solution they developed considerably and appeared to contain ribosomes and swollen organelles such as dictyosomes, mitochondria and profiles of endoplasmic reticulum. Some of the inclusions were delimited by a membrane. In carbon-starved cultures of Euglena gracilis, Brandes

et al., (1964) have found vacuoles containing portions of cytoplasm including mitochondria. Sievers (1966) found mitochondria, vesicles and remains of other membranes within vacuole-like compartments in the tips of rhizoids of Chara foetida. The inclusions were found predominantly in older or damaged cells. Gifford and Stewart (1968) investigated membrane bounded inclusions found in vacuoles and proplastids in shoot tips of Bryophyllum and Kalenchoë. They presented evidence that the inclusions arose within the proplastid and were then transferred to the vacuole. The contents of the inclusions were found to be predominantly lipid. Matile and Moor (1968) reported intra-vacuolar vesicles in replicas of frozen-etched root tips of Zea. They showed that the inclusions were formed as a result of the tonoplast engulfing portions of the cytoplasm. Buvat and Coulomb have recently studied vacuolar inclusions in various tissues and species (Buvat, 1968a & b; Coulomb, 1968a & b, 1969; Coulomb & Buvat, 1968). Some of these inclusions were multilamellar bodies resembling myelin figures. The cytosomes described by Mollenhauer et al., (1966) apparently also correspond to vacuoles containing inclusions.

The character of most vacuolar inclusions has not been studied intensively. However, some workers suggest on the basis of appearance that vacuoles containing inclusion bodies may be lysosomes (Poux, 1963a; Sievers, 1966; Thornton, 1967; Buvat, 1968a & b; Ducreux, 1968; Matile & Moor, 1968) in that they resemble the lysosomes or autophagic vacuoles of animal cells (see de Duve & Wattiaux, 1966; Gahan, 1967, 1968). From other studies, the histochemical demonstration of hydrolytic enzymes - in particular acid phosphatase - has been used to establish the lysosomal-like nature of certain vacuoles (Poux, 1963b; Brandes et al., 1964; Matile & Wiemken, 1967; Berjack, 1968; Coulomb & Buvat, 1968; Coulomb, 1968b, 1969; Matile, 1968).

This chapter presents preliminary observations on the occurrence, structure, origin and development of vacuolar inclusions in root tips of the five species studied. The structure of the inclusions is described first so as to form a basis for the outline of their origin and development. The developmental sequences presented are largely inferential, but in some instances it has been possible to study the stages in successively differentiating cells. The work is based on thin sections and freeze-etch replicas of fresh root tips. The investigation was confined to those inclusion bodies (abbreviated IBs) which are bounded by a membrane, at least in early stages of development.

7. 2 Observations

7.2. 1 Occurrence

Inclusion bodies are found in all species studied (e.g. Figs. 175, 184, 198, 242, 261). In seedling roots of Avena, Triticum and Zea IBs are more prominent than in adventitious roots of Lonicera and Mentha. Although species differ in the type and development of IBs, greater variation may occur within a species depending on the differentiation of the root tip. Inclusion bodies are more abundant in actively growing roots (Fig. 243) than in those where growth is slow or inactive (Fig. 250). Greater elaboration of the IBs is shown in vacuoles of enlarging cells than in those of promeristem and fully differentiated cells. Inclusion bodies usually attain maximum development in small vacuoles (e.g. Figs. 175, 232, 263). As vacuoles enlarge IBs continue to differentiate up to a certain point (e.g. Figs. 190, 253) and then degenerate (Figs. 206, 215-217, 254-256).

Only a small proportion of vacuoles in a cell normally contain IBs. This has been shown also by Poux (1963a) in leaf cells of Triticum. Vacuolar inclusions range in size from structures as small as golgi and ER vesicles to those as large as proplastids; most remain smaller than

mitochondria. Within a single vacuole, IBs of various dimensions and stages of differentiation may occur (Figs. 175, 228). Either one or several IBs may be visible in a sectioned or cross fractured vacuole (Figs. 176, 232, 243). Except in early stages of development, most IBs are randomly distributed within the vacuole although occasionally they are associated in pairs (Figs. 175, 184). Inclusion bodies are generally spherical (Figs. 175, 177, 185) but other forms also exist (Figs. 176, 181, 202). Usually only a small proportion of a vacuole is occupied by an IB (Figs. 175, 184, 209, 260) but in some small vacuoles the IB may dominate (Figs. 203, 209). In extreme examples, the tonoplast and limiting membrane of the IBs are closely apposed so that the vacuole superficially resembles an organelle bounded by a double membrane (Fig. 213).

7.2. 2 Types of Inclusion Bodies

In the present material IBs comprise a diversity of structures. A feature characteristic of all those investigated, however, is the presence of a single limiting membrane in early stages of development. For descriptive purposes two main morphological categories of IBs are recognised. These are designated types A and B.

Type A inclusion bodies comprise simple vesicles containing a homogeneous matrix without internal membranes (Figs. 175-178, 198-203, 209-213, 218-232, 235-239, 242-252, 260-262). Inclusion bodies of this form are illustrated elsewhere in several papers (e.g. Moor & Mühlethaler, 1963; Diers, 1965; Berjak, 1968; Buvat, 1968b; Gifford & Stewart, 1968; Matile & Moor 1968).

Type B. inclusion bodies are distinguished from type A by the presence of an internal membranous organization. The membranes occur as vesicles (Figs. 181, 190, 191, 205, 214-217, 220, 253), short profiles (Fig. 204) or extensive lamellar systems (Figs. 263-277) some of which superficially resemble myelin figures (Figs. 263, 264). So far these myelin-like forms

have not been seen in freeze-etch preparations. Structures corresponding to type B inclusions are shown in other papers (Poux, 1963a; Brandes et al., 1964; Sievers, 1966; Maniruzzaman et al., 1967; Villiers, 1967; Buvat, 1968b; Coulomb & Buvat, 1968; Coulomb, 1968a; Ducreux, 1968). Present observations suggest that type B inclusion bodies occur less frequently in root tips than do type A structures.

Inclusions other than those described as Types A and B are also found in vacuoles but their structure has not been studied fully. One form seen occasionally in $KMnO_4$ fixed cells of Triticum is partially electron transparent and surrounded by vacuolar sap precipitated as dense material (Fig. 241). Vacuoles in which these forms occur resemble those described by Paulson and Srivastava (1968) as containing proteinaceous material. Another inclusion found in dormant cells of Lonicera consists of dense granules at the periphery of the vacuole which are similar to the phenolic deposits shown in other tissues (Wardrop & Cronshaw, 1962; Esau, 1963).

7.2. 3 Structure of Inclusion Bodies

A. Membranes: In cross fractured vacuoles in which the contents of the IB exhibit etching a limiting membrane is apparent (Figs. 177, 178, 190, 191). Surface fracturing exposes the outer and inner faces of the membrane which in early stages of development is covered by particles (Figs. 181, 182, 185) similar to those found on the tonoplast. In a few IBs plaques instead of particles cover the surface of the membrane (Fig. 179). The plaques resemble those found on the tonoplast in prefixed material (see Figs. 150-152). Internal membranes of type B inclusion bodies are revealed occasionally in freeze-etch preparations. Some enclosed vesicles exhibit a surface similar to that of golgi vesicles (Figs. 181, 193). Particles are seen only

occasionally on the internal membranes.

The clarity of the membrane in sectioned material depends on the reaction between the fixative and the matrix of the IB. In KMnO_4 -preserved cells a limiting membrane is difficult to demonstrate if the matrix stains densely; but its presence may sometimes be inferred when there is a sharp separation of the IB from the vacuolar sap (Fig. 198). During later stages of differentiation the demarcation becomes less distinct (Figs. 199-203). In type B inclusion bodies the internal membranes are partially obscured when the matrix is dense (Fig. 253) but are visible when the matrix is electron transparent (Figs. 214-217, 254-256). When IBs are preserved in GA/OsO_4 the membranes are usually clearer than when they are fixed in KMnO_4 . This is because the matrix is often electron transparent (Figs. 220, 224, 257-259, 263-277) or contracts from the limiting membrane (Figs. 218-232) in GA/OsO_4 preserved cells.

B. Matrix: In freeze-etch preparations, the degree of etching of the IB indicates the amount of water present in the matrix. Type A inclusion bodies at early stages of development exhibit etching similar to that of the groundplasm. (Figs. 177, 178) which suggests their recent origin from the latter. The small amount of etching in older IBs shows that they possess a low water content (Figs. 175, 176, 183). In type B IBs etching of the matrix and the vacuolar sap is often similar (Figs. 181, 190, 191), indicating a comparable water content. However, some enclosed vesicles show little etching (Fig. 190).

In chemically fixed cells the appearance of the matrix depends on the fixative used and on the type of IB and its state of differentiation. The matrix of certain IBs resembles groundplasm and in some GA/OsO_4 preserved material ribosomes are visible (Figs. 233,

234, 287, 288) but absent in others (Figs. 207, 208). This type of matrix occurs in both A and B IBs but is more common in type B where it surrounds the internal membranes and vesicles. Matrix which resembles groundplasm is not found in the enclosed vesicles (Figs. 205, 220, 258). In other types of IB the matrix is electron transparent so that the IBs appear as membrane bounded "ghosts" within the vacuole (Gifford and Stewart, 1968). These IBs are seen clearly in GA/OsO_4 fixed material (Figs. 220, 224, 257-262) and in some KMnO_4 preserved cells (Figs. 240, 247-252).

In some type A inclusion bodies the matrix is electron dense. The matrix is either mainly uniformly dense (Figs. 198-203, 209-212) or interspersed with electron transparent material (Figs. 213, 227-232, 246-249). These conditions are shown in both KMnO_4 and GA/OsO_4 preserved specimens. Inclusion bodies with a dense matrix are found usually in actively growing root tips (Figs. 189-203, 209-212, 218-226).

7.2. 4 Origin of Inclusion Bodies

Observations from thin sections and freeze-etch replicas show that IBs originate as invaginations of the tonoplast into the vacuole (Figs. 186-188, 192, 193, 269-286, 289-291). The IB begins as a shallow inpushing which deepens, eventually being pinched off as a separate structure suspended within the vacuole. As early stages in the formation of IBs are found only occasionally, invaginations may develop comparatively rapidly. The initiation of IBs occurs mainly during stages of active cellular differentiation. As vacuoles enlarge fewer new IBs are formed. In small vacuoles IBs are usually initiated one at a time (see Figs. 163-165) but in larger vacuoles several invaginations may develop simultaneously (see Figs. 166, 170). The size of the invagination varies depending on

the type of IB being initiated. Surface views of both sides of the tonoplast reveal that IBs develop as circular depressions (see Figs. 163-170). The membrane differentiates at these points; this is shown by the greater concentration of particles here than on the rest of the tonoplast (Figs. 163-174; see Chapter 7).

Because IBs develop as invaginations of the inner membrane, the outer surface of the IB membrane represents the inner surface of the tonoplast. Conversely the inner surface of the IB corresponds to the outer surface of the tonoplast. This is confirmed by comparing the density of particles on the different membrane surfaces at early stages of development (Figs. 181, 182, 188).

While some invaginations of the tonoplast appear to be pinched off immediately as vesicles, others apparently remain attached to the tonoplast with their contents communicating with the cytoplasm (Figs. 259, 269-286). However, such protuberances seem to be eventually detached into the vacuole. When IBs are pinched off, scars are left on both the tonoplast and the IB (Matile & Moor, 1968). In the present frozen-etched material, scars occur on both surfaces of the tonoplast (Figs. 189, 194, 195, 197) and on the outer surface of the IB (Figs. 184, 185). The scars probably heal quickly as few are observed on fully differentiated IBs or on tonoplasts of large vacuoles.

The contents of IBs are derived from the cytoplasm surrounding the vacuole. Various components of the cell contribute depending on the particular IB. In some type A structures, groundplasm is incorporated directly into the invagination of the tonoplast (Figs. 192, 286, 289, 291) but in others, the groundplasm first differentiates into electron transparent material without ribosomes (Figs. 284, 285). In type B inclusion bodies the matrix also differentiates in either of these ways (Figs. 269-277, 290).

During the formation of type B inclusion bodies, vesicles and other membranes are incorporated into the protuberance before it is detached from the tonoplast. Golgi vesicles have been identified as contributing to IBs (Figs. 193, 280, 281, 290), with one or several vesicles being engulfed at a time. Mitochondria (Figs. 282, 283) and plastids (Figs. 276-279) are also associated with the formation of IBs. Figure 279 shows a proplastid abutting a small vacuole and extruding a portion of its contents into it. The complex membranous systems in some IBs are apparently derived from the envelope and internal thylakoids of amyloplasts (Figs. 276, 277). From other plastids less complicated structures may be formed. Figure 278 shows a single vesicle, apparently enclosing a starch grain, which has developed within the IB.

Developing IBs exhibiting elaborate internal membranes are sometimes associated with ER (Figs. 269-272). The ER seems to invade protuberances of the tonoplast and proliferates into various patterns (Figs. 265-272), the most distinctive being myelin-like configurations (Figs. 263, 264). Ribosomes are not usually present among the membranes of these IBs. The contents of some IBs are apparently formed by proliferations of the nuclear envelope (Figs. 273-275). Both outer and inner membranes of the envelope may be involved. This association of the nuclear envelope with vacuoles has been noted mainly in small vacuoles (Figs. 273-275).

In some instances whole organelles, rather than portions seem to be taken into vacuoles by the invaginations of the tonoplast. Mitochondria (Figs. 215, 254-256), ribosomes (Fig. 287), lipid droplets (Figs. 215, 216, 233), vesicles of various forms (Figs. 214-217, 253, 288) and cisternae resembling golgi lamellae (Fig. 204) have been observed within IBs.

7.2. 5 Development of Inclusion Bodies after Separation from the Tonoplast

Once the tonoplast invagination is pinched off, subsequent development of the IB is independent of the cytoplasm. Within the vacuole, most IBs

continue to increase in size and internal differentiation (Figs. 219-232) up to about the time cell enlargement ceases. However, growth and internal differentiation of the IBs can vary depending on the structure of the IB being formed, the size of the tonoplast invagination and the time of its initiation in relation to cell differentiation. Inclusion bodies arising late in the differentiation of root tip cells normally grow less than those initiated early.

During differentiation of root cap cells, the number of IBs within a vacuole increases through the formation of new IBs and apparently through the addition of IBs when vacuoles fuse. Occasionally the number of certain type A IBs decreases as a result of fusion (Figs. 201, 202, 251).

In most IBs, changes in the organization of the limiting membrane occur during differentiation and the membrane eventually breaks down. Other workers also note the disappearance of membranes from IBs (Berjak, 1968; Gifford & Stewart, 1968; Matile & Moor, 1968). The density of particles on both surfaces of the limiting membrane decreases until the membrane appears almost smooth (Fig. 185). Eventually the plane of fracture between the IB and the vacuolar sap becomes ill-defined (Fig. 180) presumably indicating dissolution of the membrane. Breakdown therefore seems to be an active process and is not due to a passive stretching of the membrane as the IB enlarges. The stage at which the membrane disappears varies in different IBs and apparently also depends on the metabolism of the vacuole.

In type B IBs the internal membranes become variously developed. In some, the membranes are tightly wound (Fig. 269) forming myelin-like structures (Figs. 263, 264) but in others they are less regularly arranged, (Figs. 265, 266, 269-271). Subsequent differentiation of the internal membranes in some IBs seems to involve the transformation of cisternae

into vesicles (Figs. 265-268). Eventually all cisternae and vesicles disappear.

During the differentiation of IBs, different types of changes occur in the matrix. In IBs which contain groundplasm and ribosomes (Fig. 287), the ribosomes become progressively more indistinct (Fig. 288) and eventually disappear (this indicates a change in the function of cytoplasmic contents once they become engulfed by an IB). The contents of some type A IBs lose water on differentiation (Figs. 177, 178, 183). These IBs often develop a dense matrix (Figs. 198-203, 209-212, 218-226) presumably as a result of the synthesis of new substances. Subsequent differentiation of the dense matrix of these IBs varies: the matrix either disintegrates and disperses throughout the IB (Figs. 296, 254), differentiates centrifugally into electron transparent material (Figs. 219-232, 244-250) or scatters as coarse electron dense material (Figs. 236-239). These changes in the matrix probably indicate that different reactions may occur within vacuolar inclusions.

After IBs become fully enlarged, most enter a phase of degeneration (Figs. 206, 215-217, 254, 256) and eventually disappear. Although degeneration of IBs seems to occur mainly towards the end of cellular differentiation, many IBs disappear from vacuoles in actively growing cells. Inclusion bodies at various stages of development and disintegration may occur within one vacuole (Figs. 206, 254). Disintegration of IBs with dense contents normally occurs after the disappearance of the limiting membrane (Figs. 200, 254). In contrast, the internal membranes and matrix of certain type B IBs may become disorganized while still surrounded by the limiting membrane. This indicates that for some IBs there is a breakdown in internal organization before the whole structure disappears within the vacuole. The fate of some IBs is not known.

7.3 Discussion

The present survey shows that IBs are a feature of some vacuoles in the roottips of all species studied. They comprise a diversity of form but resemble those found in other root tips (Hršel, 1965a; Matile & Moor, 1968; Northcote & Lewis, 1968) and in different material (Poux, 1963a; Moor & Mühlethaler, 1963; Brandes et al., 1964; Diers, 1965; Sievers, 1966; Villiers, 1967; Berjak, 1968; Buvat, 1968a & b; Coulomb, 1968a; Coulomb & Buvat, 1968; Ducreux, 1968; Gifford & Stewart, 1968). That IBs comprise a heterogeneous collection is shown particularly by their contents which are derived from various components of the cell. However, IBs have one feature in common; they possess a single limiting membrane derived from the tonoplast. The disappearance of this membrane during later stages of differentiation is another feature characteristic of most IBs.

In view of the fragility of the tonoplast under certain conditions of fixation (see Chapter 4), the possibility that some IBs might be artifacts of fixation can not be excluded. Lipid droplets have been observed protruding into vacuoles poorly fixed in GA/OsO₄. Some of the IBs lying against the tonoplast in figure 235 may have arisen in this way although their contents are not identical in appearance with the lipid bodies of the cytoplasm. It is possible that the myelin-like configurations and similar protuberances into vacuoles might be formed as a result of post-mortem changes. However, the regular arrangement of many myelin-like structures and the undisturbed appearance of the surrounding cytoplasm suggest that they are probably normal components of the cell. Hršel and Juráková (1964) have discussed the nature of similar protuberances into vacuoles of Lilium and Triticum and concluded that the structures were not

artifacts. Although certain IBs in the present material can not always be distinguished from cytoplasmic debris entrapped within vacuoles which have collapsed during fixation, most seem to be authentic vacuolar inclusions. The fact that certain type A IBs (e.g. Fig. 209) are not found elsewhere in the cell indicates that these components have differentiated with the vacuole. The most convincing proof that most IBs in sectioned material are normal components is demonstrated by the occurrence of similar structures in freeze-etch preparation of fresh root tips (e.g. Fig. 175). The similarity of IBs in frozen-etched prefixed root tips (Matile & Moor, 1968) and sectioned specimens (Buvat, 1968b; Coulomb & Buvat, 1968; Gifford & Stewart, 1968) to those in frozen-etched fresh root tips shows that the fundamental structure of IBs is maintained after chemical fixation.

In frozen-etched yeast cells Moor and Mühlethaler (1963) have found IBs which they consider to represent the "metachromatin granules described by Guilliermond". Certain of the type A ~~IBs~~ IBs found in the present study (Figs. 175-193) resemble the IBs described by Moor and Mühlethaler which suggests that some type A IBs may correspond to these metachromatin granules. Metachromatin granules or "corpuscles" have often been mentioned by light microscopists (see Zirkle, 1937; Guilliermond, 1941) some of whom regarded them as artifacts of fixation and staining. However, the fact that IBs resembling them occur in frozen-etched fresh cells indicates that the metachromatin granules are probably real structures.

Inclusion bodies have been described in senescent and dormant cells (Moor & Mühlethaler, 1963; Sievers, 1966; Villiers, 1967), in cells grown in culture (Poux, 1963a; Brandes et al., 1964) and in meristematic and differentiating tissues (Poux, 1963a; Thornton, 1967; Buvat, 1968a & b; Coulomb, 1968a; Coulomb & Buvat, 1968; Gifford & Stewart, 1968; Matile

& Moor, 1968). Observations from this study indicate that IBs usually arise in actively differentiating tissue. In differentiated cells the IBs are usually at an advanced stage of development. Therefore the initiation and early differentiation of IBs is considered to reflect cellular activity. Some workers have suggested that structures corresponding to the present IBs are directly involved in differentiation (Thornton, 1967). Alternatively IBs might be by-products of cellular activity.

Various interpretations are held regarding the origin of vacuolar inclusions. Brandes et al., (1964) suggest that groups of golgi vesicles surround pockets of cytoplasm and then fuse to isolate the pocket. In contrast, Buvat (1968b) considers such pockets are isolated by ER. Buvat and Coulomb (Buvat, 1968b; Coulomb & Buvat, 1968) also find that some IBs represent protrusions of the cytoplasm into the vacuole (e.g. Figs. 273-277); these protrusions may appear discontinuous depending on the plane of sectioning. These authors suggest that the protuberances might become detached towards the end of differentiation and come to lie within the vacuolar cavity. Observations by Hřšel and Juráková (1964), Matile and Moor (1968) and those from the present study show that membrane bounded IBs are initiated as invaginations of the tonoplast. In no instance have IBs been found arising de novo within vacuoles or as enclaves in the cytoplasm isolated by ER or other membrane-bounded organelles. Furthermore, vesicles and other cellular components have so far not been seen entering vacuoles without first being enclosed by the tonoplast.

The engulfing of cytoplasm by vacuoles probably represents a general phenomenon of cell metabolism. This is shown by the diversity of organelles associated with the process. Gifford and Stewart (1968) observed proplastids extruding portions of their contents into vacuoles on formation of

IBs. Matile and Moor (1968) have noted the incorporation of golgi vesicles. However, in plant tissues few instances have been reported where mitochondria, ER and the nuclear envelope apparently directly contribute to the contents of IBs.

In the absence of cytochemical studies, the composition of IBs can be inferred only from their reaction with different fixatives and in freeze-etch material by the degree of etching. Observations from these sources show that their composition is often variable, among different IBs and in the same IB, depending on their state of differentiation (e.g. Figs. 219-232, 237-239, 244-250). Some of the type A IBs appear to contain lipid-like material. In cross fractured IBs, the etching of the contents is similar to that of lipid droplets in the cytoplasm (Fig. 183). Inclusion bodies fixed in $KMnO_4$ (Fig. 198) and in GA/OsO_4 (Figs. 232, 235, 236, 280) also resemble lipid bodies. Gifford and Stewart (1968) have found predominantly lipid material in the structures they investigated. The metachromatin granules seen with the light microscope contain fatty material (see Zirkle, 1937; Guilliermond, 1941). Other compounds have been identified in IBs. In yeast cells polymetaphosphates have been found (Wiame, 1947). There is a possibility that some IBs may contain phenolic compounds (Wardrop & Cronshaw, 1962; Gifford & Stewart, 1968). However, in most instances the composition of IBs has not been determined.

Inclusion bodies are not mere fragments of cytoplasm trapped within vacuoles but dynamic components of the cell. Just as golgi vesicles continue to develop after leaving the golgi apparatus and become involved in various cellular processes (see Mollenhauer & Morré, 1966), many IBs continue to differentiate after being pinched off from the tonoplast. An important function of some IBs is apparently the synthesis of material not found elsewhere in the cell. This occurs particularly in those IBs

which develop electron dense contents (Figs. 198-203, 218-229). Synthesis may involve the conversion of toxic or unwanted by-products of metabolism into insoluble compounds which can then be stored within the vacuole. Presumably, this conversion stops sometime prior to the disappearance of the limiting membrane.

The eventual breakdown of most IBs appears to make material available for redistribution within the vacuole and later presumably within the cell (Matile & Moor, 1968; Matile, 1968). Vacuoles containing IBs may thus function as lysosomes or autophagic vacuoles. While lytic structures have been studied intensively in animal cells (see du Duve & Wattiaux, 1966; Gahan, 1967) it is only recently that comparable structures have received attention in plant cells. Autophagic systems are reported in lower plants (Buckley & Sommer, 1967; Thornton, 1967; Villiers, 1967) and in tracheophytes (Poux, 1963a & b; Berjak, 1968; Buvat, 1968b; Coulomb & Buvat, 1968; Gifford & Stewart, 1968; Matile & Moor, 1968). Various hydrolytic enzymes have also been isolated (Matile, 1965, 1966; Matile et al., 1965; Matile & Wiemken, 1967; Berjak, 1968; Coulomb & Buvat, 1968; Matile, 1968; Coulomb, 1968b) thus demonstrating a digestive role for certain components of the protoplast. It seems reasonable to assume that in the present material vacuoles containing IBs function as lysosomes. This lysosomal role is, however, not a simple one. While some IBs are engulfed apparently for immediate digestion (e.g. Figs. 215-217, 255) others continue to develop before eventually disintegrating. The vacuole therefore provides an environment for the differentiation of IBs as well as for their eventual breakdown.

While individual vacuoles may behave as lysosomes there is evidence to suggest that some IBs themselves may be lysed before the structure as a whole is digested by the vacuole. Figure 256 shows such an example. The

object contained within the IB appears to be a mitochondrion in which the outer membrane has been digested leaving the inner membrane and cristae; these eventually presumably disappear too.

In conclusion, this study has shown that IBs of the types investigated are a multifarious component of the cell. Their contents come from various sources but all IBs are bounded initially by a limiting membrane derived from the tonoplast. The initiation of IBs is considered to reflect cellular activity. The formation of IBs undoubtedly provides a means whereby unwanted portions of cytoplasm are digested and ultimately returned to the cell. Some IBs provide sites for the synthesis of products which are later broken down.

7.4 Summary

Vacuoles of plant cells often contain inclusions which at early stages of development are surrounded by a single limiting membrane. The IBs comprise a diversity of forms and various stages of differentiation are recognisable. For descriptive purposes IBs are divided into two categories: those containing mainly homogeneous contents (Type A) and those which exhibit an internal membranous organization (Type B). The internal membranes may be in the form of vesicles or tightly coiled membranes. Occasionally whole cytoplasmic organelles are found within the IB.

IBs initially arise as invaginations of the tonoplast which become detached into the vacuole. The IBs are initiated mainly during active cellular growth but may persist in modified form in differentiated cells. Various cellular components contribute to the contents of IBs: ER, nuclear envelope, excluded portions of plastids and mitochondria, ribosomes and groundplasm. The limiting membrane and contents of IBs eventually disappear within the vacuole.

Some IBs form sites for the synthesis of substances apparently not found

elsewhere in the cell. The disappearance of contents within IBs and the eventual loss of the whole structure from the vacuole suggests that such vacuoles behave as lysosomes.

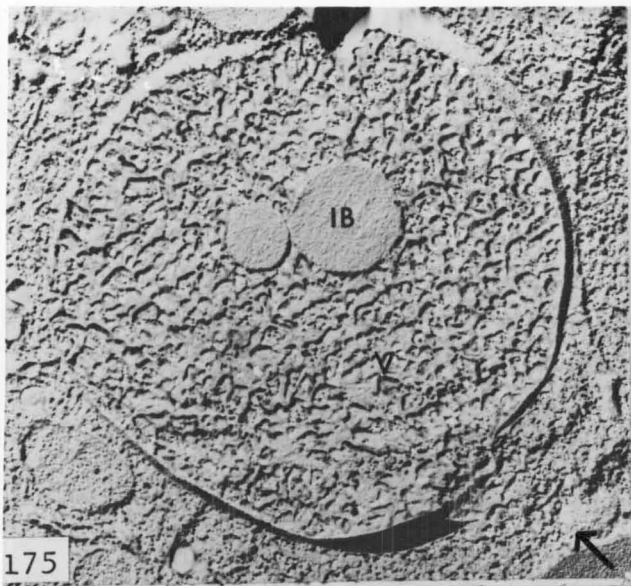
Figures 175 - 197 Vacuolar Inclusion bodies
 (IBs) in frozen-etched fresh root tips.

Figures 175 - 183 Avena sativa.

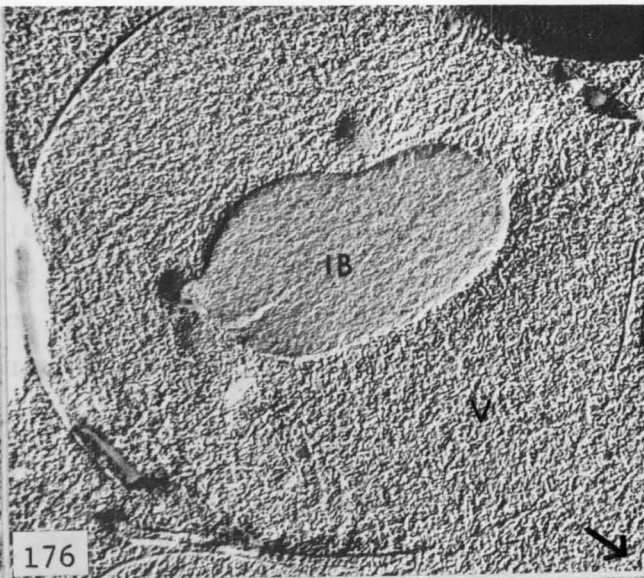
Figs. 175 & 176 Differentiated Type A IBs (for definition see text p.102) in small vacuoles. Fig. 175 shows two rounds IBs lying close together. Fig. 176 illustrates an elongated form. In both vacuoles the contents of IBs show less etching than the vacuolar sap (Fig. 175, x 25,500; Fig. 176, x 50,500).

Figs. 177 & 178 Type A IBs at early stages of development in small cross-fractured vacuoles. The contents of the IBs and the groundplasm exhibit similar freezing; this suggests recent origin of the IBs from the cytoplasm. (Fig. 177, x 71,000; Fig. 178, x 54,000).

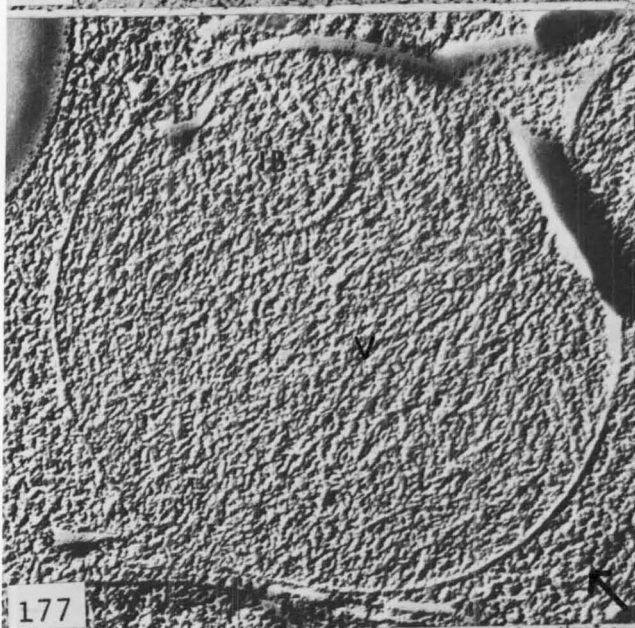
Figs. 179 & 180 The surface appearance of IBs. Fig. 179 shows the surrounding membrane covered with plaques - a condition noted occasionally. In Fig. 180 the plane between the IB and vacuole sap is indistinct suggesting a breakdown of the membrane at this stage of differentiation. (Fig. 179, x 105,500; Fig. 180, x 138,000).



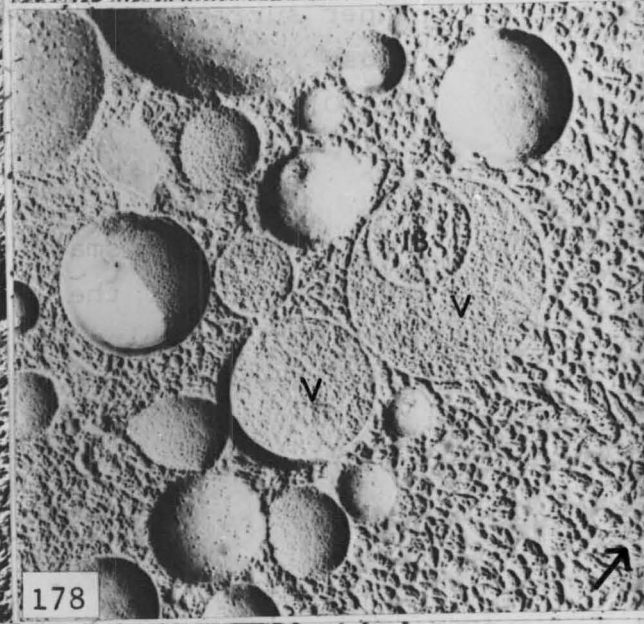
175



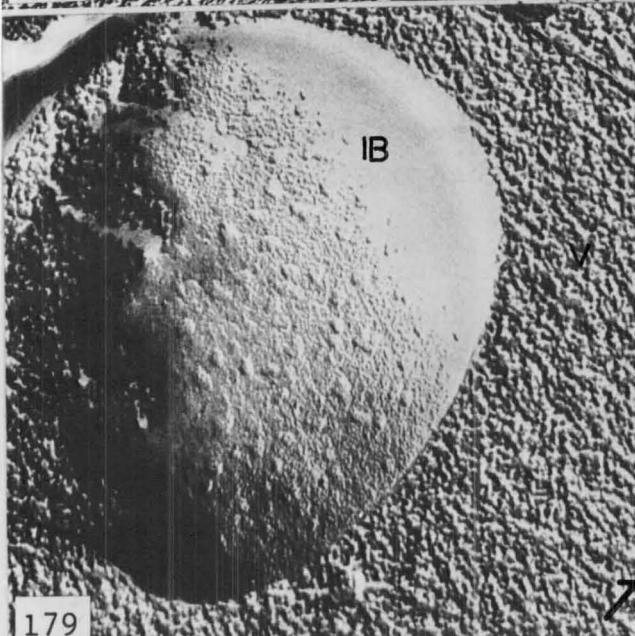
176



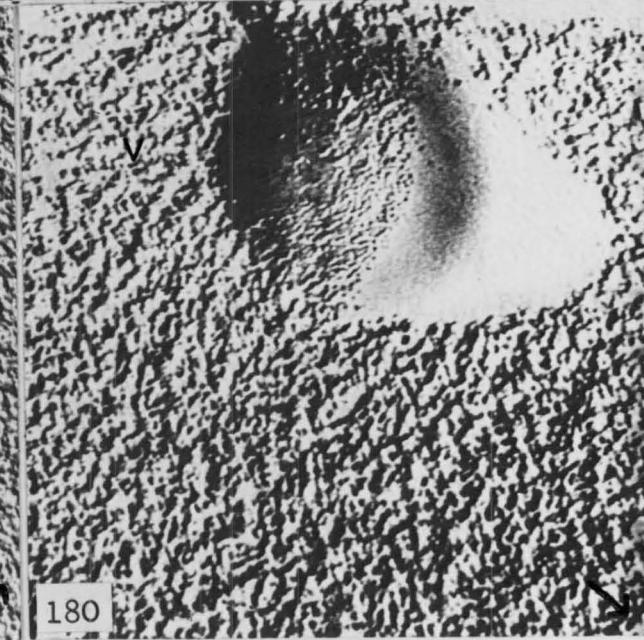
177



178



179



180

Fig. 181 A small vacuole containing a Type B IB (for definition see text p. 102). The structure is crescent-shaped; at one end a vesicle of unknown nature is shown in surface view (x 54,000).

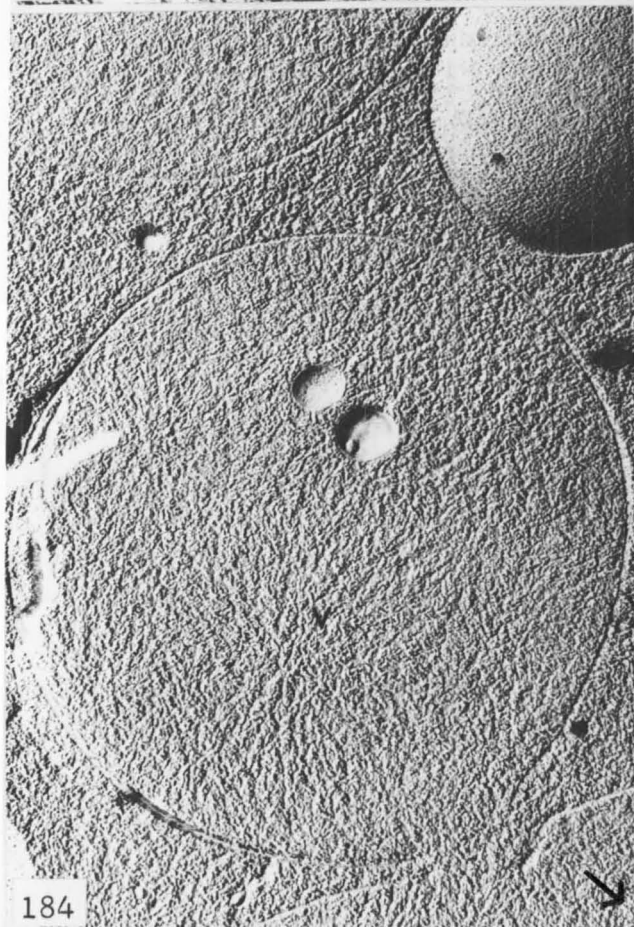
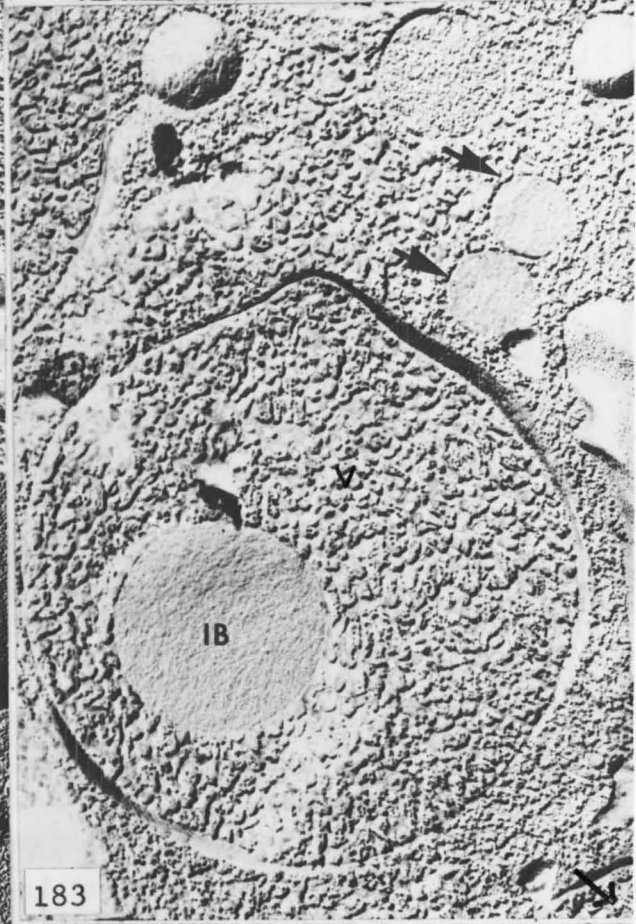
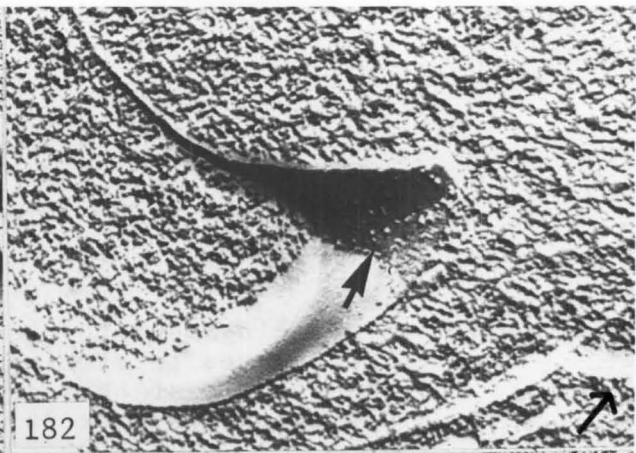
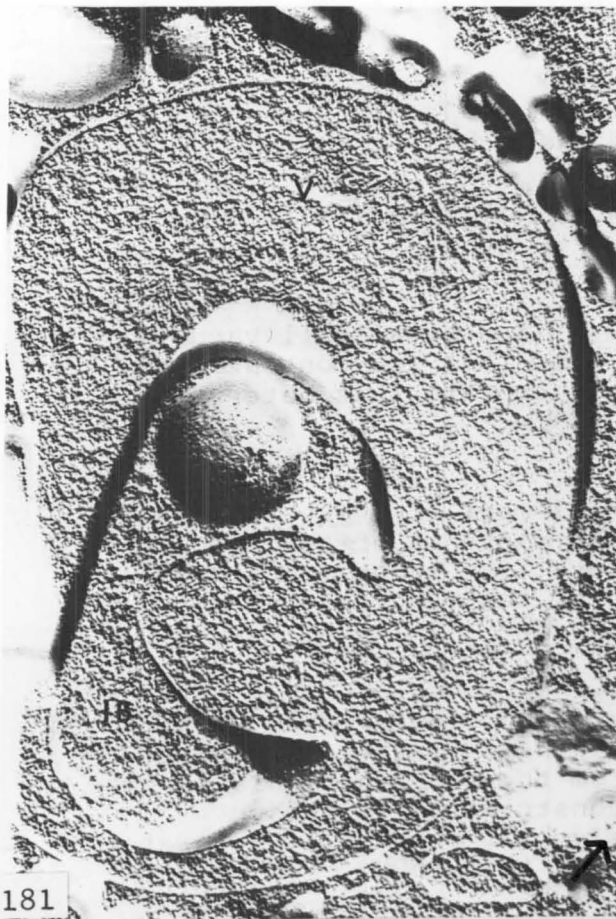
Fig. 182 Enlarged portion of the membrane surrounding the IB illustrated in Fig. 181. The particles on the inner surface of the membrane (arrows) resemble those found on the outer face of the tonoplast. (x 101,000).

Fig. 183 Cross-fractured small vacuole showing a single IB. The contents of the IB exhibit similar, but not identical, etching to that of the two lipid droplets (arrows) in the cytoplasm. (x 31,500).

Figures 184 & 185 Triticum vulgare.

Fig. 184 A vacuole containing two small IBs seen in surface view. The depression in one of the structures possibly represents the scar formed after separation of the IB from the tonoplast (x 46,000).

Fig. 185 Higher magnification of the IBs shown in Fig. 184. Particles are visible on the upper IB but are poorly represented on the other (x 138,000).

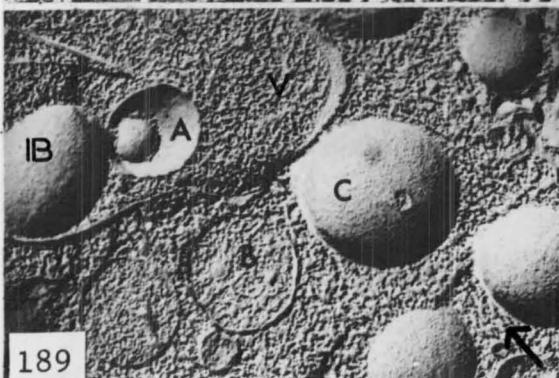
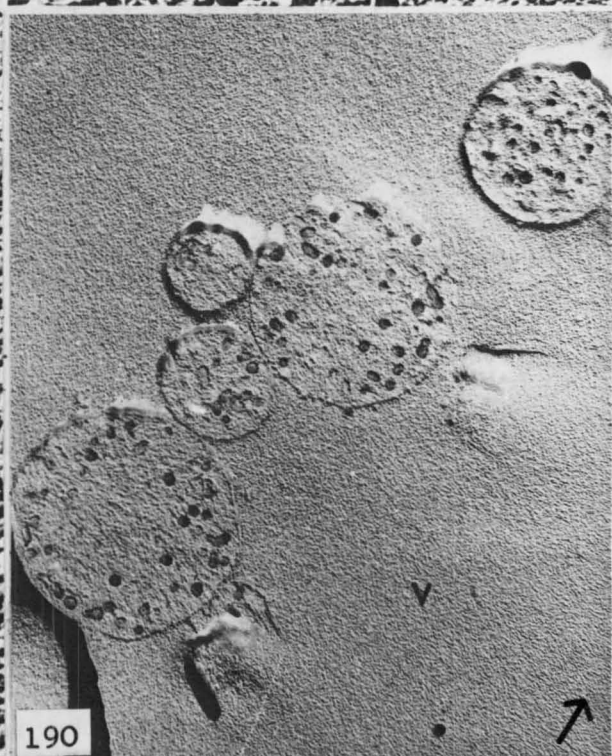
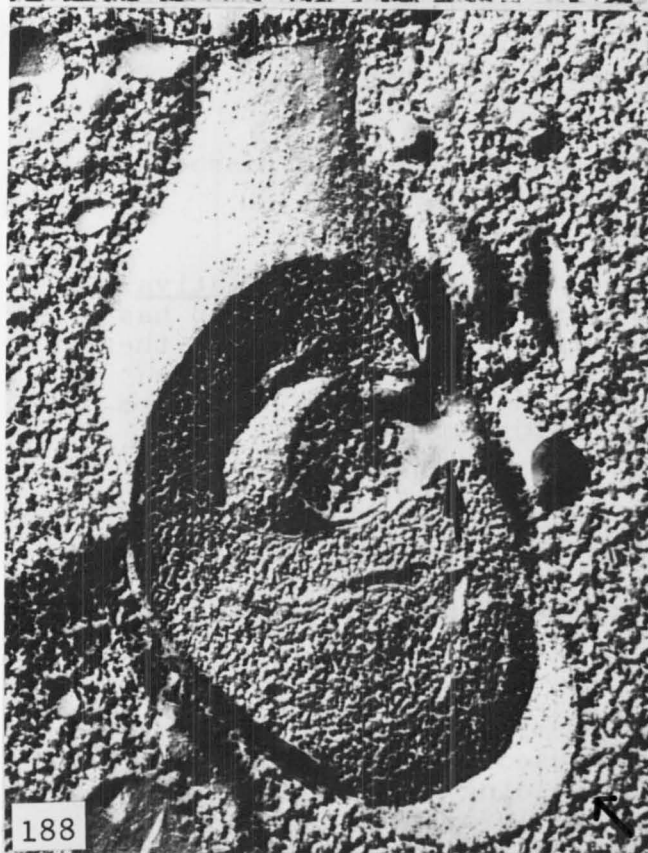
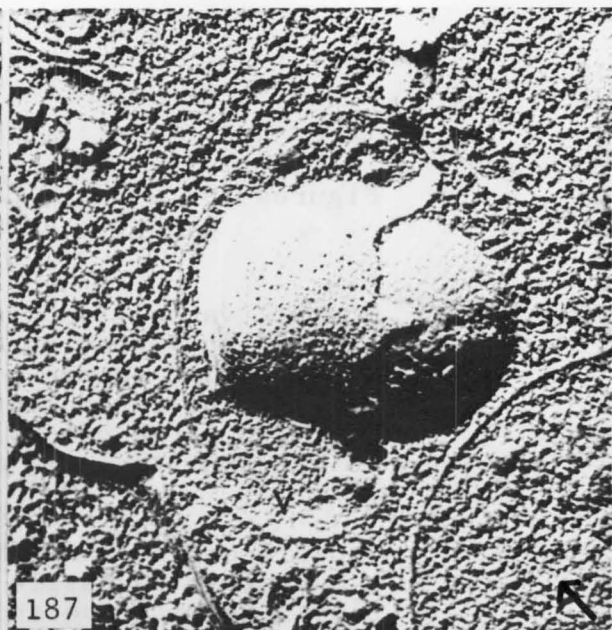


Figures 186 - 189 Early development of IBs in A.sativa.

- Fig. 186 A vesicle about to be engulfed by a small vacuole. Differences in etching show that the contents of the vacuole at this stage contain less water than the groundslam. (x 24,500).
- Fig. 187 A later stage in the engulfment of a cytoplasmic vesicle by a vacuole. The contents of the vacuole are seen in cross-fracture; the inner surface of the invaginated portion of the tonoplast is shown in surface view (x 65,700).
- Fig. 188 A surface and cross fractured view of what seems to be an elongated vacuole showing a further stage in development of an IB from the tonoplast. At the base of the invagination a constriction is present (arrows) which apparently will eventually separate the IB from the tonoplast. (x 89,500).
- Fig. 189 A group of small vacuoles showing IBs at various stages of formation. IB A lies within a larger vacuole adjacent to another IB. The invaginated portion of the tonoplast in A probably corresponds to the scar shown in Fig. 185. B shows IBs within the vacuole. The scar on C marks the site of a developing IB (x 9,500).

Figures 190 & 191 Type B IBs in large vacuoles of T.vulgare.

- Fig. 190 A group of IBs seen in cross fracture. Each is surrounded by a single membrane and contains numerous vesicles. The contents of the vesicles show less etching than the surrounding matrix of the IB and the vacuolar sap (x 35,500).
- Fig. 191 Portion of a vacuole showing indistinct IBs. Two are cross fractured and contain vesicles. The inner membrane surface of another is also visible (arrow) (x 43,000).



Figures 192 & 193 Early development of IBs in
A.sativa (cont.)

Fig. 192 A small vacuole showing an invaginated tonoplast (arrow) on formation of a Type A IB. Invaginations of this kind become pinched off from the tonoplast as vesicles resembling those shown in figs. 289 & 291. Two large cross-fractured vacuoles lie adjacent to the small vacuole (x 12,500).

Fig. 193 Cross fracture through portion of a vacuole showing a cytoplasmic vesicle being engulfed by the tonoplast. Details are shown in the insert. The vesicle is comparable to those shown in the cytoplasm which from their surfaces are identifiable as golgi vesicles (x 11,500; insert x 19,500).

Figures 194 - 197 Scars on the tonoplast associated with developing IBs.

Fig. 194 Scar on the outer surface of a vacuole in A.sativa. Details are shown in the insert. Whether the IB has been pinched off or whether the contents within the scar represent groundplasm communicating with the diverticulum of the developing IB can not be determined. (x 77,000; insert, x 101,000).

Fig. 195 Scar on the inside of a tonoplast in T.vulgare. The sharp rim surrounding the scar suggests that it represents the broken-off stalk of an IB prior to separation from the tonoplast (specimen prefixed). (x 73,500).

Fig. 196 A false scar on the inside of a tonoplast in A.sativa. The scar is an artifact caused by a plucked-out portion of the tonoplast folding back on itself (see insert). The particles visible on the flap correspond to those seen on the outside of the tonoplast (x 80,500; insert, x 105,500).

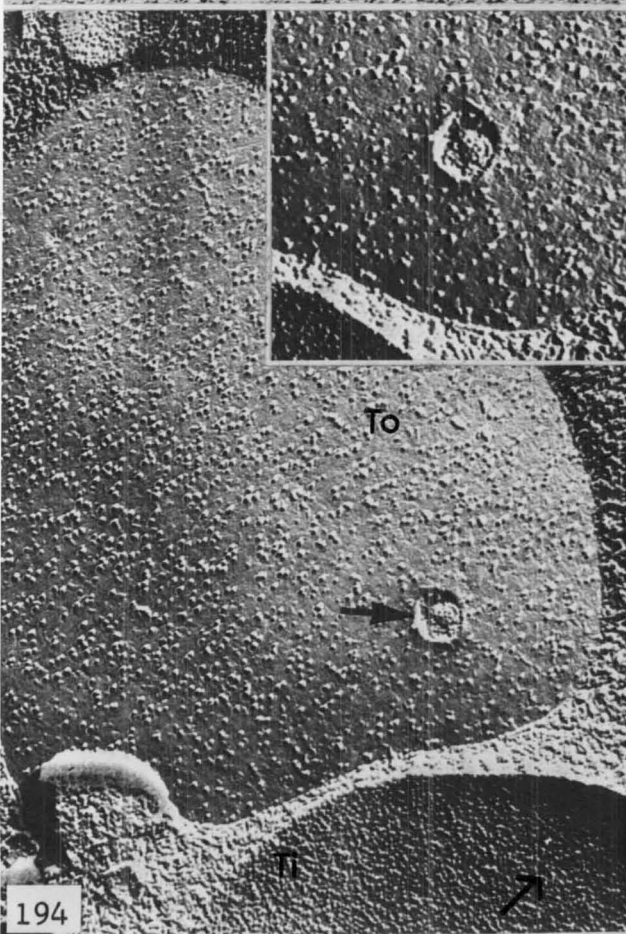
Fig. 197 A rounded scar found on the inside of a tonoplast in A.sativa after an IB has been pinched-off. (x 78,500).



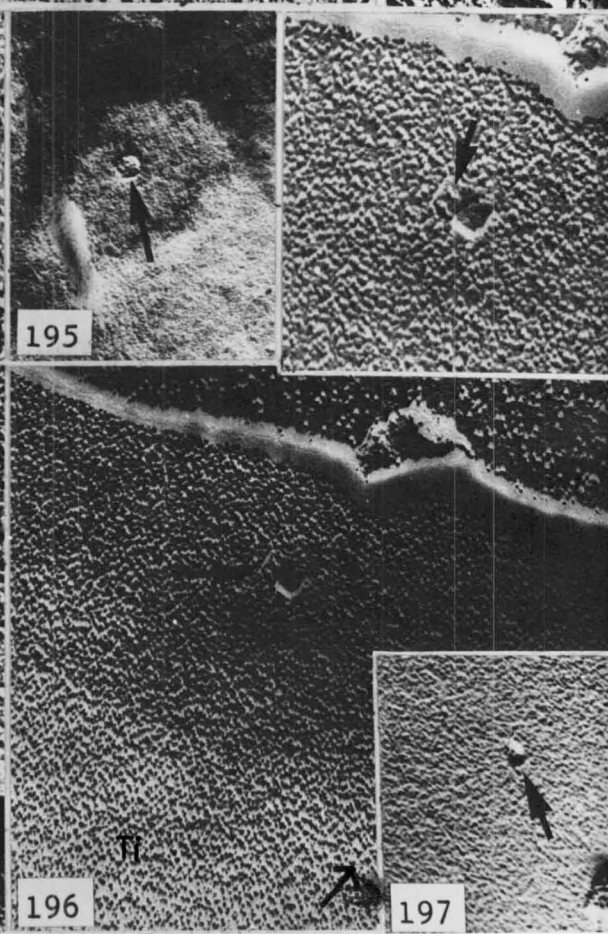
192



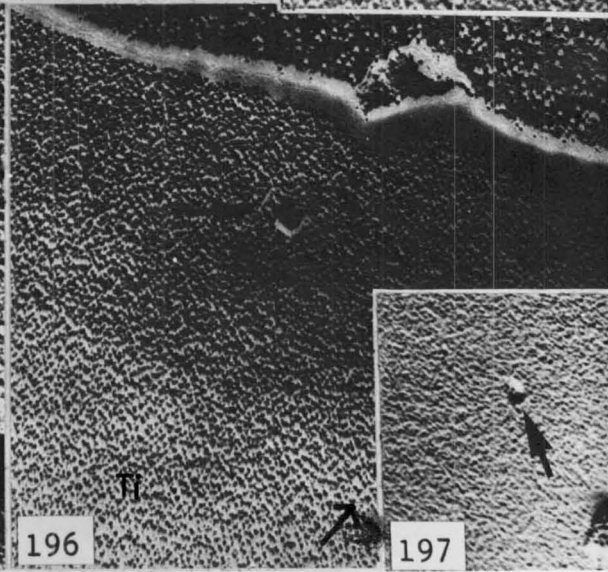
193



194



195



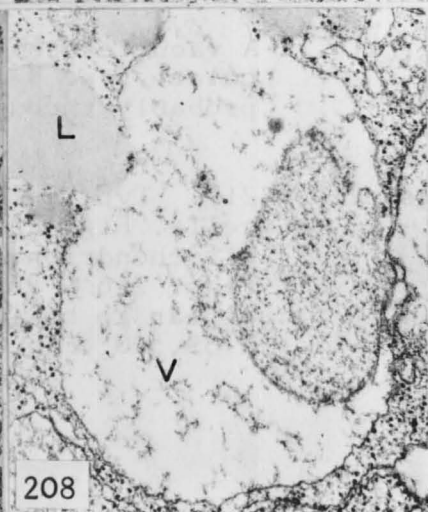
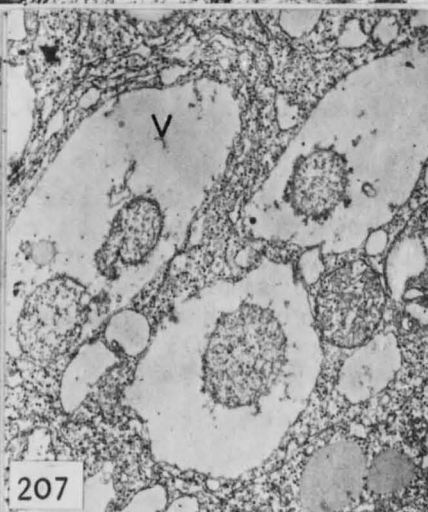
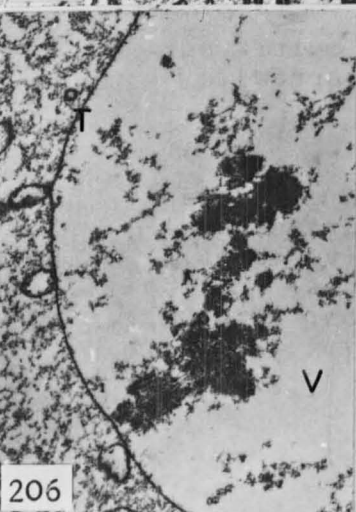
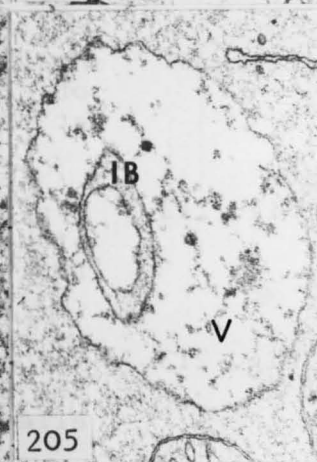
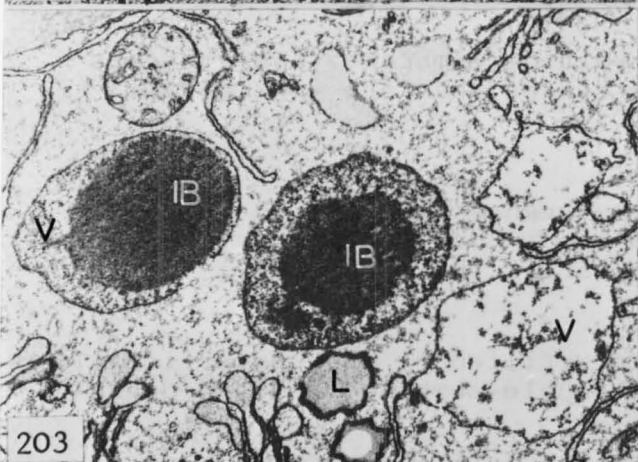
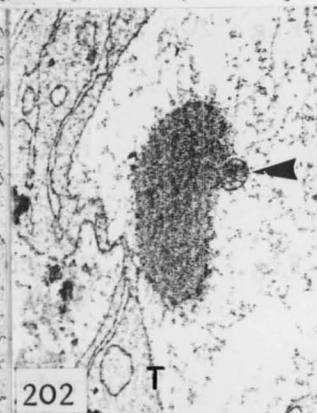
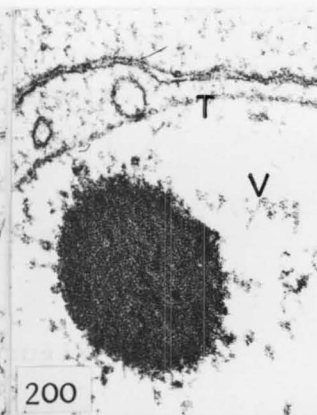
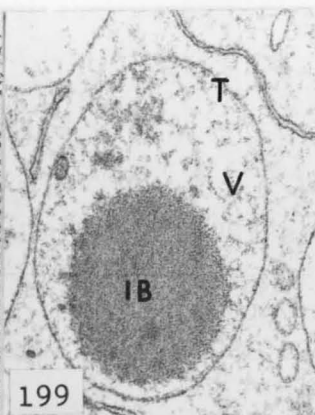
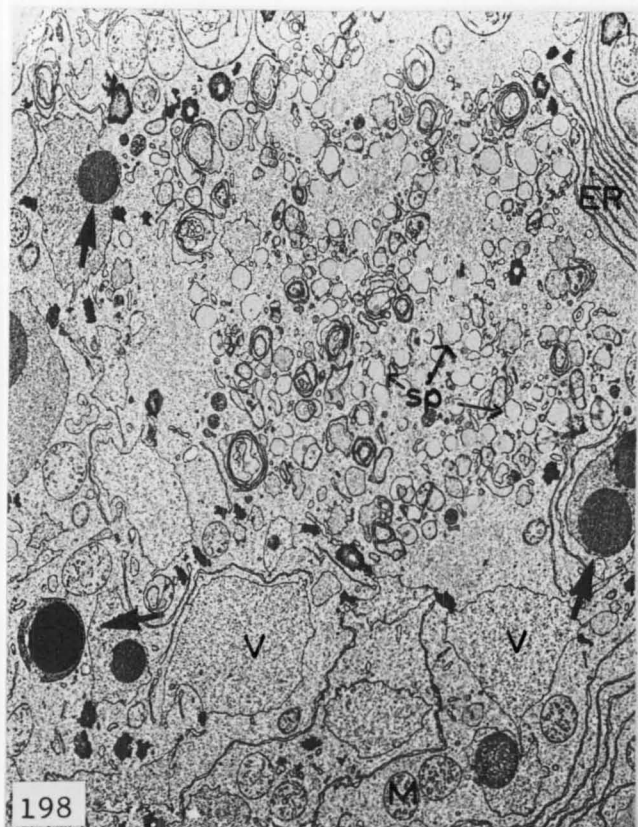
196

197

Figures 198 - 208 IBs in outer root cap cells of
Zea mays.

Figures 198 - 205 KMnO_4 fixation.

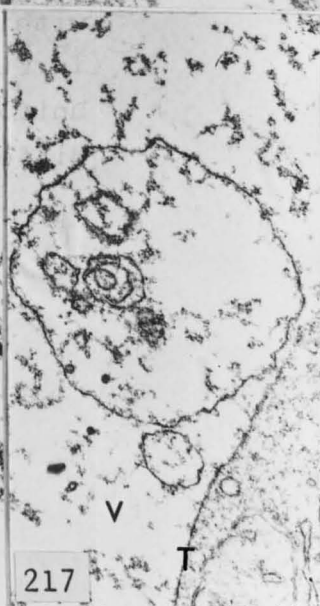
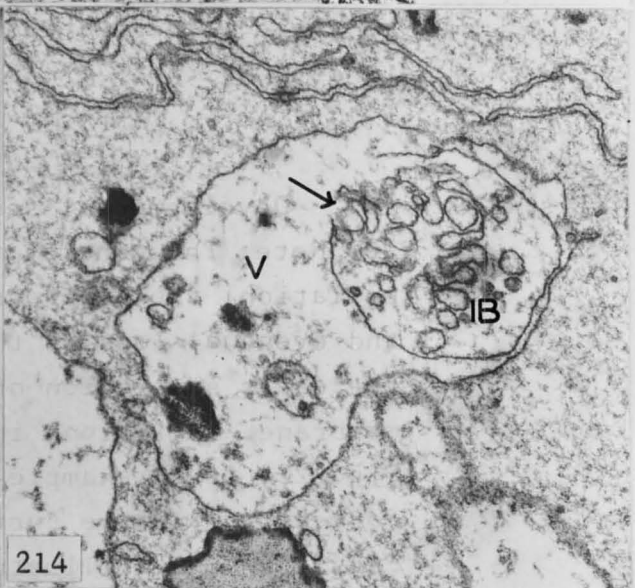
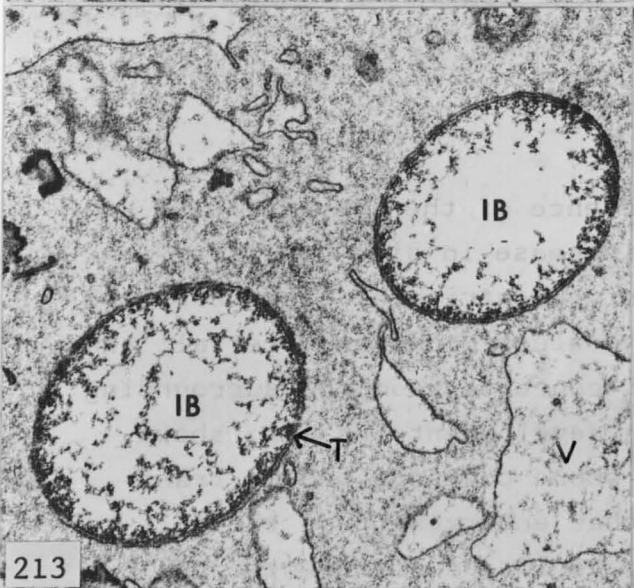
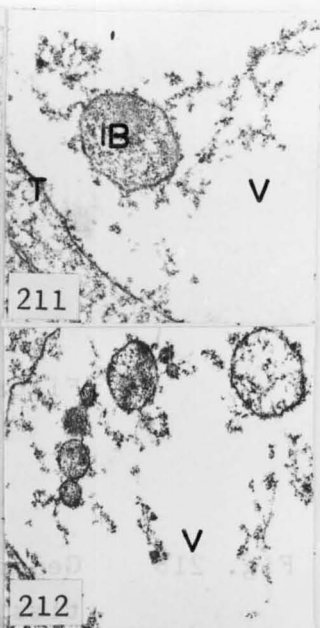
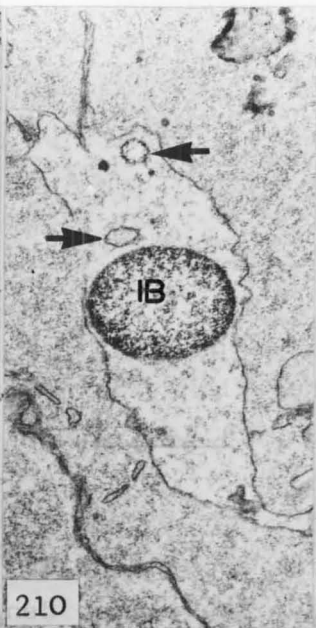
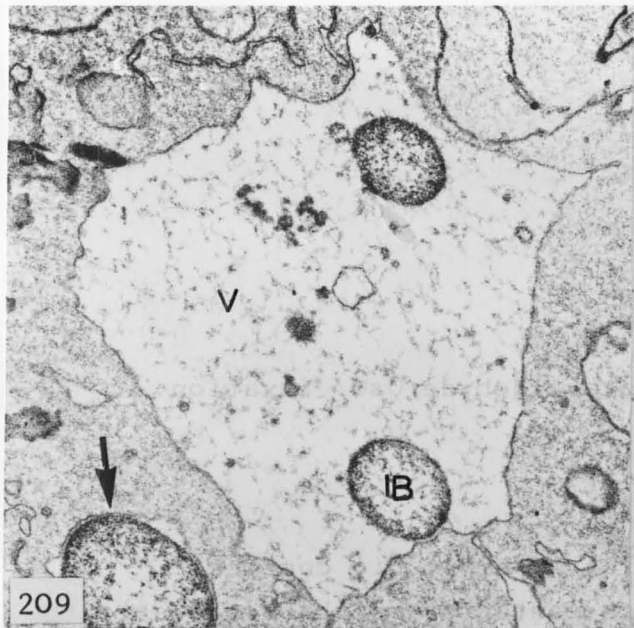
- Fig. 198 General appearance of vacuoles containing electron dense Type A IBs. The membrane is not visible surrounding the IBs but the sharp separation from vacuolar sap suggests that one is present. (x 6,500).
- Figs. 199 & 200. Typical finely granular texture of IB contents of the type shown in Fig. 198. A bounding membrane is absent at this stage of development (Fig. 199, x ca. 45,000; Fig. 200, x 46,000).
- Fig. 201 A small vacuole containing IBs. The shape of the upper IB suggests recent fusion of two IBs. The tonoplast has ruptured at one point. (x 27,400).
- Fig. 202 Fusion of a small IB with a larger one. A membrane surrounds the small structure but appears to be absent around the larger IB (x 30,000).
- Fig. 203 Vacuoles in which IBs occupy a large proportion of the volume (x 28,500).
- Fig. 204 A vacuole with portions of IBs visible. One of the structures contains membranes resembling golgi cisternae (x 49,500).
- Fig. 205 Type B inclusion body. The matrix surrounding the internal vesicle resembles that of the groundplasm (x 41,000).
- Fig. 206 Portion of a large vacuole showing a group of IBs. The diffuse shape of these suggest that they are undergoing digestion (x 41,000).
- Figs. 207 & 208 Type A IBs after fixation in glutaraldehyde/ OsO_4 . The structures apparently correspond to those shown in Figs. 198 - 203, fixed in KMnO_4 . The outline of an indistinct bounding membrane⁴ is visible around the IBs (Fig. 207, x 26,500; Fig. 208, x 41,000).



Figures 209-234 Inclusion bodies in root tips
of Avena sativa.

Figures 209 - 217 KMnO_4 fixation

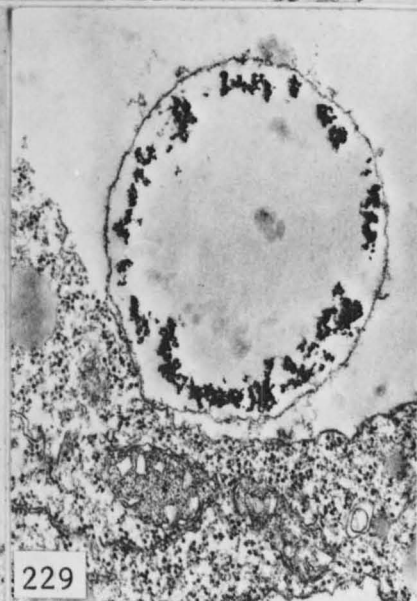
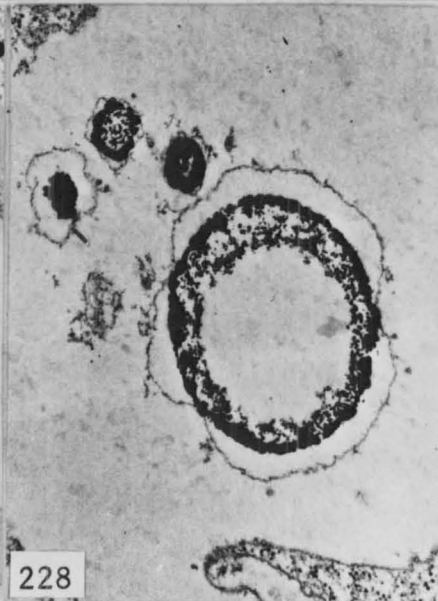
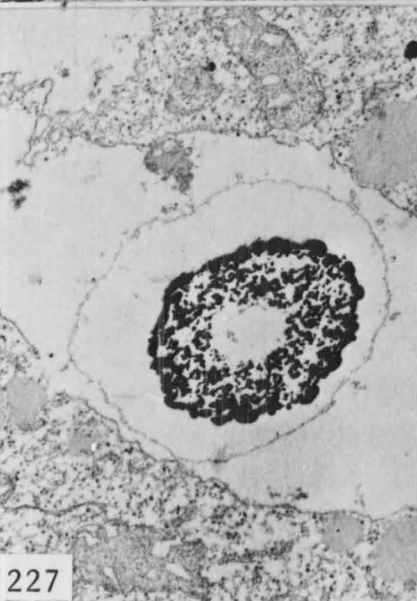
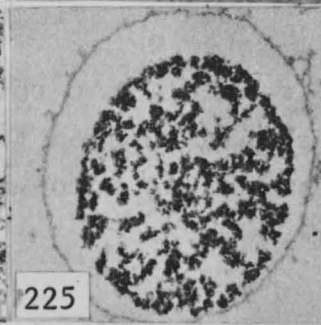
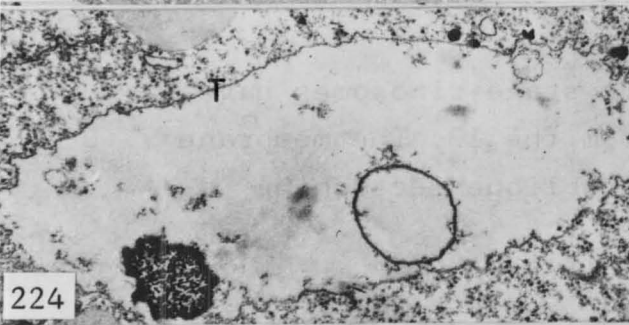
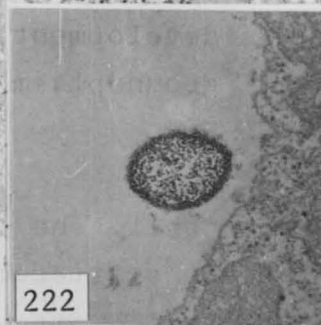
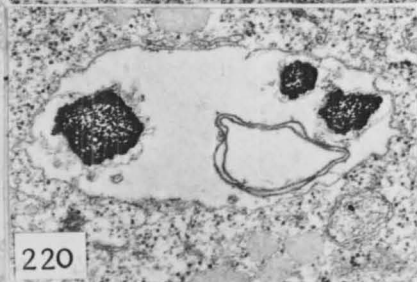
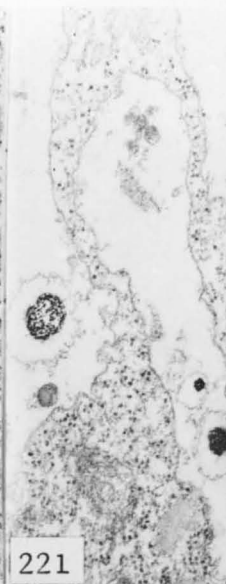
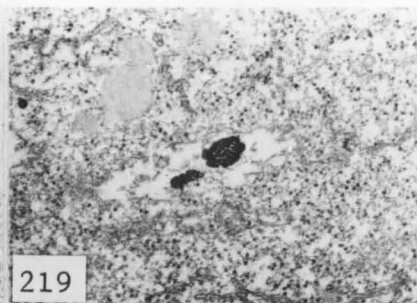
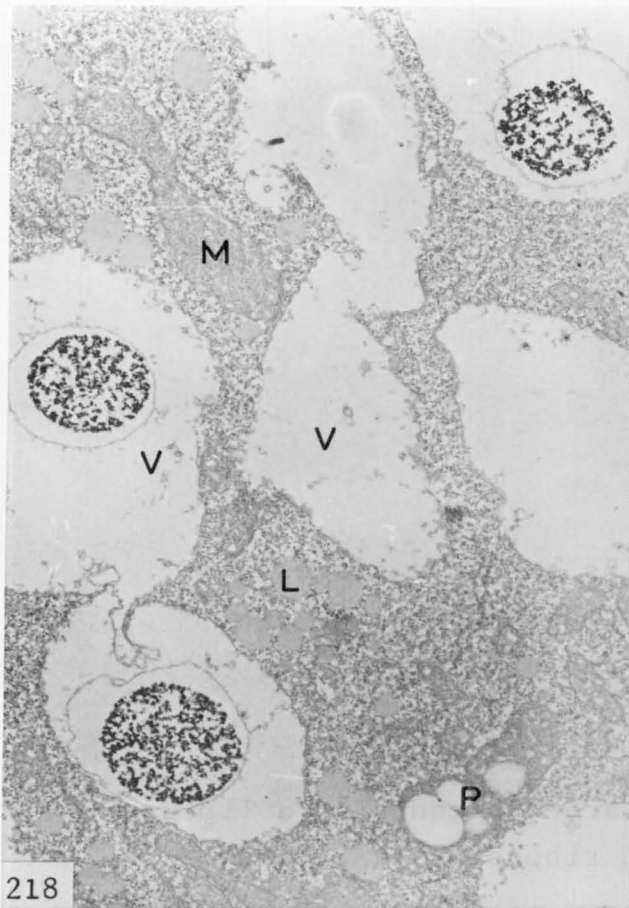
- Fig. 209 General appearance of IBs in root tip tissue at an early stage of differentiation of root cap. Contents of IBs are mainly electron dense at this stage of development. In the small vacuole (arrow) note that a large proportion of the vacuole is occupied by the IB. (ca. x 40,000).
- Fig. 210 A prominent IB with dense contents and two smaller ones (arrows) with little contents in embryonic cortex. The IB with dense contents probably corresponds to those seen in this region after glutaraldehyde fixation (Figs. 207, 208, 218) (x 36,000).
- Fig. 211 An IB surrounded by a limiting membrane. The contents of the structure become denser than the groundplasm at this stage. (x 43,000).
- Fig. 212 A group of IBs showing membranes surrounding the structures. Note the change in density of contents from the smaller to the larger IB. (x 42,000).
- Fig. 213 Vacuoles almost completely filled by IBs. Superficially the structures resemble double-membrane bounded organelles. (x 43,500).
- Fig. 214 A vacuole from the outer cap showing a multi-vesiculate Type B IB. The IB has ruptured (arrow), probably through fixation. (x 42,000).
- Figs. 215 - 217 Type B IBs from large vacuoles in outer cap cells. Fig. 215 shows a ruptured IB containing a mitochondrion, lipid droplet and other vesicles. Figs. 216 & 217 depict similar structures. The IBs appear to be in the process of digestion. (Fig. 215, x 59,500; Fig. 216, x 57,500; Fig. 217, x 41,500).



Figures 218 - 234 Glutaraldehyde/OsO₄ fixation:
A.sativa.

Fig. 218 General appearance of IBs in embryonic cortical tissue. The coarse dense contents are characteristic of these with fixation in glutaraldehyde/OsO₄ (x 15,000).

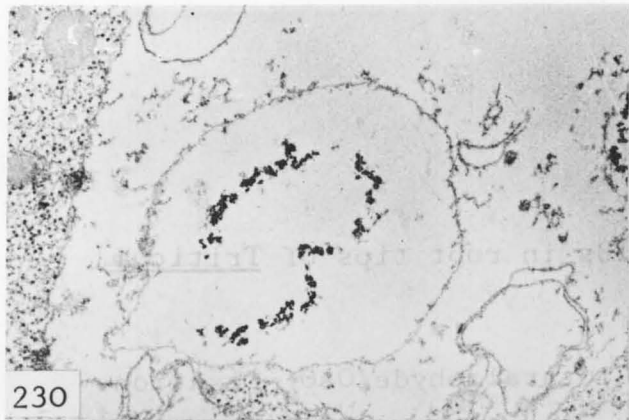
Figs. 219 - 232 Developmental sequence of the above type of IB. Note: The general increase in size on differentiation; the progressive centrifugal transformation and eventual loss of the electron dense material; and the early separation of contents from the surrounding membrane. Variations in development are also shown. Fig. 223 is an example where contents become transformed with little further enlargement of the IB. Figs. 220 & 224 illustrate IBs of different form within the same vacuole (see also Figs. 254 & 255). In Figs. 225 & 232 the dense contents show a more uniform transformation compared with the centrifugal differentiation (all x 41,000).



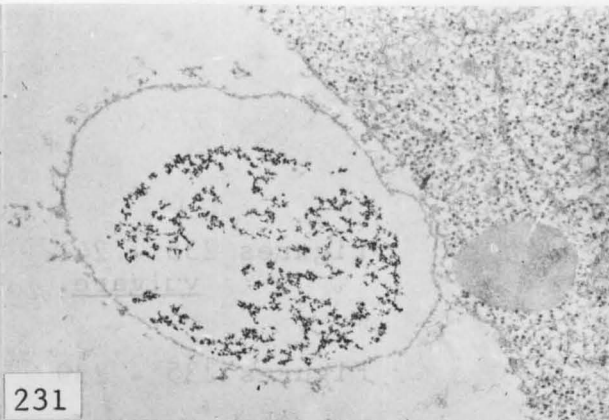
Figures 233 & 234 IBs at early stages of development showing remnants of groundplasm and ribosomes.

Fig. 233 Outer cap cell. The large IB contains a lipid droplet and scattered ribosomes (x 29,500).

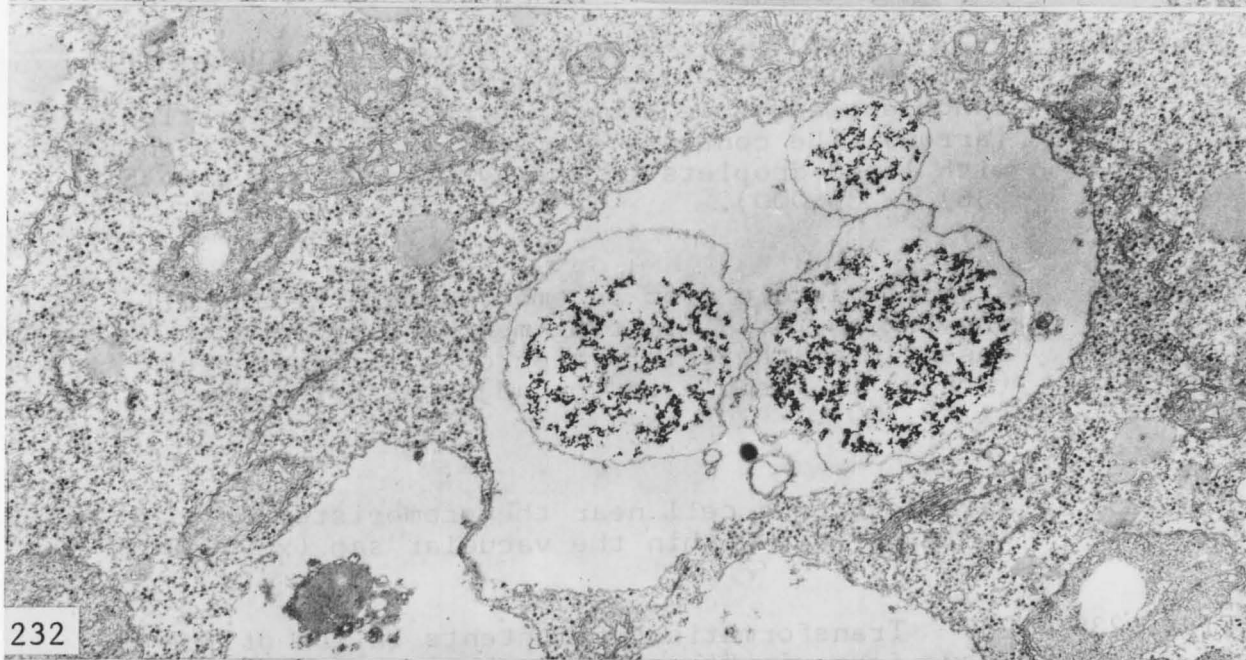
Fig. 234 Root cap cell. At this stage ribosomes have almost disappeared from the IB. The membrane appears broad through oblique sectioning (x 44,000).



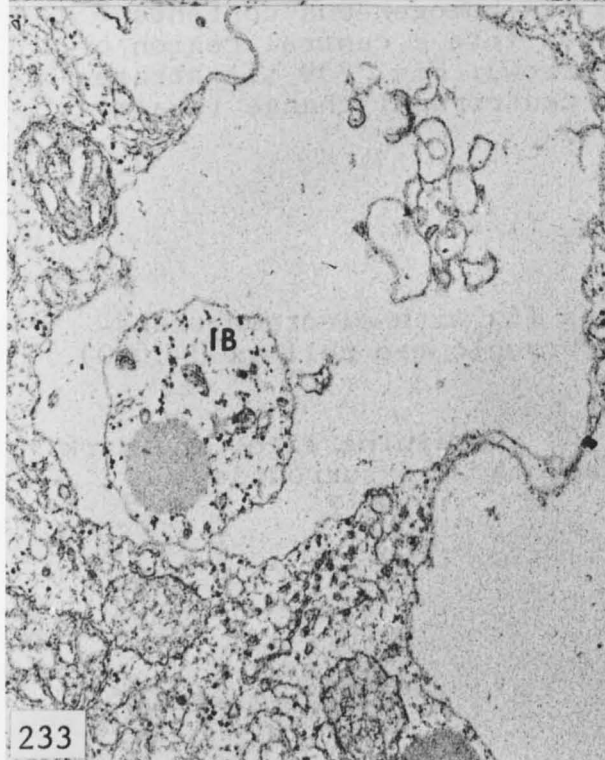
230



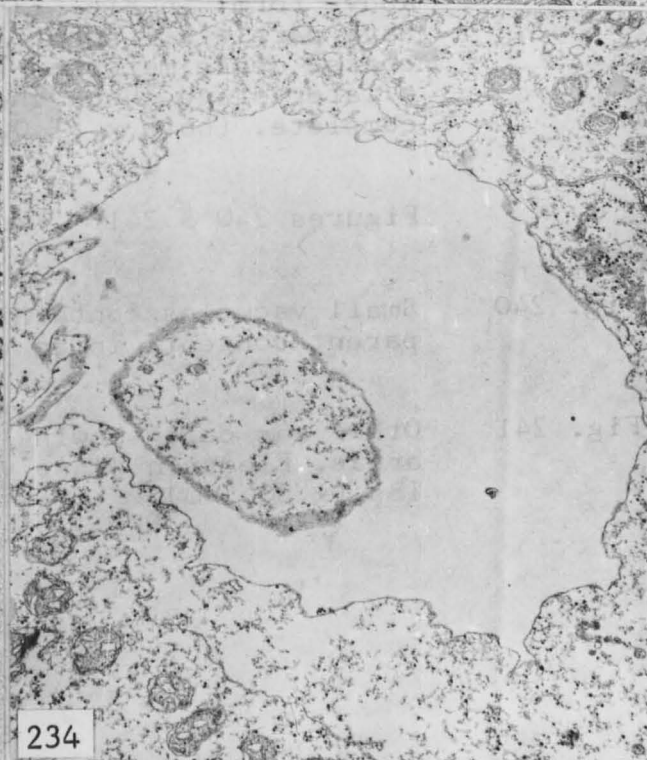
231



232



233



234

Figures 235 - 241 IBs in root tips of Triticum vulgare.

Figures 235 - 239 Glutaraldehyde/OsO₄ fixation.

Fig. 235 Root cap cell with a group of vacuoles containing what appear to be IBs. Several lie against the tonoplast and some show internal differentiation (arrow). The contents are similar (but not identical) with lipid droplets in the cytoplasm (see also Fig. 236) (x 36,000).

Fig. 236 A small vacuole from an embryonic cortical cell showing an IB. A limiting membrane appears to be absent from the IB. Portion of a lipid droplet (arrow) is visible in the adjacent cytoplasm (x 46,500).

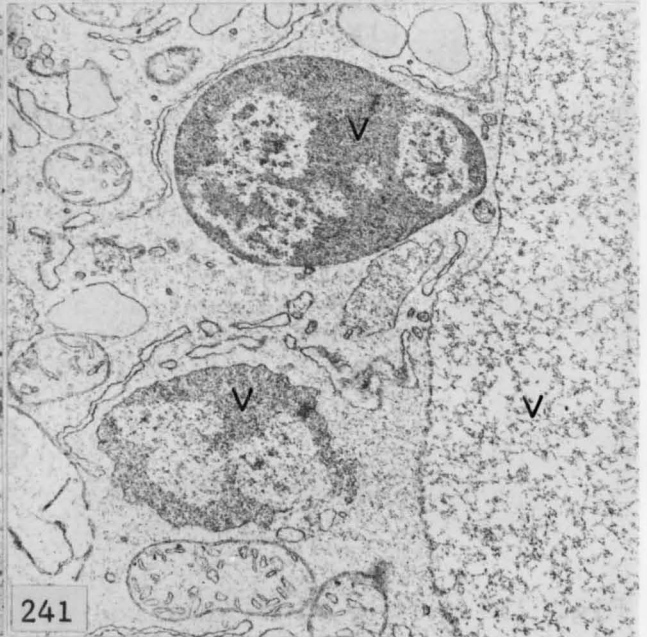
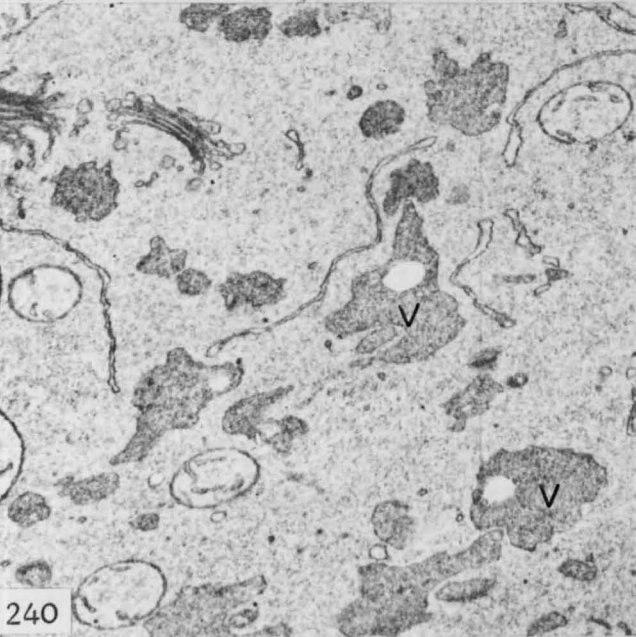
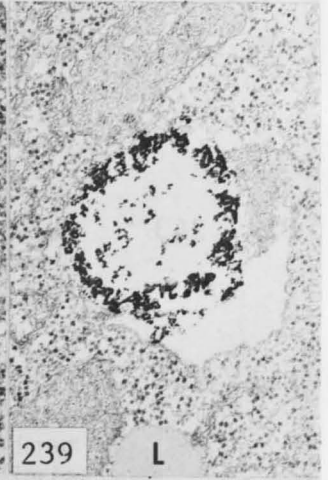
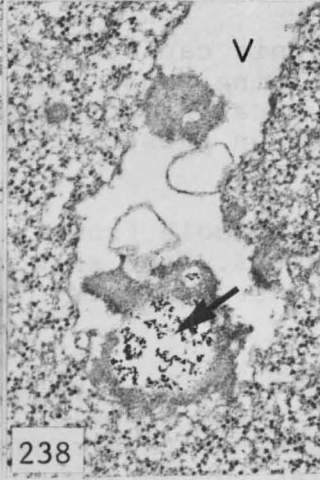
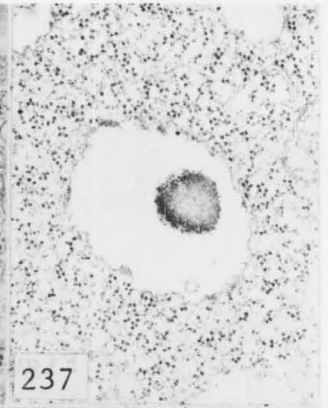
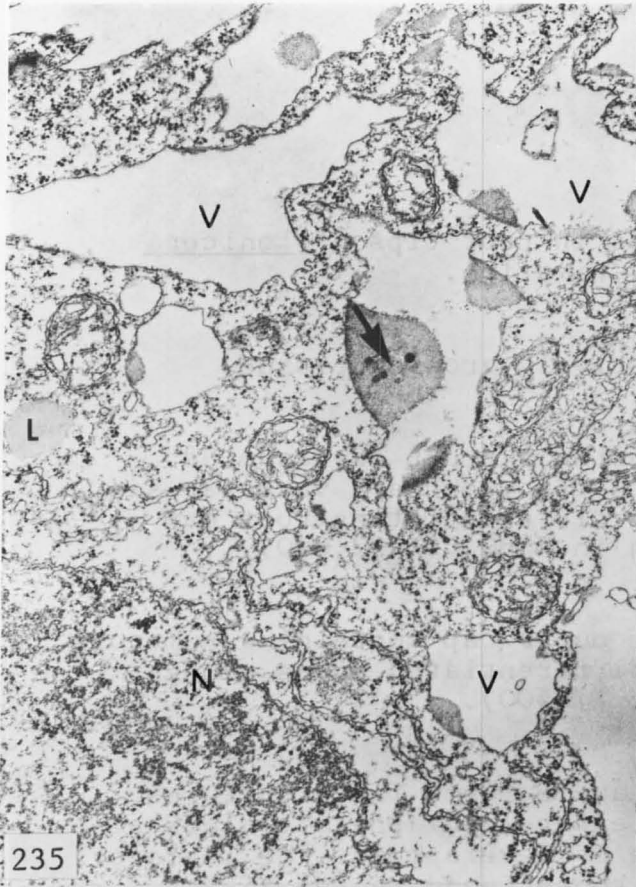
Fig. 237 Vacuole from a cell near the promeristem showing an IB lying free within the vacuolar sap (x 44,500).

Figs. 238 - 239 Transformation of contents in IBs of the above form. In Fig. 238 the homogeneous contents have partly differentiated into a central region of coarse dense material (arrow). Fig. 239 illustrates a later stage where the centrifugal change is almost complete. (both x 51,000).

Figures 240 & 241 KMnO₄ fixation.

Fig. 240 Small vacuoles containing IBs with electron transparent contents in an embryonic cap cell (x 34,500).

Fig. 241 Other cap cell showing IBs containing light and dark areas. Electron dense vacuolar sap surrounds the IBs (x 30,500).



Figures 242 - 256 IBs in root tips of Lonicera nitida: KMnO_4 fixation.

Figures 242 - 249 Actively growing tissue.

- Fig. 242 Embryonic cap cell showing a group of vacuoles containing dense Type A IBs. The dark vacuolar sap is typical of actively differentiating root cap tissue on preservation in KMnO_4 (x 23,000).
- Fig. 243 Large vacuole from the outer cap containing numerous dense IBs. Some show differentiated areas amongst the dense contents. (x 17,500).
- Fig. 244 Details of IBs of the above form. The structures have a fine granular texture and appear to lack a membrane at this stage of development. The IBs are similar to certain of those found in Zea on fixation in KMnO_4 (c.f. Figs. 198-203) (x 38,000).
- Figs. 245 - 249 Differentiation of contents in IBs of above form. Fig. 245 shows light areas (arrows) beginning to develop amongst the dark contents. Figs. 246 - 248 illustrate progressive enlargement of the light areas and restriction of dense material to the periphery of the IBs. In Fig. 249 the dense contents have disappeared; a limiting membrane is apparently absent also. (Fig. 245, x 33,000; Fig. 246, x 26,000; Fig. 247, x 29,000; Fig. 248, x 6,500; Fig. 249, x 25,500).

Figures 250 - 253 Roots less actively growing than above.

- Fig. 250 Low magnification of tissue near the promeristem showing vacuoles containing IBs. Most of the dark contents of the IBs have differentiated into electron transparent material at this stage. (x 5,000).

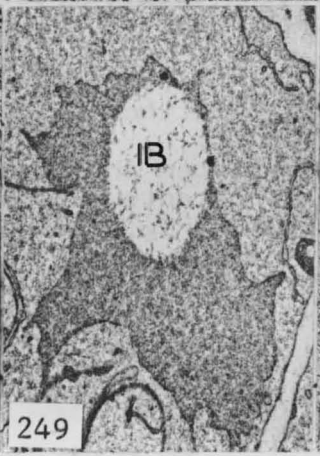
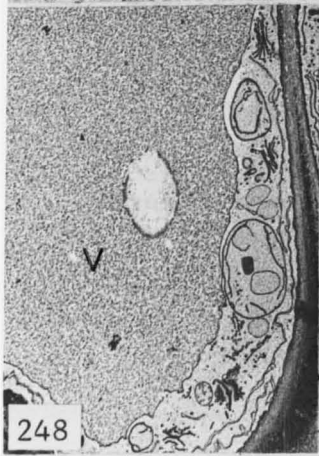
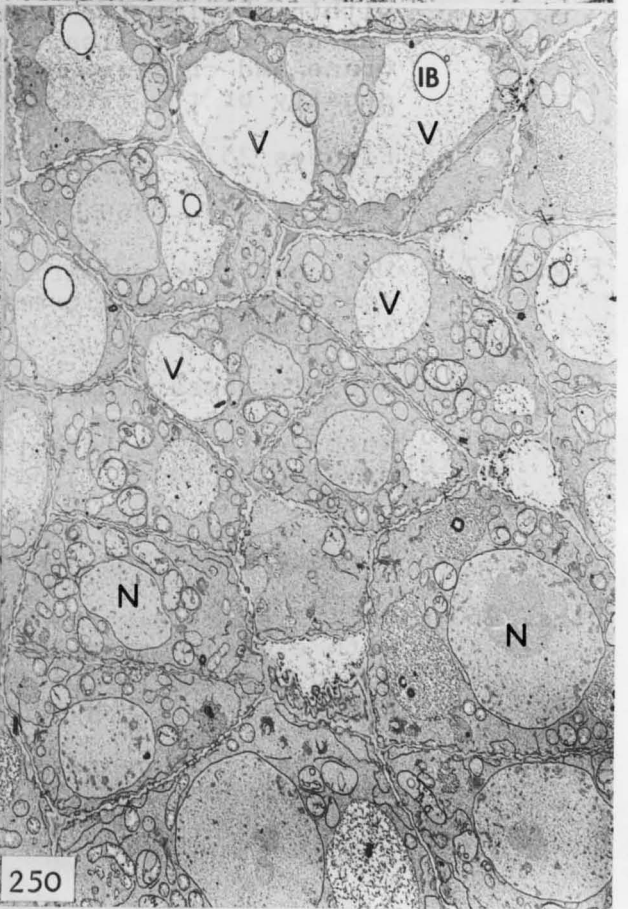
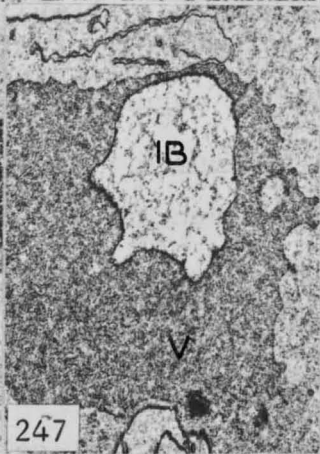
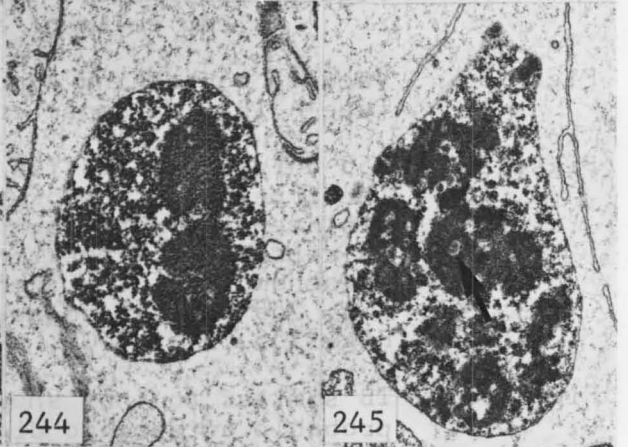
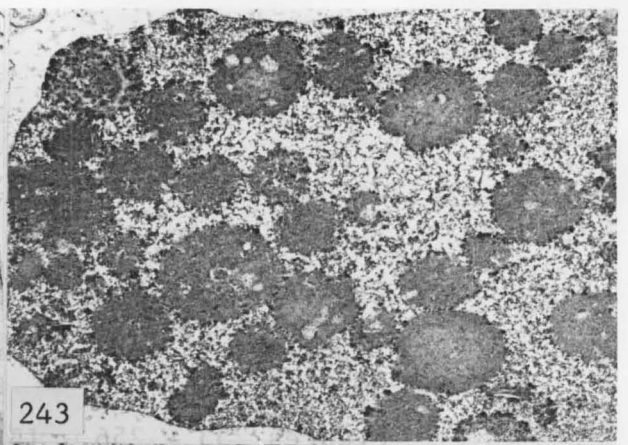


Fig. 251 Fusion of three IBs in a large vacuole (x 31,500).

Fig. 252 Typical appearance of an enlarged IB from tissue similar to that shown in Fig. 250. The electron transparent contents are surrounded by a dense rim (x 31,500).

Figures 253 - 256 IBs in large vacuoles of outer cap cells of actively growing root tips.

Fig. 253 Vacuole containing a multivesiculate Type B, and a Type A IB with dense contents (arrow) (x 31,500).

Figs. 254 & 255 Type A and B IBs within the same vacuole. The irregular form of the Type A IB (arrow) suggests disintegration through digestive action. The Type B IB represents a portion of cytoplasm containing a mitochondrion; the identity of this is seen better in Fig. 255 from an adjacent section (Fig. 254, x 23,000; Fig. 255, x 52,500).

Fig. 256 IB similar to that shown in Fig. 254 illustrating probable autophagic digestion of a mitochondrion within the IB. The outer membrane of the mitochondrion has apparently disappeared leaving only the inner membrane and cristae. Note also the decreased electron density of the mitochondrial matrix. (x 55,000).

Figures 257 - 262 IBs in Mentha root tips.

Figures 257 - 259 Glutaraldehyde/OsO₄ fixation

Fig. 257 Cell near the promeristem showing a small vacuole containing a single membrane bounded Type A IB (arrow) (x 27,000).

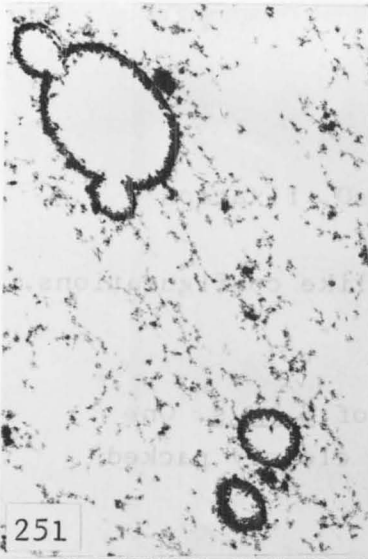
Fig. 258 Vacuole containing a Type B IB (x 36,300).

Fig. 259 Vacuole in a differentiating cortical cell showing a developing IB still attached to the tonoplast (x 46,000).

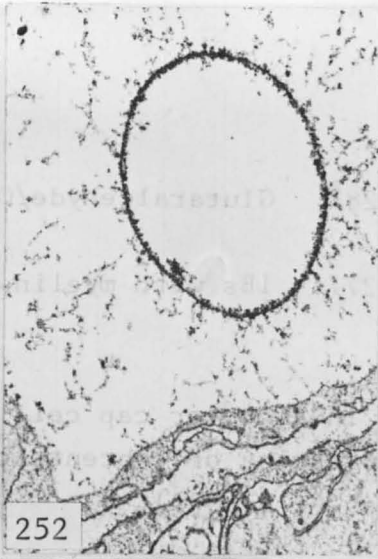
Figures 260 - 262 KMnO₄ fixation

Figs. 260 & 261 Cells near the promeristem showing Type A IBs with electron transparent contents. A membrane clearly surrounds the IB in Fig. 261 (Fig. 260, x 57,500; Fig. 261, x 27,000).

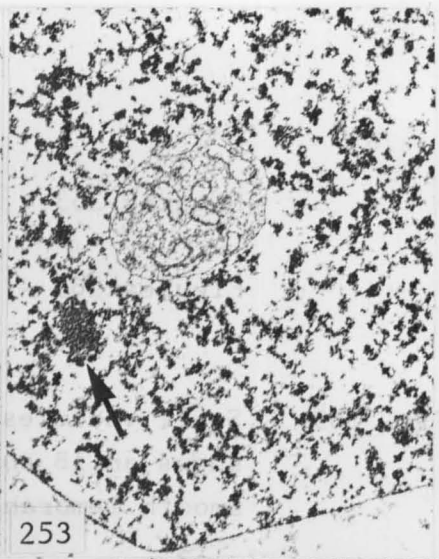
Fig. 262 Root cap cell containing IBs, one of which shows an unusual form (x 31,500).



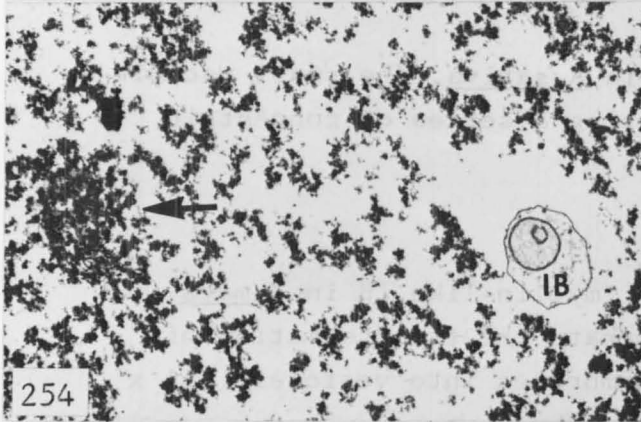
251



252



253



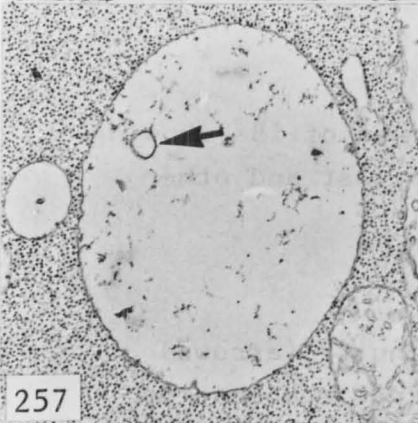
254



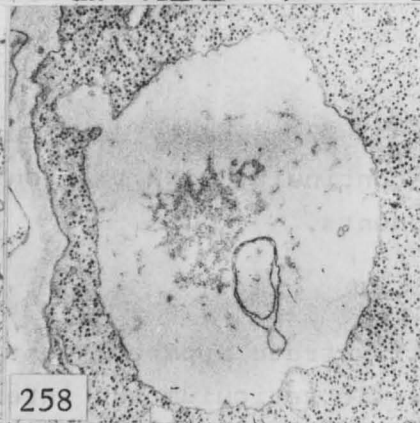
255



256



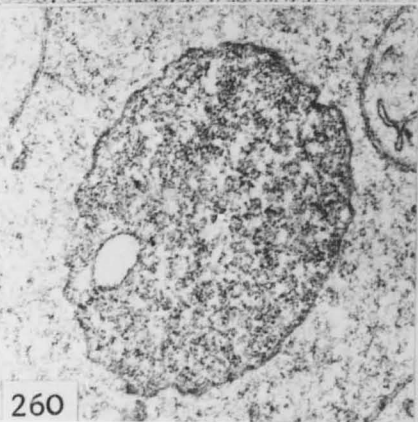
257



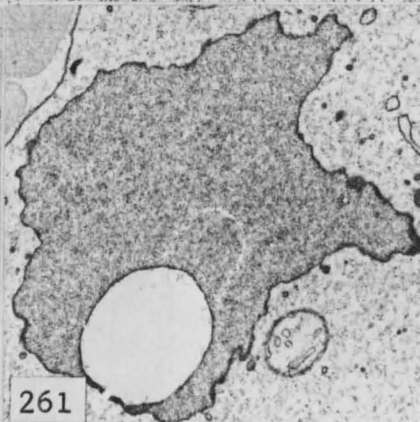
258



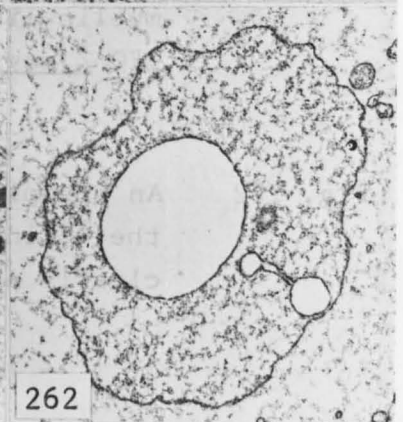
259



260



261



262

Figures 263 - 287 Glutaraldehyde/OsO₄ fixation

Figures 263 - 277 IBs with myelin-like configurations.

Fig. 263 Small vacuoles in an outer cap cell of Z.mays. One shows an IB consisting of concentric closely packed smooth membranes (x 52,500).

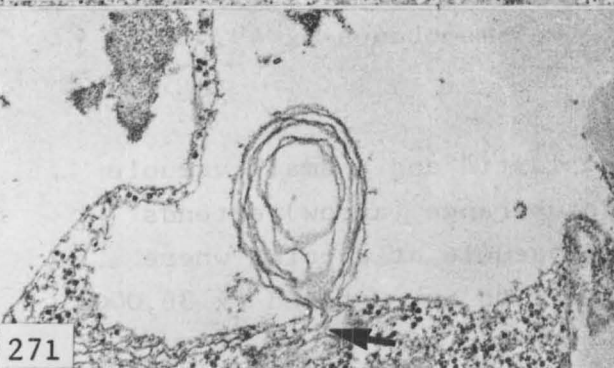
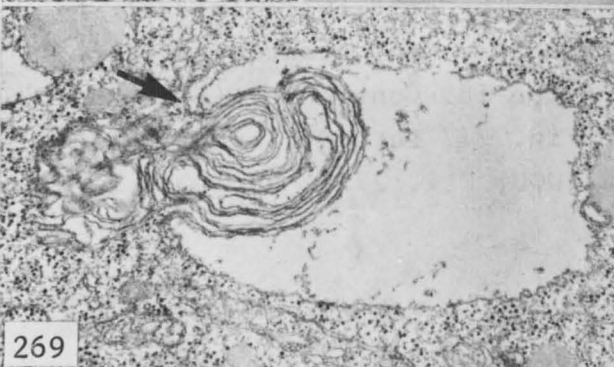
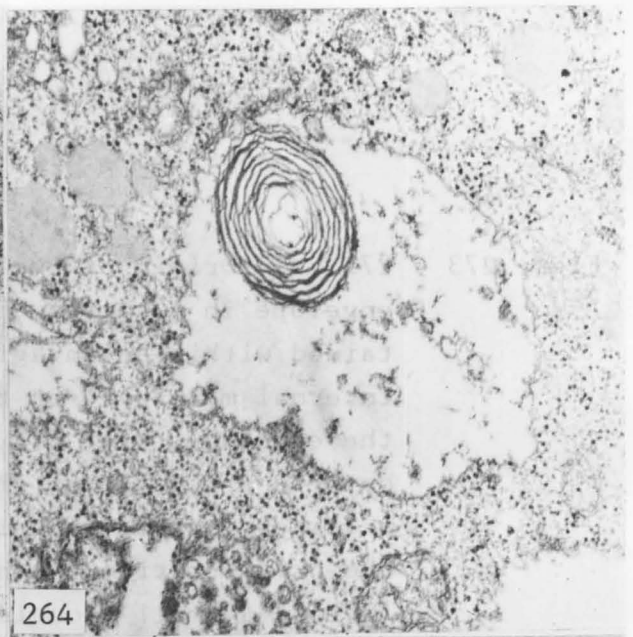
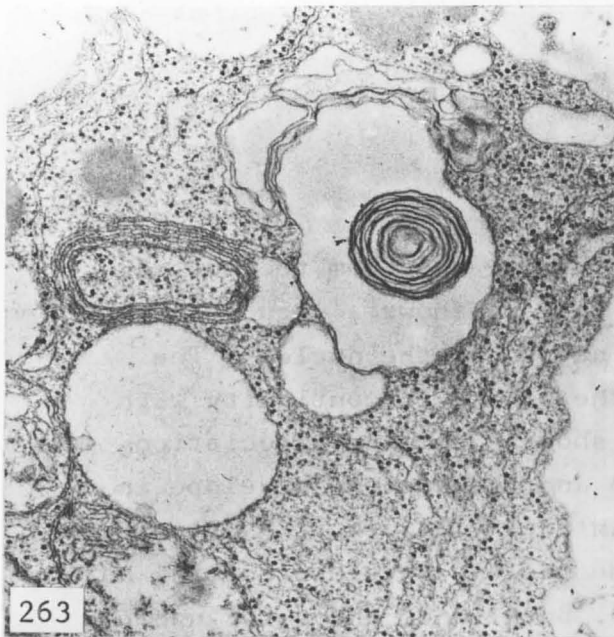
Fig. 264 Differentiating cell in A.sativa. The small vacuole contains an IB comprising a series of concentric membranes (x 52,500).

Figs. 265 - 268 Various forms of myelin-like IB in Z.mays. Figs. 266 - 268 illustrate the transformation of concentric internal membranes into vesicles (all x 46,500).

Figures 269 - 277 Early development of IBs showing stages in continuity with the tonoplast and other cell components.

Figs. 269 - 271 Illustrate apparent continuity (arrows) of endoplasmic reticulum and internal membrane of myelin-like IBs (Fig. 269 A.sativa, x 41,000; Fig. 270 Z.mays, x 74,000; Fig. 271 Z.mays, x 57,500).

Fig. 272 An example in Mentha where the relationship between the IB internal membranes and the cytoplasm is not clearly shown (x 36,700).

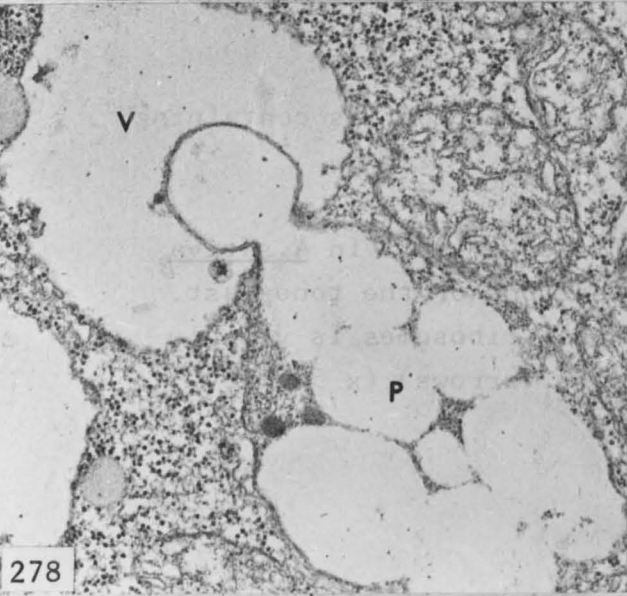
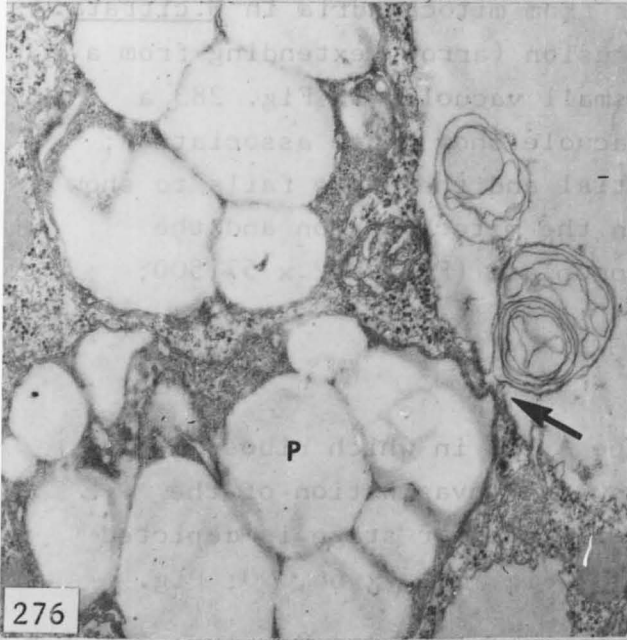
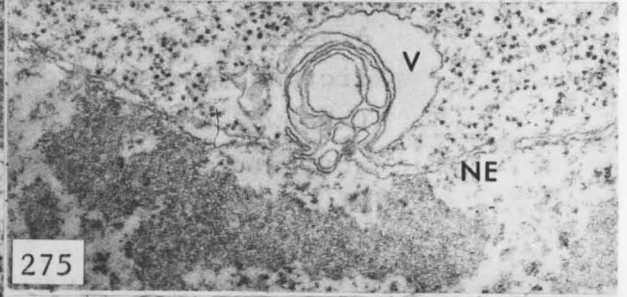
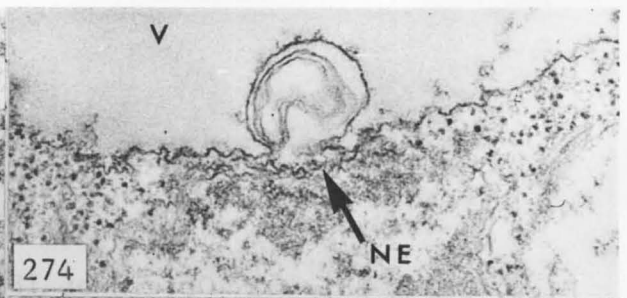
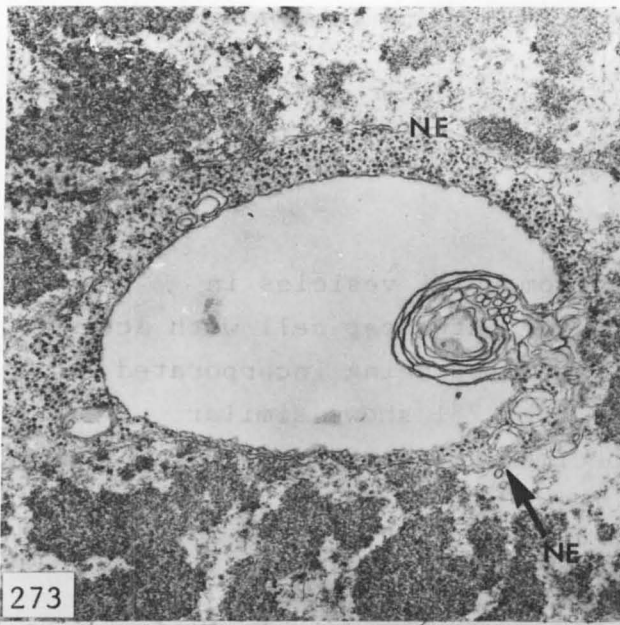


Figs. 273 - 275 Origin of myelin-like IBs from the nuclear envelope in A.sativa. Fig. 273 shows a vacuole contained within an invagination of the nucleus. The internal membranes of the IB are in continuity with the envelope. Fig. 274 shows a similar association between a developing IB and the nuclear envelope in a larger vacuole than in fig. 273. Fig. 275 illustrates the proliferation of the nuclear envelope forming an IB into a small vacuole. Both inner and outer nuclear membranes appear to be involved in formation of this IB. A tonoplast surrounds the IB in all of these figures (Fig. 273 x 41,000; Fig. 274 x 62,500; Fig. 275 x 49,500).

Figs. 276 & 277 Origin of myelin-like IBs from plastids. Fig. 276 shows close association (arrow) between an IB and an amyloplast in Z.mays. The section is lateral and does not show contact between the plastid envelope and internal membranes of the IB. Continuity (arrows) is indistinctly shown in Fig. 277 in a different IB of Z.mays. (Fig. 276 x 41,000; Fig. 277 x 99,000).

Fig. 278 An amyloplast in Z.mays showing a protuberance extending into a vacuole on formation of a Type B IB without elaborate internal membranes (x 49,500).

Fig. 279 Association between a proplastid and a small vacuole in M.citrata. A small protuberance (arrow) extends from the plastid into the vacuole at a point where the plastid envelope appears to be ruptured (x 36,000).



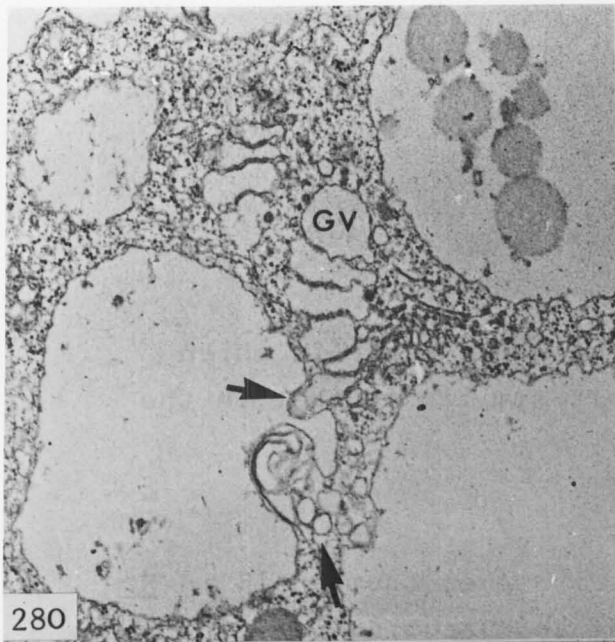
Figs. 280 & 281 Origin of IBs from golgi vesicles in A.sativa. Fig. 280 shows an outer cap cell with active dictyosomes, and golgi vesicles being incorporated (arrows) into vacuoles. Fig. 281 shows similar activity in another specimen (Fig. 280 x 32,500; Fig. 281 x 41,500).

Figs. 282 & 283 Origin of IBs from mitochondria in M.citrata. Fig. 282 shows a protrusion (arrow) extending from a mitochondrion into a small vacuole. In Fig. 283 a mitochondrion and a vacuole show close association; the section is tangential and therefore fails to show direct contact between the mitochondrion and the protrusion from the tonoplast (Fig. 282 x 57,500; Fig. 283 x 49,500).

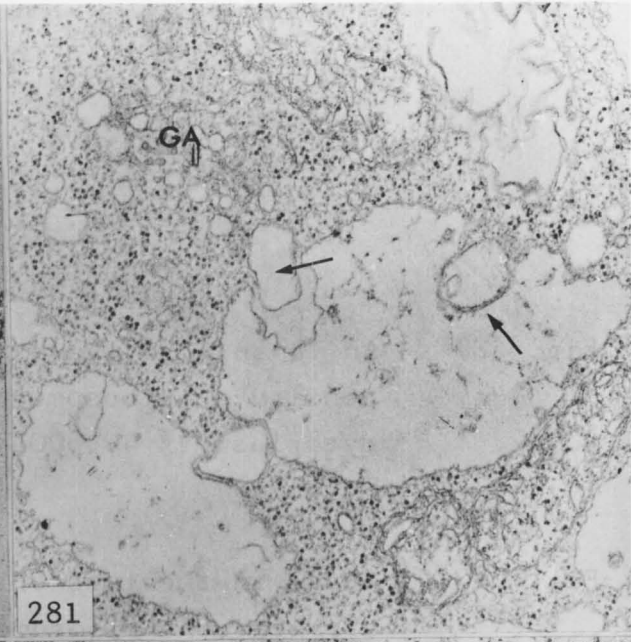
Figs. 284 & 285 Origin of Type A IBs in which ribosomes are excluded. Fig. 284 shows an invagination of the tonoplast in M.citrata. A similar stage is depicted in Fig. 285 in A.sativa (Fig. 284, x 66,000; Fig. 285, x 80,500).

Figures 286 - 288 Development of IBs containing ribosomes.

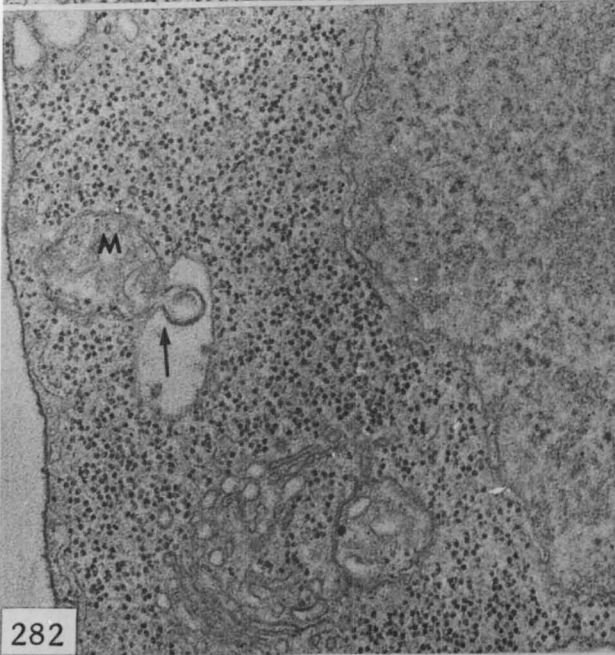
Fig. 286 Small vacuole from a protoderm cell in A.sativa showing various invaginations of the tonoplast. Groundplasm with prominent ribosomes is visible in one of the protrusions (arrows) (x 54,000).



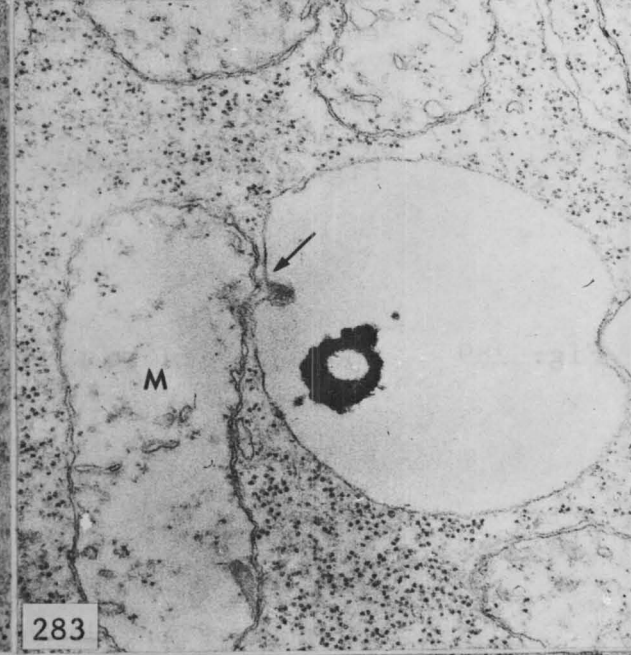
280



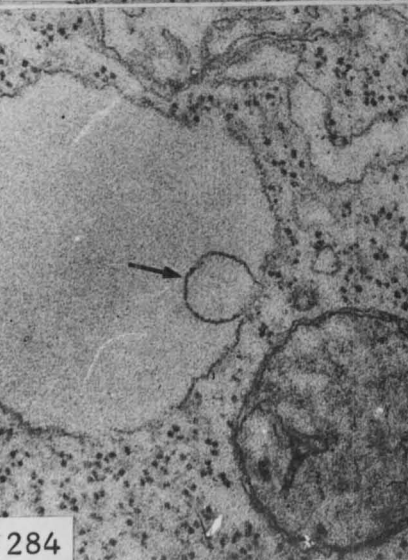
281



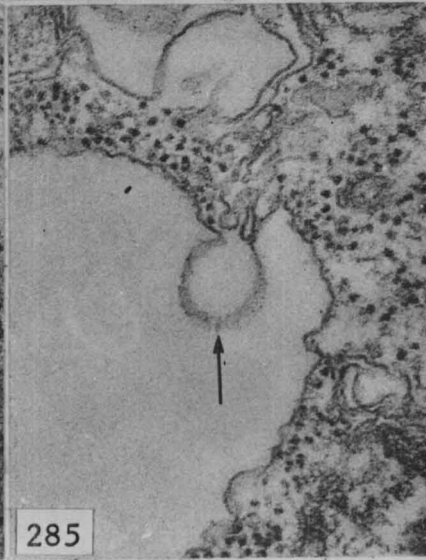
282



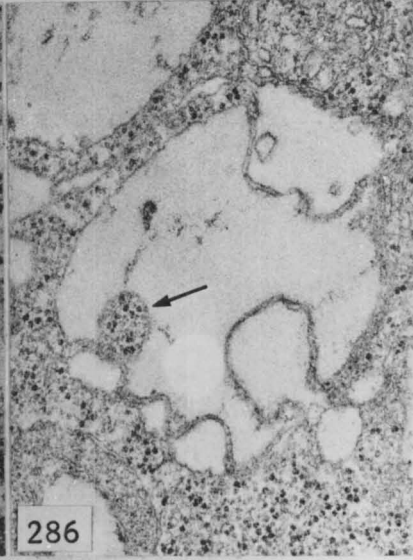
283



284



285



286

Fig. 287 An IB in A.sativa at an early stage in which density of ribosomes is similar to that in the cytoplasm (x 40,500).

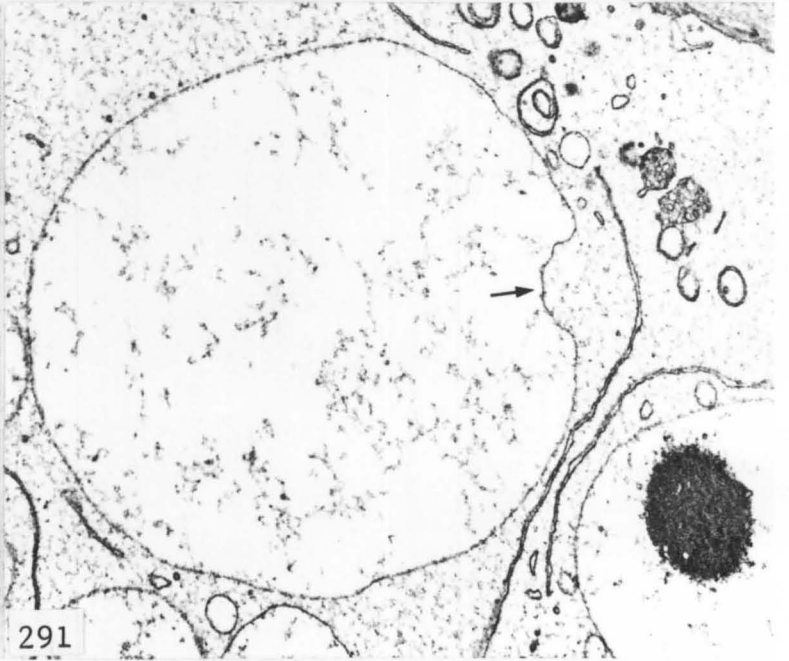
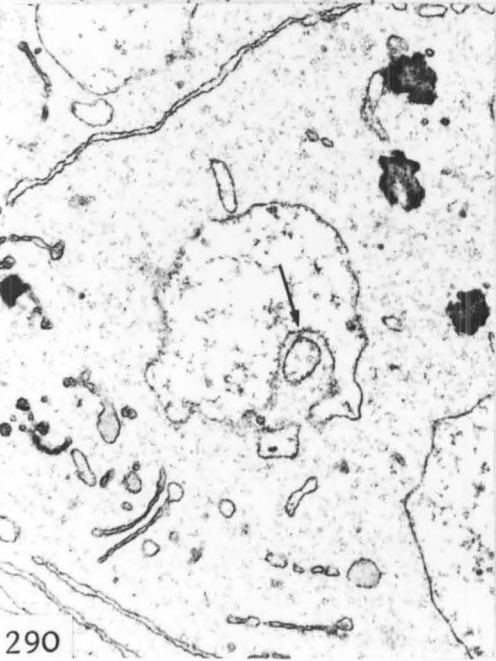
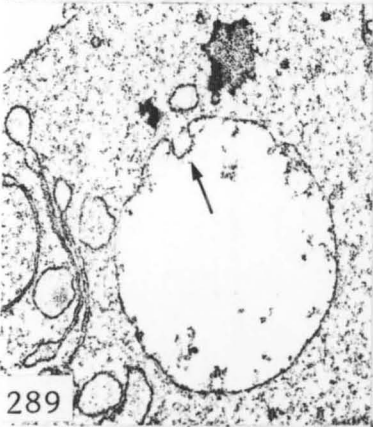
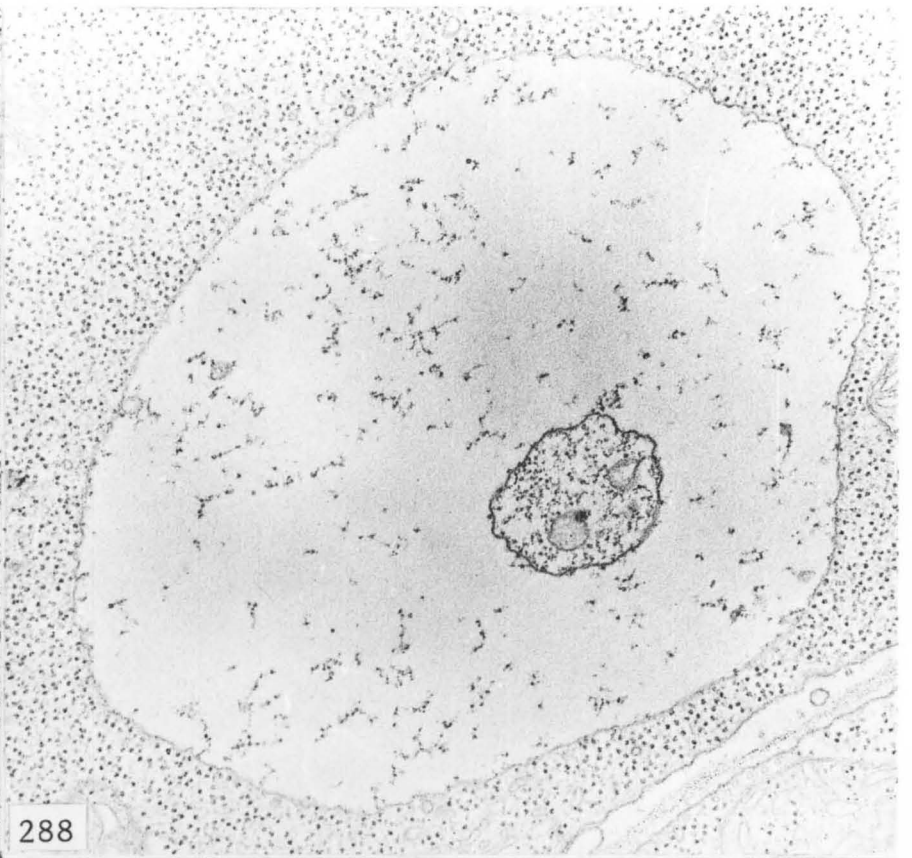
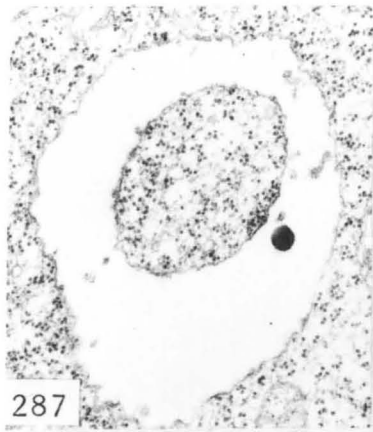
Fig. 288 Stage in the loss of ribosomes from an IB in M.citrata. The ribosomes are becoming less distinct compared with those in the cytoplasm (x 52,000).

Figures 289 - 291 Early development of IBs in root cap cells of Z.mays.KMnO₄ fixation.

Fig. 289 Shows a vacuole with a small invagination on formation of a Type A IB (x 42,000).

Fig. 290 Illustrates a small vacuole with development of a Type B IB containing a vesicle similar to that shown in fig. 193 (x 35,000).

Fig. 291 Depicts the invagination of the tonoplast on formation of an IB (c.f. Fig. 171) (x 30,000).



8. SUMMARY AND GENERAL CONCLUSIONS

Selected aspects of the ultrastructure of vacuoles in root tips of five species have been studied using thin sectioning and freeze-etching techniques. The topics investigated include: (a) the preparation of root tips for freeze-etching, (b) an evaluation of the shape of vacuoles in thin sections and freeze-etch replicas, (c) the effect of various conditions of fixation on the preservation of vacuoles for thin sectioning, (d) the ultrastructure of the tonoplast and (e) a survey of cytoplasmic inclusions found within vacuoles. The main points arising from this work are as follows:-

1. For successful freeze-etching of fresh root tips, specimens must be grown in glycerol to prevent the formation of ice crystals on freezing. Most established roots absorb glycerol with difficulty. However, roots of seedlings placed in contact with 20% glycerol with aeration give mainly good freeze-fixation.
2. When fresh root tips are not required for freeze-etching, roots may be prefixed in GA/glycerol. This gives uniform freezing but may cause slight shrinkage of some organelles.
3. The angular irregular shape of vacuoles often seen in chemically fixed and sectioned material is considered to be an artifact of fixation. These irregular shapes are caused through the shrinkage of mainly spherical vacuoles. Frozen-etched fresh root tips show that vacuoles are spherical or, if irregular, with a rounded outline.
4. The quality of preservation of vacuoles for thin sectioning depends on the fixative used and on the composition and concentration of the vacuolar sap. Owing to the diverse composition of vacuoles within and among cells, it is often difficult to achieve satisfactory preservation of all vacuoles using a given fixative. KMnO_4 causes greater shrinkage of vacuoles than does GA/OsO_4 fixation.

The presence of buffer ions in $KMnO_4$ fixation often helps reduce vacuolar shrinkage.

5. Freeze-etch studies show that the tonoplast is a dynamic membrane with a high degree of organization. At points where the tonoplast invaginates to form inclusion bodies there is a local differentiation of the membrane. This is shown particularly on the outer surface of the tonoplast where there is a greater concentration of particles at these points than elsewhere on the membrane.
6. In frozen-etched fresh root tips fracturing appears to expose the true surfaces of the tonoplast. Both outer and inner faces of the membrane are revealed. When root tips are prefixed in GA/glycerol the plane of fracture within the membrane may be modified and various levels within the membrane are revealed.
7. Inclusions of cytoplasmic origin are common features of many vacuoles in root tip tissue. Various components of the cell are incorporated within these inclusion bodies which are surrounded initially by a single unit membrane derived from the tonoplast. Most inclusion bodies seem to be initiated during periods of cellular activity and eventually disappear from vacuoles probably as a result of digestion. Vacuoles containing inclusion bodies therefore appear to function as lysosomes.

This study has been largely exploratory. Several aspects could now be investigated in greater detail on the basis of information obtained from this work.

Little is known about the interaction of different variables of fixation on the preservation of vacuoles and this might prove an interesting area of research. At the structural level, the organization of the tonoplast at different stages of development requires further investigation and results

from such a study would be a valuable contribution towards an understanding of membrane development. More work of a fundamental nature is required to establish with certainty where the plane of fracture within the tonoplast (and in other frozen-etched membranes) occurs, how this is affected by the organization of the membrane and how it may be modified by various kinds of treatment. The origin and differentiation of inclusion bodies needs to be followed more fully. The conditions which determine their formation, fate and their role in the economy of the cell need to be placed on a firmer basis.

In addition to topics investigated in this thesis, many other fundamental problems of vacuole structure and function could be examined. How membranes fuse when vacuoles coalesce and what factors control this process are still largely unknown. There is also very little knowledge of the composition of individual vacuoles within a cell. How vacuole composition changes during development and whether or not an adjustment is made in sap concentration before vacuoles fuse could also be investigated.

In conclusion, this thesis has shown that the vacuole is a dynamic organelle of the cell. It can no longer be regarded as a passive cell inclusion but as a centre for vital activities of the cell. Furthermore, not only are vacuoles variable in composition among themselves, both in space and time, but also it is now apparent that their function changes during their development. In early stages many vacuoles apparently function as lysosomes but after they enlarge to fill the greater part of the cell their main role seems to be that of maintaining the turgor pressure of the cell.

LITERATURE CITED

- AFZELIUS, B.A. 1957. Electron microscopy on the basophilic structures of the sea urchin egg. *Z. Zellforsch. mikrosk. Anat.* 45: 660-675.
- 1959. Electron microscopy of the sperm tail. Results obtained with a new fixative. *J. biophys. biochem. Cytol.* 5: 269-278.
- 1962. Chemical fixatives for electron microscopy. In HARRIS, R.J.C. (ed.) *The Interpretation of Ultrastructure: 1-19.* Academic Press.
- BAILEY, I.W. 1930. The cambium and its derivative tissues V. A reconnaissance of the vacuome in living cells. *Z. Zellforsch. mikrosk. Anat.* 10: 651-682.
- BAKER, J.R. 1958. *Principles of Biological Microtechnique.* Methuen, London. 357pp.
- 1960. *Cytological Technique (2nd ed.).* Methuen, London. 211pp.
- 1965. The fine structure produced in cells by fixatives. *Jl. R. microsc. Soc.* 84: 115-
- & McRAE, J.M. 1966. The fine structure resulting from fixation by formaldehyde; the effects of concentration, duration and temperature. *J. roy. microsc. Soc.* 85: 391-399.
- BAMBERGER, E.S. & PARK, R.B. 1966. Effect of hydrolytic enzymes on the photosynthetic efficiency and morphology of chloroplasts. *Pl. Physiol.* 41: 1591-1600.
- BARTON, R. 1965. Electron microscope observations on the origin and development of the vacuole in root tip cells of Phaseolus. *Cytologia* 30: 266-273.
- BERJAK, P. 1968. A lysosome-like organelle in the root cap of Zea mays. *J. Ultrastruct. Res.* 23: 233-242.
- BERNARD, G.R. & WYNN, G.G. 1965. The behaviour of tissue slices in chromate solutions: a study of the comparative importance of the tonicity and osmotic concentration of fixative systems. *Q. Jl. microsc. Sci.* 106: 23-30.
- BERTAUD, W.S. 1962. Visit to Australia. Dominion Physical. Lab. Report No. R. 301. 15pp. (D.S.I.R. Lower Hutt).
- BOUCK, G.B. 1963. Stratification and subsequent behaviour of plant cell organelles. *J. Cell Biol.* 18: 441-457.
- & CRONSHAW, J. 1965. The fine structure of differentiating sieve tube elements. *J. Cell Biol.* 25: 79-96.
- BOWES, B.G. 1965a. The ultrastructure of the shoot apex and young shoot of Glechoma hederacea L. *La Cellule* 65: 351-356.
- 1965b. The origin and development of vacuoles in Glechoma hederacea L. *La Cellule* 65: 359-364.

- BOWES, J.H. & CATER, C.W. 1966. The reaction of glutraldehyde with proteins and other biological material. *Jl. R. microsc. Soc.* 85: 193-200.
- BRADBURY, S. & MEEK, G.A. 1960. A study of potassium permanganate 'fixation' for electron microscopy. *Jl. microsc. Sci.* 101: 241-250.
- BRANDES, D., BUETOW, D.E. & BERTINI, F. 1964. Role of lysosomes in cellular lytic processes. 1. Effect of carbon starvation in Euglena gracilis. *Experimental & Molecular Pathology*. 3: 583-609.
- BRANTON, D. 1966. Fracture faces of frozen membranes. *Proc. nat. Acad. Sci.* 55: 1048-1056.
- 1967. Fracture faces in frozen myelin. *Expl. Cell Res.* 45: 703-707.
- & MOOR, H. 1964. Fine structure in freeze-etched Allium cepa L. root tips. *J. Ultrastruct. Res.* 11: 401-411.
- & PARK, R.B. 1967. Subunits in chloroplast lamellae. *J. Ultrastruct. Res.* 19: 283-303.
- & SOUTHWORTH, D. 1967. Fracture faces of frozen Chlorella and Saccharomyces cells. *Expl. Cell Res.* 47: 648-653.
- BUCKLEY, P.M. & SOMMER, N.F. 1967. Cytosomes and vacuoles in fungus spores. *J. Ultrastruct. Res.* 21: p 163.
- BUVAT, R. 1957. Relations entre l'ergastoplasme et l'appareil vacuolaire. *C.r. Acad. Sci. (Paris)* 245: 350-352.
- 1958. Recherches sur les infra-structures du cytoplasme dans les cellules du méristème apical, des ébauches, foliaires et des feuilles développées d'Blodea canadensis. *Annls. Sci. Nat. Botanique. et Biol. Vegetale* 19: 121-161.
- 1960. Origine et évolution du système vacuolaire dans la racine de Triticum vulgare; relation avec l'ergastoplasme. *C.r. Acad. Sci. (Paris)* 251: 3051-3053.
- 1961. Le réticulum endoplasmique des cellules végétales. *Ber. dt. bot. Ges.* 74: 261-267.
- 1963a. Electron microscopy of Plant Protoplasm. *Int. Rev. Cytol.* 14: 41-155. New York.
- 1963b. Infrastructure et différenciation des cellules criblées de Cucurbita pepo. Evolution du tonoplaste et signification du contenu cellulaire final. *C.r. Acad. Sci. (Paris)* 256: 5193-5195.
- 1968a. Différenciation des cellules criblées du protophloème dans les jeunes d'Orge (Hordeum sativum). *C.r. Acad. Sci. (Paris)* 267: 406-408.
- 1968b. Diversité de vacuoles dans les cellules de la racine d'Orge (Hordeum vulgare). *C.r. Acad. Sci. (Paris)* 267: 296-298.

- & MOUSSEAU, A. 1960. Origine et évolution du système vacuolaire dans la racine de Triticum vulgare; Relation avec l'ergastoplasme. C.r. Acad. Sci. (Paris) 251: 3051-3053.
- CAULFIELD, J.B. 1957. Effects of varying the vehicle for OsO₄ tissue fixation. J. biophys. biochem. Cytol. 3: 827-830.
- CLAUDE, A. 1961. Problems of fixation for electron microscopy. Results of fixation with osmium tetroxide in acid and alkaline media. Path. et Biol. 9: 933-947.
- CLOWES, F.A.L. 1961. Apical meristems. Blackwell Scientific Publications, Oxford. 217pp.
- & JUNIPER, B.E. 1964. The fine structure of the quiescent center and neighbouring tissues in root meristem. J. exp. Bot. 15: 622-630.
- & ----, 1968. Plant Cells. Blackwell Scientific Publications, Oxford. 546pp.
- CÔTÉ, W.A. (ed.) 1965. Cellular Ultrastructure in Woody Plants. Syracuse University Press 603 pp.
- COULOMB, P. 1968a. Sur la présence de structures lamellisées dans les cellules méristématiques du bourgeon de Solanum tuberosum. C.r. Acad. Sci. (Paris) 267: 1373-1374.
- 1968b. Etude préliminaire sur l'activité phosphatasique acide des particules analogues aux lysosomes des cellules radiculaires jeunes de la Courge (Cucurbita pepo L. Cucurbitacée). C.r. Acad. Sci. (Paris) 267: 2133-2136.
- 1969. Mise en évidence de structures analogues aux lysosomes dans le méristème radiculaire de la courge (Cucurbita pepo L. Cucurbitacée). J. de Microscopie 8: 123-138.
- & BUVAT, R. 1968. Processus de dégénérescence cytoplasmique particielle dans les cellules de jeunes racines de Cucurbita pepo. C.r. Acad. Sci. (Paris) 267: 843-844.
- CRONSHAW, J. & BOUCK, G.B. 1965. The fine structure of differentiating xylem elements. J. Cell Biol. 24: 415-431.
- CUNNINGHAM, W.P. & CRANE, F.L. 1966. Variations in membrane structure as revealed by negative staining technique. Expl. Cell Res. 44: 31-45.
- DAINTY, J. 1968. The structure and possible function of the vacuole. In PRIDHAM, J.B. (ed.) Plant Cell Organelles: 40-46. Academic Press.
- DANGEARD, P. 1956. Le vacuome de la cellule végétale morphologie. Protoplasmatologica Bd, III/D: 1-41 Springer-Verlag.
- DAVIS, J.M. 1959. Preparation and sectioning of tissue embedded in 'araldite' for electron microscope examination. Nature 183: 200-201.
- DAVISON, H. & DANIELLI, J.F. 1952. The Permeability of Natural Membranes. Cambridge University Press, Lond. & New York. 365pp.

- DEAMER, D.W. & BRANTON, D. 1967. Fracture planes in an ice-bilayer model membrane system. *Science* 158: 655-657.
- DIERS, L. 1965. Elektronenmikroskopische Beobachtungen zur Archegonienentwicklung des Lebermooses Sphaerocarpus donnellii Aust. Die Entwicklung des jungen Archegons bis zum Stadium des ausgebildeten sekundären Zentralzelle. *Planta* 66: 165-190.
- & SCHÖTZ, F. 1966. Über die dreidimensionale Gestaltung des Thylakoidsystems in den Chloroplasten. *Planta* 70: 322-343.
- DILLEY, R.A., PARK, R.B. & BRANTON, D. 1967. Ultrastructure studies of light-induced chloroplast shrinkage. *Photochem. & Photobiol.* 6: 407-412.
- DRAWERT, H. 1955a. Der pH-wert des Zellsaftes. *In* W. Ruhland (ed.) *Encycloped. Pl. Physiol.* 1: 627-648. Springer-Verlag.
- 1955b. Die Entstehung der Vacuolen. *In* W. Ruhland (ed.) *Encycloped. Pl. Physiol.* 1: 661-667. Springer Verlag.
- DUCREUX, G. 1968. Sur L'ultrastructure des apex des bourgeons, principal et axillaire de Chara vulgaris. *C.r. Acad. Sci. (Paris)* 267: 163-166.
- De DUVE, C. & WATTIAUX, R. 1966. Functions of Lysosomes. *Ann. Rev. Physiol.* 28: 435-492.
- EGGMANN, H. 1966. Untersuchungen zur Kern-und Chromosomenstruktur pflanzlicher Zellen. Sonderabdr. aust. (Vierteljahrsschrift der Naturforschenden Gesellschaft in Zürich). Jahrgang III, Schlussheft s 281-307 Prow Nr 3819.
- ENRLICH, H.G. 1958. Electron microscope studies of Saintpaulia ionantha Wendl. pollen walls. *Expl. Cell Res.* 15: 463-474.
- ELFVIN, L.G. 1962. Electron microscope studies on the effect of anisotonic solutions on the structure of unmyelinated splenic nerve fibers of the cat. *J. Ultrastruct. Res.* 7: 1-38.
- ERICSSON, J.L.E., SALADINO, A.J. & TRUMP, B.F. 1965. Electron microscopic observations of the influence of different fixatives on the appearance of cellular ultrastructure. *Z. Zellforsch. mikrosk. Anat.* 66: 161-181.
- ESAU, K. 1963. Ultrastructure of differentiated cells of higher plants. *Am. J. Bot.* 50: 495-506.
- 1965. Anatomy and cytology of Vitis phloem. *Hilgardia* 37: 17-72.
- 1965c. Fixation of sieve element plastids in Beta. *Proc. nat. Acad. Sci.* 54: 429-437.
- & CRONSHAW, J. 1968. Plastids and mitochondria in the phloem of Cucurbita. *Can. J. Bot.* 46: 877-880.
- ETTLINGER, K., HERRMANN, C. & STAEBELIN, A. 1965. Morphologie und Teilungsmodus von Pediococcus cerevisiae. *Pathologia. Microbiol.* 28: 561-566.
- EVERT, R.F. & MURMANIS, L. 1965. Ultrastructure of the secondary phloem of Tilia americana. *Am. J. Bot.* 52: 95-106.

- , ---- & SACHS, I.B. 1966. Another view of the ultrastructure of Cucurbita phloem. *Ann. Bot.* 30: 563-585
- FALK, H. & SITTE, P. 1963. Zellfeinbau bei Plasmolyse. I Der Feinbau der Elodea Blattzellen. *Protoplasma* 57: 290-303.
- FAWCETT, D.W. 1964. In histology and cytology. In SIEGEL, B.M. (ed.) *Modern developments in Electron Microscopy*. Academic Press. 257-333.
- FINERAN, B.A. 1966. Fine structure of meristem and differentiating root cap cells in Ranunculus hirtus Hook. f. *Phytomorphology* 16: 1-16.
- FREY-WYSSLING, A. 1966. Freeze-etching, a method of obtaining true-to-life electron micrographs. *Advm. Sci.* 23: 109-117.
- 1967. The Organelles of the cell, their comparative ultrastructure and physiology. *Aust. J. Sci.* 29: 458-462.
- & MÜHLETHALER, K. 1965. *Ultrastructural plant cytology*. Elsevier, Amsterdam. 377pp.
- & SCHWEGLER, F. 1965. Ultrastructure of the chloroplasts in the carrot root. *J. Ultrastruct. Res.* 13: 543-559.
- FRIEDERICI, H.H.R. 1968a. Freeze-etch observations on interstitial connective tissue. *J. Ultrastruct. Res.* 24: 269-285.
- 1968b. The tridimensional ultrastructure of fenestrated capillaries. *J. Ultrastruct. Res.* 23: 444-456.
- GAHAN, P.B. 1967. Histochemistry of lysosomes. *Int. Rev. Cytol.* 21: 2-53.
- 1968. Lysosomes. In FRIDHAM, J.B. (ed.) *Plant Cell Organelles*: 228-238. Academic Press.
- GALEY, F.R., NILSSON, S.E. 1966. A new method for transferring sections from liquid surface of the trough through staining solutions to the supporting film. *J. Ultrastruct. Res.* 14: 405-410.
- GIESBRECHT, P. & DREWS, G. 1966. "Über die Organisation und die macromolekulare Architektur der Thylakoide lebender Bakterien. *Arch. Microbiol.* 54: 297-330.
- GIFFORD, E.M. & STEWART, K.D. 1967. Ultrastructure of the shoot apex of Chenopodium album and certain other seed plants. *J. Cell Biol.* 33: 131-142.
- 1968. Inclusions of the proplastids and vacuoles in the shoot apices of Bryophyllum and Kalenchoe. *Am. J. Bot.* 55: 269-279.
- GLAUERT, A.M. 1966. The fixation and embedding of biological specimens. In KAY, D.H. (ed.) *Techniques for Electron Microscopy*: 166-212. Blackwell Scientific Publ. Oxford.
- & GLAUERT, R.H. 1958. Araldite as an embedding medium for electron microscopy. *J. biophys. biochem. Cytol.* 4: 191-194.
- 1968. Electron microscopy of lipids and membranes. *Jl. R. Microsc. Soc.* 88: 49-70.

- GRAY, P. 1954. The Microtome's Formulary and Guide. Constable & Co. Ltd, London. 794pp.
- GRUN, P. 1963. Ultrastructure of plant plasma and vacuolar membranes. J. Ultrastruct. Res. 9: 198-208.
- GUERIN-DUMARTRAIT, E. 1968. Étude en cryodécoupage de la morphologie des surfaces lamellaires chloroplastiques de Chlorella pyrenoidosa, en cultures synchrones. Planta 80: 96-110.
- GUILLIERMOND, A. 1941. The cytoplasm of the plant cell. Chronica Botanica 247pp.
- GREENHAM, C.G. 1966. The relative electrical resistances of the plasmalemma and tonoplasts in higher plants. Planta 69: 150-157.
- HAIRSTON, M.A. 1956. Osmium tetroxide fixation of fine plant structures for electron microscopy. Cytologia 21: 179-182.
- HALL, C.E. 1950. A low temperature replica method for electron microscopy. J. appl. Phys. 21: 61.
- 1966. Introduction to electron microscopy. McGraw-Hill, New York. 397pp.
- HALL, D.M. 1967. Wax microchannels in the epidermis of white clover. Science 158: 505-506.
- HAWKER, L.E. & HENDY, R.J. 1963. An electron microscope study of germination of conidia of Botrytis cinerea. J. gen. Microbiol. 33: 43-46.
- HERRMANN, C. & STAEBELIN, A. 1965. Licht-und elektronenmikroskopische Untersuchungen an Pediococcus cerevisiae. Schweiz. Brau-Rdsch. 76: 76-79.
- HESS, W.M. 1966. Fixation and staining of fungus hyphae and host plant root tissue for electron microscopy. Stain Technol. 41: 27-35.
- 1968. Ultrastructural comparisons of fungal hyphae cells using frozen-etched replicas and thin sections of the fungus Pyrenochaeta terrestris. Can. J. Microbiol. 14: 205-210.
- , SASSEN, M.M.A. & REMSEN, C.C. 1966. Surface structures of frozen-etched Penicillium conidiospores. Naturwissenschaften 24: 708.
- , ---- & ---- 1968. Surface characteristics of Penicillium conidia. Mycologia 60: 290-303.
- , ----, ---- & STOCKS, D.L. 1968. Surface structures and organelle characterization of frozen-etched Aspergillus conidiospores. Am. J. Bot. 54: p.637.
- HOHL, H.R. 1960. "Über die submikroskopische Struktur normaler und hyperplastischer Gewebe von Datura stramonium L. 1. Normalgewebe. Ber. dt. bot. Ges. 70: 395-439.
- HOLMAN, R.M. & ROBBINS, W.W. 1954. A Textbook of General Botany (4th ed.). John Wiley, New York. 664pp.

- HOLT, S.J. & HICKS, R.M. 1961a. Use of veronal buffers in formalin fixatives. *Nature* 191: 832.
- & ---- 1961b. Studies on formalin fixation for electron microscopy and cytochemical staining purposes. *J. biophys. biochem. Cytol.* 11: 31-45.
- HRSEL, I. 1961a. Histochemische Studium Golgi-ähnlicher Einschlüsse im Wurzelmeristem von Fagopyrum. *Acta histochem. Suppl.* 11: 243-247.
- 1961b. Golgi-Apparat und Golgi-ähnliche Inklusionen. *Ber. dt. bot. Ges.* 74: 257-260.
- 1965a. The classic golgi apparatus and vacuoles. *Biologia Pl.* 7: 136-144.
- 1965b. The electron microscopic classic and golgi apparatus. *Biologia Pl.* 7: 293-307.
- 1965c. Morphology and function of the golgi apparatus. *Biologia Pl.* 7: 437-448.
- 1966. The morphology and function of vesicles and of vesicle related formations in cell ultrastructure. *Biologia Pl.* 8: 263-272.
- 1967. Golgi apparatus and golgi zones. *Biologia Pl.* 9: 157-167.
- & JURÁKOVÁ, J. 1964. Elektronenmikroskopische Untersuchungen über das Eindringen plasmatischer Partikel in Vakuolen der pflanzlichen Zelle. *Biologia Pl.* 6: 17-21.
- , ---- & BENEŠ, K. 1960. Cytochemical and electron-microscopic investigations of the golgi apparatus and golgi resembling inclusions in the root meristem of Fagopyrum esculentum Moench. *Biologia Pl.* 2: 252-268.
- HURD-KARRER, A.M. 1939. Hydrogen-ion concentration of leaf juice in relation to environment of plant species. *Am. J. Bot.* 26: 834-846.
- INTERNATIONAL BIBLIOGRAPHY OF ELECTRON MICROSCOPY. Vol. 1, 1950-1955. Vol. II, 1956-1961. Published by the New York Society of Electron Microscopists.
- JENSEN, W.A. 1963. Cell development during plant embryogenesis. *In* Meristems and Differentiation. *Brookhaven Symp. Biol.* 16: 179-202.
- 1964. Observations on fusions of nuclei in plants. *J. Cell Biol.* 23: 669-672.
- 1965. The ultrastructure and histochemistry of the synergids of cotton. *Am. J. Bot.* 52: 238-256.
- JOHNSON, R.P.C. 1966. Potassium permanganate fixative and the electron microscopy of sieve tube contents. *Planta* 68: 36-43.
- 1968. Microfilaments in pores between frozen etched sieve elements. *Planta* 81: 314-322.
- JOHNSON, U.G. & PORTER, K.R. 1968. Fine structure of cell division in Chlamydomonas reinhardtii. *J. Cell Biol.* 38: 403-425.

- LUFT, J.H. & WOOD, R.L. 1963. The extraction of tissue protein during and after fixation with osmium tetroxide in various buffer systems. *J. Cell Biol.* 19: 46a.
- MACHADO, B.M. 1967. Straight osmium tetroxide versus glutaraldehyde - OsO₄ in sequence as fixatives for the granular vesicles in sympathetic axons of the rat pineal body. *Stain Technol.* 42: 293-300.
- MALHOTRA, S.K. 1962a. Experiments on fixation for electron microscopy 1. Unbuffered osmium tetroxide. *Q. Jl. Microsc. Sci.* 103: 5-15.
- 1962b. Experiments on fixation for electron microscopy 2. Alkalized osmium tetroxide. *Q. Jl. Microsc. Sci.* 103: 287-296.
- 1963. Experiments on fixation for electron microscopy 4. Sodium veronal buffer. *Q. Jl. Microsc. Sci.* 104: 155-167.
- MANIRUZZAMAN, A.F.M., BENES, K. & LUDVIK, J. 1967. Cytoplasmic components of the broad bean root tip cells. *Biologia Pl.* 9: 454-461.
- MANTON, I. 1961. Plant cell structure. In Contemporary Botanical Thought MACLEOD, A.M. & COBLEY, L.S. (eds.) Edinburgh. 197pp.
- 1962. Observations on stellate vacuoles in the meristem of Anthoceros. *J. expl. Bot.* 13: 161-167.
- MARINOS, N.G. 1963a. Vacuolation in plant cells. *J. Ultrastruct. Res.* 9: 177-185.
- 1963b. Studies on submicroscopic aspects of mineral deficiencies II. Nitrogen, potassium, sulfur, phosphorus and magnesium deficiencies in the shoot apex of barley. *Am. J. Bot.* 50: 998-1005.
- MARUYAMA, K. 1965. Cyclic changes of the golgi body during microsporogenesis in Tradescantia paludosa. *Cytologia* 30: 354-374.
- MASER, M.D., POWELL, T.E. & PHILPOTT, C.W. 1967. Relationships among pH, osmolarity and concentration of fixative solutions. *Stain Technol.* 42: 175-182.
- MATILE, P. 1965. Intrazelluläre Lokalisation proteolytischer Enzyme von Neurospora crassa. 1. Funktion und subzelluläre Verteilung proteolytischer Enzyme. *Z. Zellforsch. mikrosk. Anat.* 65: 884-896.
- 1966. Enzyme der Vakuolen aus Wurzelzellen von Maiskeimlingen. Ein Beitrag zur funktionellen Bedeutung der Vakuole bei der intrazellulären Verdauung. *Z. Naturf.* 21b: 871-878.
- 1968. Lysosomes of root tip cells in corn seedlings. *Planta* 79: 181-196.
- , BALZ, H.P., SEMADENI, E & JOST, M. 1965. Isolation of spherosomes with lysosome characteristics from seedlings. *Z. Naturf.* 20b: 693-698.
- & MOOR, H. 1968. Vacuolation: origin and development of the lysosomal apparatus in root tip cells. *Planta* 80: 159-175.
- & WIEMKEN, A. 1967. The vacuole as the lysosome of the yeast cell. *Arch. Mikrobiol.* 56: 148-155.

- MAUNSBACH, A.B. 1966. The influence of different fixatives and fixation methods on the ultrastructure of rat kidney proximal tubule cells II. Effects of varying osmolarity, ionic strength, buffer system and fixative concentration of glutaraldehyde solutions. *J. Ultrastruct. Res.* 15: 283-309.
- , MADDEN, S.C. & LALTA, H. 1962. Variations in fine structure of renal tubular epithelium under different conditions of fixation. *J. Ultrastruct. Res.* 6: 511-530.
- McLEAN, J.D. 1960. Fixation of plant tissue. *In* BARGMANN *et al.*, (eds.), Fourth Int. Congr. Electron Microsc. Vol 2: 27-29.
- MERCER, E.H. & BIRBECK, M.S.C. 1961. *Electron Microscopy. A handbook for biologists.* Blackwell Scientific Publ. Oxford. 76pp.
- MERRILLEES, N.C.R., BURNSTOCK, G. & HOLMAN, M.E. 1963. Correlation of fine structure and physiology of the innervation of smooth muscle in the guinea pig vas deferens. *J. Cell Biol.* 19: 529-550.
- MESQUITA, J.F. 1968. Sur les modifications ultrastructurales produites par le phényl-uréthane dans les cellules méristématiques des racines d'Allium cepa L. *C.r. Acad. Sci. (Paris)* 267: 303-305.
- MICHAUX, N. 1968. Etude de cytologie du méristème apical du Pteris cretica L. *C.r. Acad. Sci. (Paris)* 267: 1442-1444.
- MILLONIG, G. 1961a. A modified procedure for lead staining of thin sections. *J. biophys. biochem. Cytol.* 2: 736-739.
- 1961b. Advantages of a phosphate buffer for osmium tetroxide solutions in fixation. *J. appl. Phys.* 32: 1637.
- 1962. Further observations on a phosphate buffer for osmium solutions in fixation. *In* BREESE, S.S. (ed.), Fifth Int. Congr. Electron Microsc. Vol. 2: P-8.
- 1966. Model experiments on fixation and dehydration. *In* UYEDA, R. (ed.), Sixth Int. Congr. Electron Microsc. Vol. 2: 21-22.
- MOHR, W.P. & COCKING, E.C. 1968. A method of preparing highly vacuolated senescent or damaged plant tissue for ultrastructural study. *J. Ultrastruct. Res.* 21: 171-181.
- MOLLENHAUER, H.H. 1959. Permanganate fixation of plant cells. *J. biophys. biochem. Cytol.* 6: 431-435.
- 1964. Plastic embedding mixtures for use in electron microscopy. *Stain Technol.* 39: 113.
- & MORRE, D.J. 1966. The golgi apparatus and plant secretion. *Ann. Rev. Pl. Physiol.* 17: 27-46.
- , ---- & KELLY, A.G. 1966. The widespread occurrence of plant cytosomes resembling animal microbodies. *Protoplasma* 62: 44-52.

- MOOR, H. 1959. Platin-Kohle-Abdruck-Technik angewandt auf den Feinbau der Milchröhren. J. Ultrastruct. Res. 2: 393-422.
- 1964. Die Gefrier-Fixation lebender Zellen und ihre Anwendung in der Elektronenmikroskopie. Z. Zellforsch. 62: 546-580.
- 1965. 'Freeze-etching' a specimen preparation technique for electron-microscopical investigations of living cells and tissues. In High Vacuum Report. No. 2: Balzers, A.G., Liechtenstein 23pp.
- 1966a. The performance of freeze-etching and the interpretation of results concerning the surface structure of membranes and the fine structure of microtubules and spindle fibres. In High Vacuum Report. No. 9: Balzers, A.G., Liechtenstein 11pp.
- 1966b. Use of freeze-etching in the study of biological ultrastructure. Int. Rev. exp. Pathol. 5: 179-216.
- 1966c. Der Feinbau der Mikrotubuli in Hefe nach Gefrierätzung. Protoplasma 64: 89-103.
- 1966d. Ultrastrukturen im Zellkern der Backerhefe. J. Cell Biol. 29: 153-155.
- 1967. Endoplasmic reticulum as the initiator of bud formation in yeast (S. cerevisiae). Arch. Mikrobiol. 57: 135-146.
- & MÜHLETHALER, K. 1963. Fine structure in frozen-etched yeast cells. J. Cell Biol. 17: 609-628.
- , ----, WALDNER, M. & FREY-WYSSLING, A. 1961. A new freeze-ultra microtome. J. biophys. biochem. Cytol. 10: 1-10.
- , C. RUSKA & H. RUSKA, 1964. Elektronenmikroskopische darstellung tierischer Zellen mit der Gefrierätzungstechnik. Z. Zellforsch. 62: 581-601.
- MOSS, G.I. 1966. Glutaraldehyde as a fixative for plant tissues. Protoplasma 62: 194-200.
- MÜHLETHALER, K. 1960. Die Entstehung des Vacuolensystems in Pflanzenzellen. In BARGMANN, W., PETERS, D. & WOLPERS, C. (eds.) Fourth Int. Congr. Electron Microsc. Vol. 2: 491-494.
- , MOOR, H., & SZARKOWSKI, J.W. 1965. The ultrastructure of the chloroplast lamellae. Planta 67: 305-323.
- NANNINGA, N. 1968. Structural feature of meosomes (chondroids) of Bacillus subtilis after freeze-etching. J. Cell Biol. 39: 251-263.
- NORTHCOTE, D.H. 1967. Electron microscopy of frozen specimens. New Scient. 33: 658-660.
- & LEWIS, D.R. 1968. Freeze-etched surfaces of membranes and organelles in the cells of pea root tip. J. Cell Sci. 3: 199-206.

- NORTHCOTE, D.H. 1968. The organization of the Endoplasmic Reticulum, the Golgi Bodies and Microtubules during cell division and subsequent growth. In J.B. PRIDHAM (ed.), Plant Cell Organelles: 179-197 Academic Press.
- NOUGAREDE, A. 1963a. Premières observations sur l'infrastructure et sur l'évolution des cellules des jeunes ébauches foliaires embryonnaires du Tropaeolum majus L. Cytologie de la déshydratation due maturation. C.r. Acad. Sci. (Paris) 257: 1335-1338.
- 1963b. Premières observations sur l'infrastructure et sur l'évolution des cellules des jeunes ébauches foliaires embryonnaires du Tropaeolum majus L. Cytologie de l'hydratation germinative et des premières étapes de la germination. C.r. Acad. Sci. (Paris) 257: 1495-1497.
- PALADE, G.E. 1952. A study of fixation for electron microscopy. J. exp. Med. 95: 285-298.
- 1956. The fixation of tissues for electron microscopy. In ROSS, R. (ed.) Proc. Third Int. Congr. Electron Microsc. 129-142.
- PALAY, S.L. & PALADE, G.E. 1955. The fine structure of neurons. J. biophys. biochem. Cytol. 1: 69-88.
- PAOLILLO, D.J., FALK, R.H. & REIGHARD, J.A. 1967. The effect of chemical fixation on the framework of chloroplasts. Trans. Am. microsc. Soc. 86: 225-232.
- PARK, R.B. & BRANTON, D. 1967. Freeze-etching of chloroplasts from glutaraldehyde fixed leaves. In Energy Conversion by the Photosynthetic Apparatus. Brookhaven Symp. Biol. 19: 341-352.
- PEASE, D.C. 1960 & 1964. Histological techniques for Electron Microscopy. 1st. ed. 1960. 2nd ed. 1964. Academic Press.
- 1967. Eutectic ethylene glycol and pure propylene glycol as substituting media for dehydration of frozen tissue. J. Ultrastruct. Res. 21: 75-97.
- PERNER, E.S. 1958. Elektronenmikroskopische Untersuchungen zur Cytomorphologie des sogenannten (Golgi-Systems) in Wurzelzellen verschiedener Angiospermen. Protoplasma 49: 407-446.
- PEYTON, G.A. & BROWN, C.C. 1963. The host-parasite interface of Peronospora manshurica on Glycine max. Am. J. Bot. 50: 787-797.
- PICKETT-HEAPS, J.D. 1967. Ultrastructure and differentiation in Chara sp. 1. Vegetative cells. Aust. J. biol. Sci. 20: 539-551.
- & NORTHCOTE, D.H. 1966. Relationship of cellular organelles to the formation and development of the plant cell wall. J. exp. Bot. 17: 20-26.
- PISEK, A. 1955. Chemie des Zellsaftes 1. Der Vacuoleninhalt und seine Bedeutung im allgemeinen. In RUHLAND, W. (ed.), Encycloped. Pl. Physiol. 1: 614-626.

- PORTER, K.R. 1964. Ultramicrotomy. In SIEGEL, B.M. (ed.), Modern Developments in Electron Microscopy: 119-144.
- & MACHADO, R.D. 1960. Studies on the endoplasmic reticulum IV. Its form and distribution during mitosis in cells of onion root tips. J. biophys. biochem. Cytol. 7: 167-180.
- POUX, N. 1961. Observations sur les rapports des vacuoles avec les structures réticulaires du cytoplasme. C.r. Acad. Sci. (Paris) 253: 2395-2397.
- 1962. Nouvelles observations sur la nature et l'origine de la membrane vacuolaire des cellules végétales. J. de Microscopie 1: 55-66.
- 1963a. Sur la présence d'enclaves cytoplasmiques en voie de dégénérescence dans les vacuoles des cellules végétales. C.r. Acad. Sci. (Paris) 257: 736-738.
- 1963b. Localisation de la phosphatase acide dans les cellules meristematiques de blé (Triticum vulgare Vill.) J. de Microscopie 2: 485-489.
- POWELL, T.E., PHILPOTT, C.W. & MASER, M.D. 1964. On the hydrogen ion concentration and osmium tetroxide osmolarity of fixative compounds. J. Cell Biol. 23: 110a-111a.
- PRIDHAM, J.B. (ed.) 1968. Plant Cell Organelles. Academic Press. 261pp.
- RANGAN, S.R.S. 1960. The effect of osmium tetroxide at two different osmolar concentrations on mouse mammary carcinoma. J. Ultrastruct. Res. 3: 392-397.
- RAYNS, D.G., SIMPSON, F.O. & BERTAUD, W.S. 1967. Transverse tubule apertures in mammalian myocardial cells: Surface arrays. Science 156: 656-657.
- REMSSEN, C.C. 1966. The fine structure of frozen-etched Bacillus cereus spores. Arch. Microbiol. 54: 266-275.
- & LUNDGREN, D.G. 1966. Electron microscopy of the cell envelope of Ferrobacillus ferrooxidans prepared by freeze-etching and chemical fixation techniques. J. Bact. 92: 1765-1771.
- , VALOIS, V.W., & WATSON, S.W. 1967a. Fine structure of the cytomembranes of Nitrosocystis oceanus. J. Bact. 94: 422-433.
- , HESS, W.M. & SASSEN, M.M.A. 1967b. Fine structure of germinating Penicillium megasporum conidia. Protoplasma 64: 439-451.
- , WATSON, S.W., WATERBURY, J.B. & TRUPER, H.G. 1968. Fine structure of Ectothiorhodospira mobilis Pelsh. J. Bact. 95: 2374-2392.
- REYNOLDS, E.S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. 17: 208-212.

- RICHTER, H. 1968a. Die Reaktion hochpermeabler Pflanzenzellen auf drei Gefrierschutzstoffe (Glyzerin, Äthylenglykol, Dimethylsulfoxid). *Protoplasma* 65: 155-166.
- 1968b. Die Gefrierresistenz glyzerinbehandelten Campanula Zellen. *Protoplasma*, 66: 63-78.
- ROBERTSON, J.D. 1959. The ultrastructure of cell membranes and their derivatives. In The Structure and Function of Subcellular Components. *Biochem. Soc. Symp.* 16: 3-43.
- ROSEN, W.G. & GAWLIK, S.R. 1966. Fine structure of lily pollen tubes following various fixation and staining procedures. *Protoplasma* 61: 181-191
- SABATINI, D.D., BENSCH, K.G. & BARNETT, R.J. 1963. Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. *J. Cell Biol.* 17: 19-58.
- SAKAI, A. 1966. Survival of plant tissue at super-low temperatures. IV. Cell survival with rapid cooling and rewarming. *Pl. Physiol.* 41: 1050-1054.
- SASSEN, M.M.A., RENSEN, C.C. & HESS, W.M. 1967. Fine structure of Penicillium megasporum conidiospores. *Protoplasma* 64: 75-88.
- SCHNEPF, E. 1959. Zur Feinstruktur der Drüsen von Drosophyllum lusitanicum. *Planta* 54: 641-647.
- SCHULTZ, R.L. & KARLSSON, U. 1965. Fixation of the central nervous system for electron microscopy by aldehyde perfusion. II. Effect of osmolarity, pH of perfusate and fixative concentration. *J. Ultrastruct. Res.* 12: 187-206.
- SEDAR, A.W. 1962. Electron microscopy of the oxyntic cell in the gastric glands of the bullfrog Rana catesbiana III. Permanganate fixation of the endoplasmic reticulum. *J. Cell. Biol.* 14: 152-156.
- SIEVERS, A. 1966. Lysosomen-ähnliche Kompartimente in Pflanzenzellen. *Naturwissenschaften* 53: 334-335.
- SITTE, P. 1957. Elektronenmikroskopische Untersuchungen an meristematischen Pflanzenzellen. *Experientia* 13: 419-420.
- 1958. Die Ultrastruktur von Wurzelmeristemzellen der Erbse (Pisum sativum). *Protoplasma* 49: 447-522.
- 1961. Die submikroskopische Organisation der Pflanzenzelle. *Ber. dt. bot. Ges.* 74: 177-206.
- SJÖSTRAND, F.S. 1953. The ultrastructure of outer segments of rods and cones of the eye as revealed by the Electron microscope. *J. cell Comp. Physiol.* 42: 15-44.
- 1956a. Recent advances in the biological applications of the electron microscope. In ROSS, R. (ed.) *Proc. Third. Int. Confr. Electron Microsc.*: 26-37.

- SJÖSTRAND, F.S. 1956b. Electron microscopy of cells and tissues. In Physical Techniques in Biological Research. Vol. 3. OSTER, G. & POLLISTER, A.W. (eds). 241-298.
- 1956c. The ultrastructure of cells revealed by electron microscopy. Int. Rev. Cytol. 5: 456-533.
- 1958. Ultrastructure of retinal rod synapses of the guinea pig eye as revealed by three-dimensional reconstructions from serial sections. J. Ultrastruct. Res. 2: 122-170.
- 1963. A comparison of plasma membrane, cytomembranes and mitochondrial elements with respect to ultrastructural features. J. Ultrastruct. Res. 9: 561-580.
- 1967. Electron microscopy of cells and tissues. Vol. 1. Instrumentation and techniques. Academic Press. 462pp.
- SORVALL, I. 1965. Thin sectioning and associated techniques for electron microscopy. (2nd ed.) Sorvall Inc. Connecticut, U.S.A. 113pp.
- SRIVASTAVA, L.M. & O'BRIEN, T.P. 1966. On the ultrastructure of cambium and its vascular derivatives. 1, Cambium of Pinus strobus L. Protoplasma 61: 257-276.
- & PAULSON, R.E. 1968. The fine structure of the embryo of Lactuca sativa II. Changes during germination. Can. J. Bot. 46: 1447-1453.
- STAEHELIN, L.A. 1966. Die Ultrastruktur der Zellwand und des Chloroplasten von Chlorella. Z. Zellforsch. mikrosk. Anat. 74: 325-350.
- 1968a. The interpretation of freeze-etched artificial and biological membranes. J. Ultrastruct. Res. 22: 326-347.
- 1968b. Ultrastructural changes of the plasmalemma and the cell wall during the life cycle of Cyanidium caldarium. Proc. R. Soc. (Lond.) B. 171: 261-272.
- STEERE, R.L. 1957. Electron microscopy of structural detail in frozen biological specimens. J. biophys. biochem. Cytol. 3: 45-60.
- SUN, C.N. 1963. Fixing and embedding of plant tissues for electron microscopy. Protoplasma 56: 374-376.
- 1964. Fine structure of parenchyma cells of the ovary wall in Aloe vera. La Cellule 64: 215-221.
- TAHMISIAN, T.N. 1964. Using of the freezing point method to adjust the tonicity of fixing solutions. J. Ultrastruct. Res. 10: 182-188.
- THORNTON, R.M. 1967. The fine structure of Phycomyces 1. Autophagic vesicles. J. Ultrastruct. Res. 21: 269-280.
- TRUMP, B.F. & ERICSSON, J.L.E. 1965. The effect of the fixative solution on the ultrastructure of cells and tissues. A comparative analysis with particular attention to the proximal convoluted tubule of the rat kidney. Lab. Investigation 14: 1245-1323.

- TUCKER, S.C. & HOEFERT, L.L. 1968. Ontogeny of the tendril in Vitis vinifera. Am. J. Bot. 55: 1110-1119.
- UEDA, K. 1966. Fine structure of Chlorogonium elongatum with special reference to vacuole development. Cytologia 31: 461-472.
- VIAL, J.D. & ORREGO, H. 1966. Effects of molar concentration of fixative upon the fine structure of oxyntic cells. Protoplasma 61: 164-180.
- VILLIERS, T.A. 1967. Cytolysomes in long-dormant plant embryo cells. Nature 214: 1356-1357.
- VITOLS, E., NORTH, R.J. & LINNANE, A.W. 1961. Studies on the oxidative metabolism of Saccharomyces cerevisiae. J. Cell Biol. 9: 689-699.
- VOELLER, B.R. 1964. The Plant Cell: aspects of its form and function. In BRACHET, J. & MIRSKY, A.E. (eds.), The Cell. 6: 245-312. Academic Press.
- VOGEL, A.I. 1946. Quantitative Inorganic Analysis. Longman, Green, Lond. 856pp.
- WALLACE, P.G., HUANG, M. & LINNANE, A.W. 1968. The biogenesis of mitochondria II. The influence of medium composition on the cytology of anaerobically grown Saccharomyces cerevisiae. J. Cell Biol. 37: 207-220.
- WARDROP, A.B. & CRONSHAW, J. 1962. Formation of phenolic substances in the ray parenchyma of angiosperms. Nature 193: 90-92.
- & FOSTER, R.C. 1964. A cytological study of the oat coleoptile. Aust. J. Bot. 12: 135-141.
- WEST, E.S. 1963. Text book of biophysical chemistry. Macmillan, New York. 435pp.
- WETZEL, B.K. 1961. Sodium permanganate fixation for electron microscopy. J. biophys. biochem. Cytol. 9: 711-716.
- WHALEY, W.G. MOLLENHAUER, H.H. & KEPHART, J.E. 1959a. The endoplasmic reticulum and golgi structures in maize root cells. J. biophys. biochem. Cytol. 5: 501-506.
- , ----- & ----- 1959b. Developmental changes in the golgi apparatus of maize root cells. Am. J. Bot. 46: 743-751.
- , ----- & LEECH, J.H. 1960a. The ultrastructure of the meristematic cell. Am. J. Bot. 47: 401-449.
- , ----- & ----- 1960b. Some observations on the nuclear envelope. J. biophys. biochem. Cytol. 8: 233-245.
- WHITE, P.R. 1968. Evidence of turgor pressure gradients within and between single plant cells grown in culture. Phytomorphology 17: 507-509.

- WIAME, J.M. 1947. Étude d'une substance polyphosphorée, basophile et métachromatique chez les levures. *Biochem. Biophys. Acta.* 1: 234-255.
- WISCHNITZER, S. 1962. Introduction to electron microscopy. Pergamon Press. 132pp.
- 1967. Current techniques in biomedical electron microscopy. *Int. Rev. Cytol.* 22: 2-47.
- WOOD, R.L. & LUFT, J.H. 1965. The influence of buffer systems on fixation with osmium tetroxide. *J. Ultrastruct. Res.* 12: 22-45.
- WOODCOCK, C.L.F. & BELL, P.R. 1968. Features of the ultrastructure of the female gametophyte of Myosurus minimus. *J. Ultrastruct. Res.* 22: 546-563.
- WOODING, F.B.P. & NORTHCOPE, D.H. 1965. An anomalous wall thickening and its possible role in the uptake of stem-fed tritiated glucose by Pinus pinea. *J. Ultrastruct. Res.* 12: 463-472.
- & ---- 1966. The fine structure of the mature resin canal cells of Pinus pinea. *J. Ultrastruct. Res.* 13: 233-244.
- ZIRKLE, C. 1928. The effect of hydrogen-ion concentration upon fixation images of various compounds of chromium. *Protoplasma* 4: 201-227.
- 1932. Vacuoles in primary meristems. *Z. Zellforsch. mikrosk. Anat.* 16: 26-47.
- 1937. The plant vacuole. *Bot. Rev.* 3: 1-30.

Postscript

Since this paper was written, other accounts of changes in fine structure of root tip tissues have appeared. Clowes and Juniper have obtained results in Zea mays similar to those in Ranunculus hirtus (Clowes & Juniper 1964; Juniper & Clowes, 1965). The ultrastructure of tissues basipetal to the promeristem has also been examined (Leech et al., 1963; Maniruzzaman et al., 1967). Observations from these studies suggest that differentiation, at the structural level, involves the development of organelles found in embryonic cells rather than the formation of new structures.

During this project, further observations have been made on root tips. The structure of promeristem and differentiating root cap cells in Avena, Lonicera, Mentha, Triticum and Zea agrees with that found in Ranunculus. Some additional information has also been noted. Golgi vesicles have now been seen accumulating against the wall in outer cap cells, particularly in actively growing roots of seedlings. Amyloplasts in growing root tips are larger and contain more starch than those in dormant material. Freeze-etching has also revealed much new information on the structure of organelles, but the technique proved limited for studies involving a linear sequence of differentiating cells. This is because fracturing is a random process and files of cells are seldom exposed.

APPENDIX I

Reprinted from PHYTOMORPHOLOGY, Vol. 16, No. 1, March 1966

Issued April 1966, pp. 1-16

PRINTED IN INDIA

FINE STRUCTURE OF MERISTEM AND DIFFERENTIATING ROOT CAP CELLS IN *RANUNCULUS HIRTUS* HOOK. F.

B. A. FINERAN*

Department of Botany, University of Canterbury, Christchurch, New Zealand

Introduction

Electron microscopy is providing a valuable tool whereby differentiation of plant tissues can be followed in greater detail than was previously possible with optical microscopic techniques. In the present study a general survey is made of the ultrastructural changes in the cellular compounds during differentiation of root cap tissue in *Ranunculus hirtus*. As a basis for comparing the ultrastructure of differentiating cap cells the fine structure of cells from the promeristem region of the root are described first.

In this study the root cap was selected in preference to other tissues of the root since differentiation can be followed over a comparatively short lineage of cells from the meristem to the tip of the cap. Compared with other tissues the cap is also comparatively simple in structure, thus making it ideally suited for preliminary investigations of fine structure differentiation. Information on the root cap, at the time this study was undertaken, was limited mainly to the studies of Whaley and Mollenhauer and their associates (Mollenhauer, Whaley & Leech, 1960, 1961; Mollenhauer & Whaley, 1962, 1963; Whaley, 1959; Whaley, Kephart & Mollenhauer, 1959; Whaley, Mollenhauer & Leech, 1960a, b; Whaley, Mollenhauer & Kephart, 1959, 1962; Whaley & Mollenhauer, 1963), and a note by Kawamatu (1962) with little information on the changes in fine structure accompanying differentiation of the tissue

(Mollenhauer, Whaley & Leech, 1960; Mollenhauer & Whaley, 1962; Whaley, 1959). During preparation of this paper Juniper & Clowes (1964) gave an account at the Tenth International Botanical Congress, Edinburgh (August, 1964), describing similar work as outlined here but for a monocotyledon, maize. At the same congress, Whaley outlined work on cell division and cell differentiation in a linear growth system in which results from fine structure were also discussed (Whaley, Mollenhauer, Stallard & Kephart, 1964). At present, however, only abstracts of these accounts are available.

Material and Methods

The plants of *R. hirtus* (stock G. 2080, Botany Division, D.S.I.R., Lincoln, New Zealand) were grown in soil in pots in a growth cabinet. Clonal material and controlled conditions were used to minimize the possibility of developmental differences attributable to genetic and environmental variation. Root tips 1-2 mm long were selected from actively growing and dormant roots and fixed for two hours at room temperature (22°C) in 2 per cent aqueous potassium permanganate (Mollenhauer, 1959). This schedule was the most suitable of the various concentrations, period of fixation, and different fixatives initially tested. Since the present work was carried out, aldehyde fixation techniques have been adopted (Ledbetter & Porter, 1963) and

*This work was carried out in 1963 while the author was on study leave at the Electron Microscope Section, Physics and Engineering Laboratory (formerly the Dominion Physical Laboratory), Department of Scientific and Industrial Research, Lower Hutt, New Zealand.

these have modified certain interpretations and demonstrated new components. Nevertheless, permanganate fixation is still useful for a general survey of the gross ultrastructural changes accompanying differentiation, provided its limitations are fully realized. During fixation for thirty minutes the specimens were subjected under partial vacuum to facilitate the removal of air from intercellular spaces. Dehydration was carried out rapidly in acetone of increasing 10 per cent concentrations up to 90 per cent, followed by one hour in absolute acetone. Specimens were embedded in Araldite (Luft, 1961) in which casting resin M was replaced by type N. Infiltration commenced in a mixture comprising equal parts by volume of absolute acetone in Araldite, the mixture being left to gently agitate for one hour. Then half the mixture was removed and replaced by fresh Araldite, giving a final mixture containing about one part acetone in eight parts Araldite. The specimens were left to agitate in this mixture overnight, and finally embedded in fresh Araldite in gelatin capsules. Blocks were polymerized at 35°C for 24 hrs followed by a similar period at 60°C. Sections were cut on a Cooke & Perkins Ultramicrotome, using glass knives prepared according to that described by André (1962), to show interference colours from pale gold to silver. Grids were stained in lead hydroxide solution (Karnovsky, 1961) followed by a brief rinse in 1 per cent potassium hydroxide solution (Lever, 1960) and then viewed under a Philips EM 100B electron microscope.

General Organization of the Root Tip

Figure 3 is an optical median longitudinal section through the root tip of *R.*

hirtus. A root cap (RC) extends distal to the meristem and down the sides of the root for a short distance. Distally the cap is many cells thick but along the sides of the root it tapers off, and is finally replaced by the epidermis as the protective tissue of the tip.

The term meristem is used here in a general sense to designate the growing region of the root (Whaley, Mollenhauer & Kephart, 1959). Within the region of meristem, at the focus of the differentiating cap and root axis tissues, is a group of cells referred to as the promeristem. In a median longitudinal section these cells are distinguished from neighbouring parts of the meristem by being roughly round in shape; those of neighbouring regions are more rectangular, becoming progressively elongated further from the promeristem. Promeristem cells were selected as representing perhaps the least differentiated ones of the meristem from which comparisons of differentiating cells could be made.

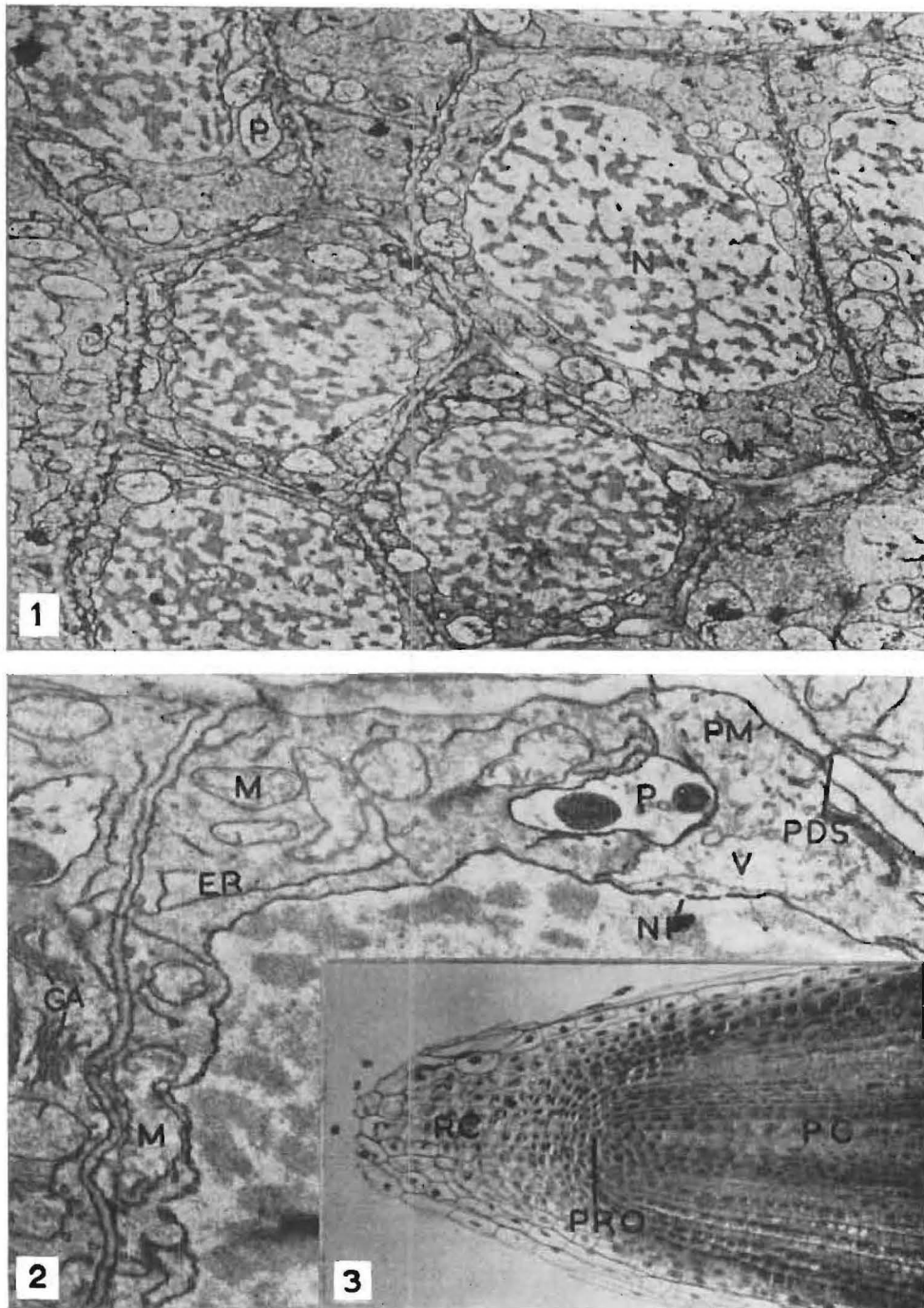
On the cap-side of the promeristem the cells are arranged roughly in files, and it is here that the cap initials and their immediate derivatives appear to be situated. From here to the tip of the root the cells become progressively enlarged reaching their final size within a few cells from the surface of the cap. Thereafter, they undergo little change and are finally sloughed from the surface as the root grows through the soil.

Fine Structure of Meristem Cells

Figure 1 shows a group of roughly round cells from the promeristem. Each cell possesses a prominent nucleus surrounded by abundant cytoplasm containing plastids, mitochondria, endoplasmic reticulum (Whaley, Mollenhauer &

—→

FIGS. 1-3 (*ER*, endoplasmic reticulum; *GA*, golgi apparatus; *M*, mitochondria; *N*, nucleus; *NP*, nuclear pore; *P*, plastid; *PC*, procambium; *PDS*, plasmodesmata; *PM*, plasma membrane; *PRO*, promeristem; *RC*, root cap; *V*, vacuole). Fig. 1. A group of cells from promeristem; the nuclei are large and contain reticulate electron-dense chromatin. $\times 4400$. Fig. 2. Portion of cytoplasm of a promeristem cell; the endoplasmic reticulum (*ER*) is comparatively short and scattered; the plastids contain small grains, presumably starch. $\times 16000$. Fig. 3. Optical section of root tip showing the position of promeristem (*PRO*), root cap (*RC*) and procambium (*PC*). $\times 116$.



FIGS. 1-3

Kephart, 1959), golgi apparatus (Whaley, Kephart & Mollenhauer, 1959; Whaley, Mollenhauer & Kephart, 1959) and embryonic or prevacuolar bodies (Whaley, Mollenhauer & Kephart, 1962) among the principal organelles. A plasma membrane surrounds each protoplast while plasmodesmata occur between the cells (Fig. 2). The organelles are described below in greater detail.

NUCLEUS — In a section the nucleus is round to oval occupying about half to two-thirds the visible portion of the cell. In interphase cells the chromatin is conspicuous appearing as an electron-dense mass surrounded by a less dense nucleoplasm (Fig. 1). Within the nucleus is the nucleolus which in these preparations usually shows as a round area with an electron density between that of the nucleoplasm and ground substance of the cytoplasm. Surrounding the nucleus is the nuclear envelope with a typical double membrane (Buvat, 1958; Setterfield, 1961; Whaley, Mollenhauer & Leech, 1960a, b) in which pores are present (Whaley, Mollenhauer & Leech, 1960a).

VACUOLES — In promeristem cells the vacuoles are usually small appearing as dense, often irregular, masses dispersed throughout the cytoplasm (Fig. 5). That these structures are in fact prevacuoles is shown when their growth is followed through successively differentiating cells from the meristem — as demonstrated elsewhere also by Manton (1962) and various other workers. Not all young vacuoles of promeristem cells have dense contents but may instead have an appearance similar to that of differentiating vacuoles (Fig. 2).

PLASTIDS — In meristematic cells the plastids are usually designated as

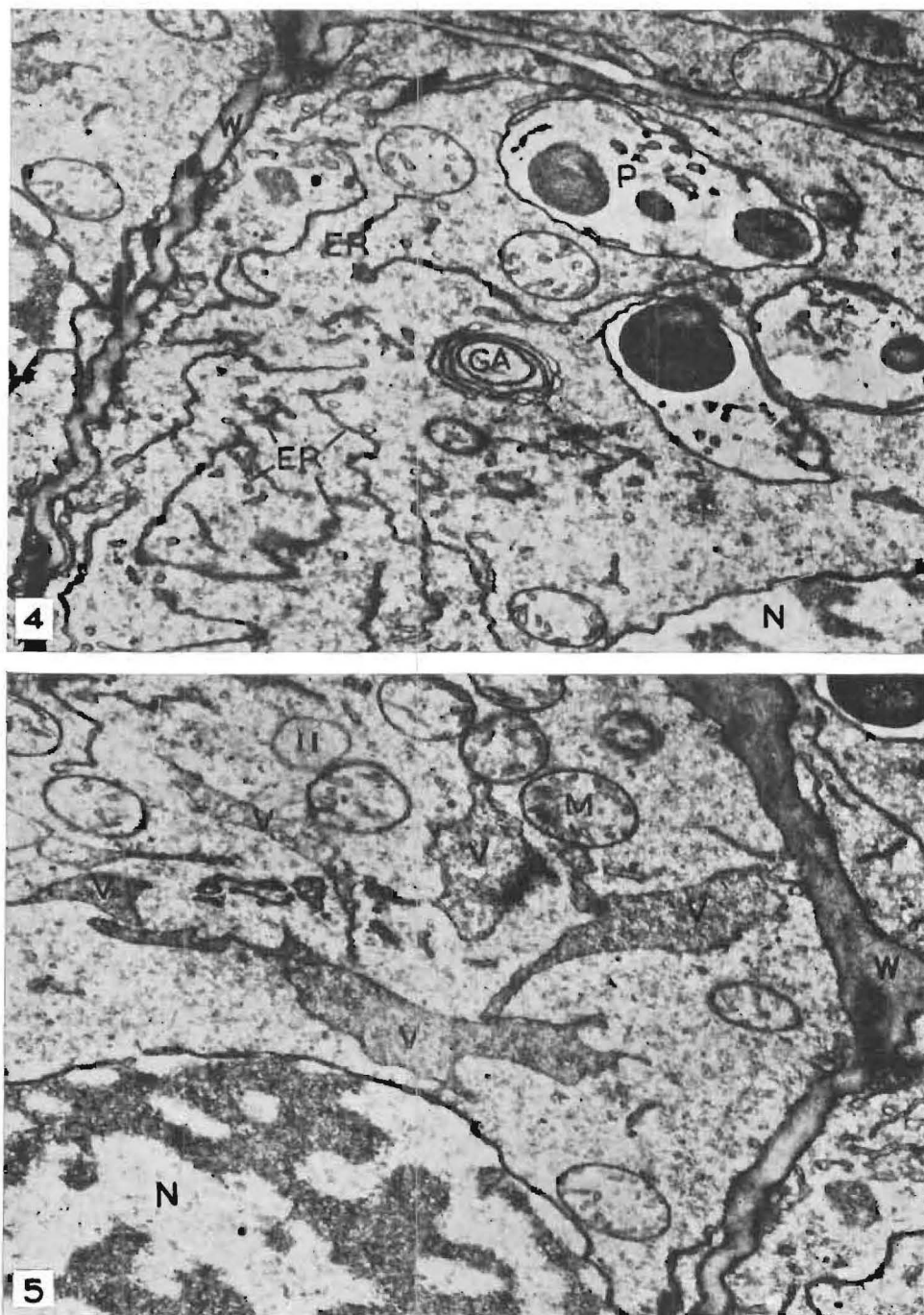
proplastids (Whaley, Mollenhauer & Leech, 1960b). Promeristem cells of the present material average about eight visible plastids per median section. They are oval to oblong, sometimes with irregular outlines (Figs. 1, 2). A double membrane surrounds each plastid enclosing an internal system of lamellae and vesicles of various configurations. The ground substance of the organelle is finely granular with an electron density slightly less than that of the cytoplasm and mitochondria (Figs. 1, 2, 4). At this stage small grains, presumably starch, are sometimes present within the plastids (Fig. 2) which are occasionally grouped near the nucleus. During differentiation of cap tissue the proplastids develop into amyloplasts (Whaley, Mollenhauer & Leech, 1960b), similar to those figured elsewhere (Figs. 4, 11) (Lance, 1958; Buttrose, 1960).

MITOCHONDRIA — Mitochondria commonly occur in promeristem cells (Figs. 1, 2) in all parts of the cytoplasm, sometimes associated with the endoplasmic reticulum. They are generally smaller than the plastids and vary from oval to oblong (Fig. 2) — sometimes with irregular outlines which is probably the result of improper fixation. The structure of each mitochondrion is basically similar to that described in other root meristems (Sitte, 1958; Buvat, 1958; Whaley, Mollenhauer & Leech, 1960b). In the current preparations most of the cristae were seen to be separated from the inner membrane of the organelle though at places they were in continuity.

GOLGI APPARATUS — In median sections of promeristem the average number of visible golgi structures per cell is about eight. Structurally each apparatus is similar to that described

—→

FIGS. 4, 5 (*ER*, endoplasmic reticulum; *GA*, golgi apparatus; *II*, unidentified cytoplasmic body; *M*, mitochondria; *N*, nucleus; *P*, plastid; *V*, vacuole; *W*, wall). Fig. 4. Cytoplasmic organization of a cell from the general region of root cap initials; compared with nearby promeristem cells (Figs. 1, 2) the *ER* has increased slightly in length and concentration of its profiles and vesicular derivatives of *ER* are also present; note the early increase in size of plastids over that of mitochondria. $\times 15000$. Fig. 5. Part of cytoplasm of an early differentiated root cap cell showing variation in the size of vacuoles; between the larger vacuoles note smaller vacuolar areas; at this stage the vacuoles are filled with material more electron-dense than the ground substance of cytoplasm. $\times 18000$.



FIGS. 4, 5

from other meristems (Mollenhauer, Whaley & Leech, 1961; Mollenhauer & Whaley, 1962; Sun, 1962; Whaley, Kephart & Mollenhauer, 1959; Whaley, Mollenhauer & Leech, 1960b). The number of cisternae varies between 4-7 which has been reported earlier (*see* Setterfield, 1961; Whaley, Mollenhauer & Leech, 1960b). They are double-membraned with an intra-membrane space similar to that of the endoplasmic reticulum. Occasionally wider spacings may occur, especially at the ends of the cisternae where small swellings are sometimes present (Fig. 2). Associated vesicles, derived from the golgi apparatus (Buvat, 1958; Whaley, Kephart & Mollenhauer, 1959; Whaley, Mollenhauer & Kephart, 1959; Whaley, Mollenhauer & Leech, 1960b), are also often present in the adjacent cytoplasm. As noted elsewhere (Whaley, Kephart & Mollenhauer, 1964), there appears to be some consistent relationship of the golgi apparatus with the endoplasmic reticulum.

ENDOPLASMIC RETICULUM (ER)—The ER (Figs. 1, 2, 4) is represented by profiles of a typical double membrane (Buvat, 1963; Porter & Machado 1960; Whaley, Mollenhauer & Leech, 1960a, b), and associated with these sometimes are small vesicles or tubules (Figs. 2, 4). The intra-membrane space of the ER is similar to that of the nuclear envelope, though wider spacings may often be observed depending on the plane of sectioning. At the ends of the membranes small swellings are occasionally present becoming more common in differentiating cells (Figs. 4, 8, 9). The ER is widely distributed throughout the cytoplasm, showing little apparent association with any particular organelle. In promeristem cells the ER is usually

sparsely represented compared with differentiating cells with short profiles seldom much longer than the larger plastids, and generally showing as discontinuous profiles except where they make contact with the nuclear envelope and occasionally with the plasma membrane (Fig. 2) (Porter & Machado, 1960; Whaley, Mollenhauer & Leech, 1960a). Nuclear connections in *R. hirtus* are more common in recently divided than in interphase cells (Porter & Machado, 1960).

ORGANELLES OF UNCERTAIN AFFINITY—In some promeristem cells one occasionally sees round to oval bodies bounded by what appear to be single membranes. Such bodies, however, are usually common in differentiating cells (Fig. 8). Their contents are finely granular with an electron density slightly more than that of the ground substance of the cytoplasm. In general form these organelles appear similar to those labelled as 'unidentified cytoplasmic inclusion bodies' by Whaley *et al.* (1960b) and Mollenhauer *et al.* (1961), or the structures figured by Frey-Wyssling *et al.* (1963) as spherosomes.

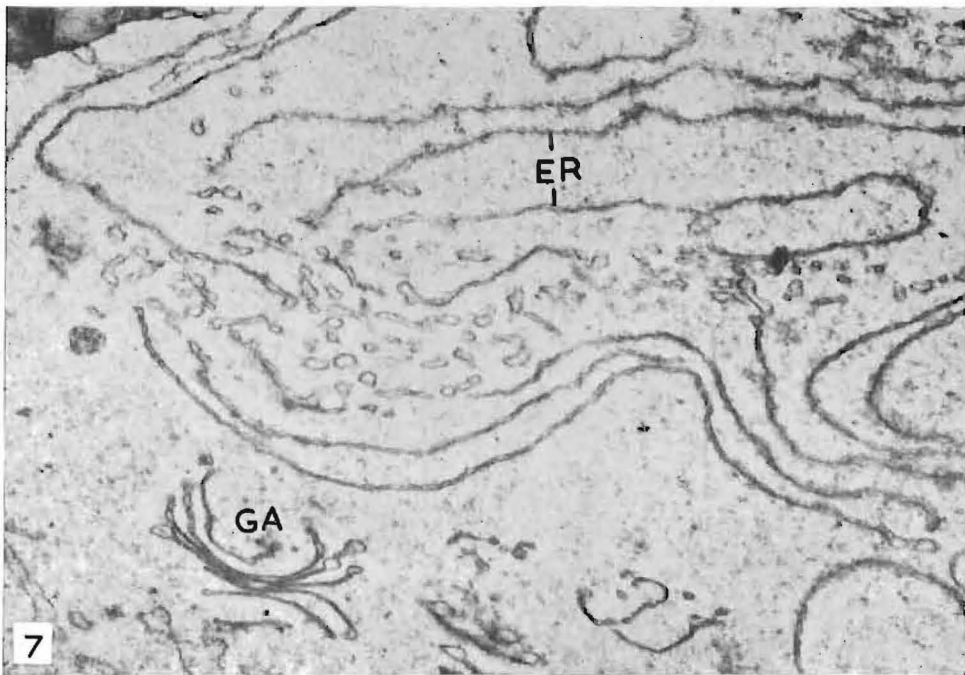
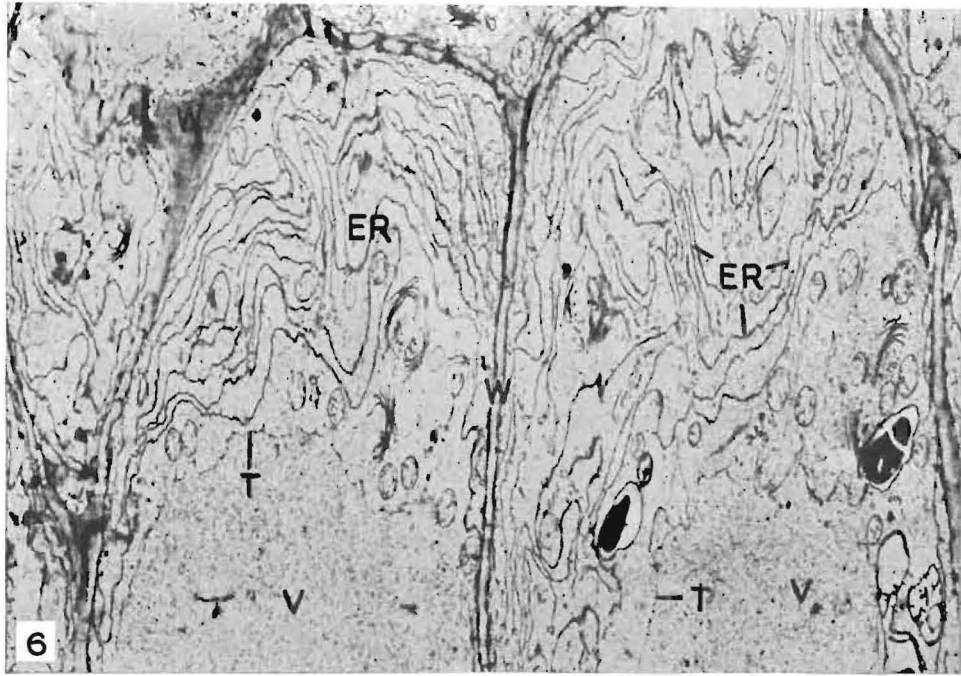
Changes in the Fine Structure of Differentiating Root Cap Cells

The following observations are based on cells from all parts of the root cap with particular reference to those in a broad zone from the promeristem to the tip of the root.

NUCLEUS—The nucleus of actively differentiating root cap cells increases in size, reaching its maximum some time prior to final enlargement of the cell. Such nuclei have been measured up to one and a half times that of nuclei visible

→

FIGS 6, 7 (ER, endoplasmic reticulum; GA, golgi apparatus; T, tonoplast; V, vacuole). Fig. 6. Portion of two cap cells from about midway between the promeristem and tip of root cap, these cells are almost fully enlarged and show a massing of ER within the cytoplasm; the contents of vacuoles (V) are about the same electron density as that of cytoplasm and are therefore less readily distinguishable, a distinct tonoplast (T) delimits the vacuole from cytoplasm and the outline of tonoplast is mainly entire but fine projections into the cytoplasm are present at places. $\times 6300$. Fig. 7. Portion of a root cap cell (after enlargement) showing the character of ER; some of the cisternoid profiles appear to have broken up into smaller derivatives; a few vesicles are visible at the ends of cisternae of nearby golgi apparatus. $\times 17000$.



FIGS. 6, 7

in promeristem cells. In less actively differentiating root tips the nucleus also increases during differentiation of the cap, but here the increase is of a smaller degree. The nucleus usually fails to increase in a linear manner from the promeristem through the cap, but in the region of the cap initials it is often smaller as a result of recent division. After enlargement of the cell it decreases (Figs. 13, 14) and eventually disappears on the death of the protoplast in outermost cap cells. In moribund cells (Fig. 14) the nucleus often comes to lie against the wall surrounded by a mass of shrinking cytoplasm in which various organelles are still visible. At the stage shown in Fig. 13 a thin peripheral mass of cytoplasm lines the cell, but later this disappears leaving only the nucleus and its immediate cytoplasm (Fig. 14). Even at this stage, however, the nucleus retains its identity with chromatin and nuclear envelope. Besides changes in size alterations also occur in the shape of the nucleus and, as differentiation proceeds, its outline becomes more oval to elliptic. Towards the end of differentiation it usually becomes irregular (Figs. 13, 14).

VACUOLES — Concerning the growth of the vacuole(s) in tissue of the root cap, the prevacuoles first become noticeably larger in the region of the cap initials. Here young vacuoles are often grouped close together (Fig. 5) and sometimes interconnected by narrow 'channels'. Usually in early stages of growth the vacuoles have irregular outlines with fine projections extending into the adjacent cytoplasm. Further out in the root cap, however, they enlarge and become entire, though occasionally fine projections can still be seen. By the time the root cap cells are fully enlarged,

the vacuole(s) occupies a prominent part of the protoplast. Thereafter it continues to enlarge and ultimately restricts the cytoplasm to a thin peripheral lining in outermost cap cells. Eventually, the cytoplasm disappears and the vacuole becomes replaced by the lumen of the cell.

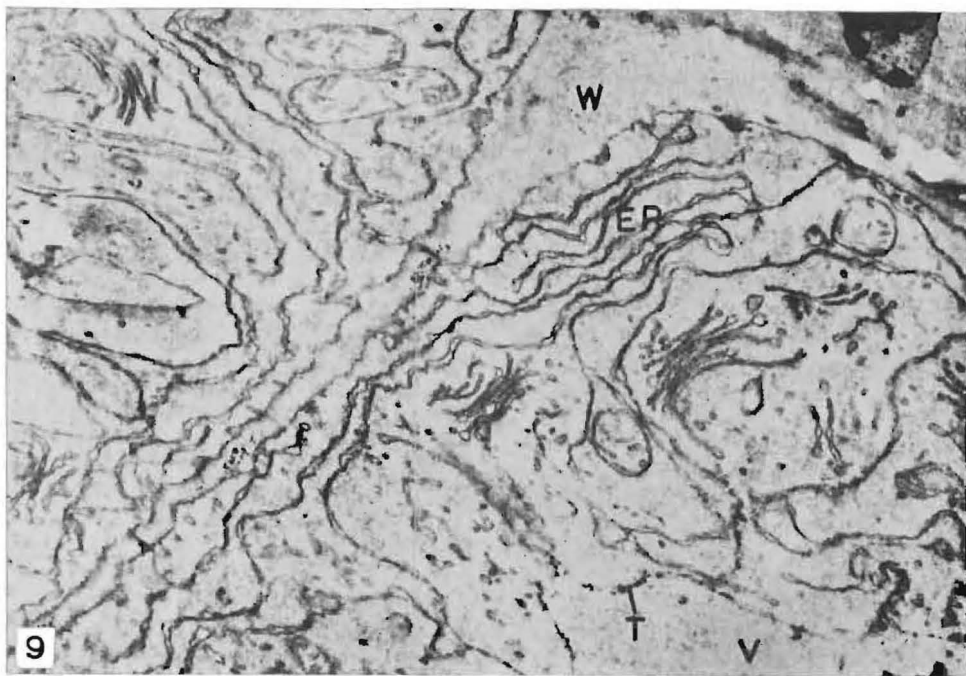
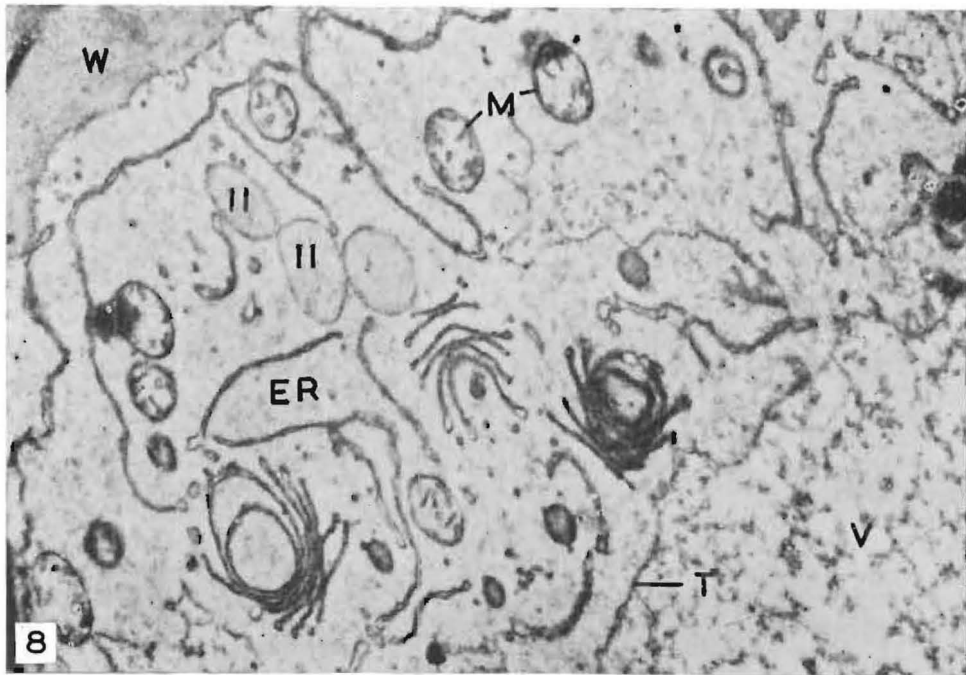
In other tissues several vacuolar areas may enlarge considerably before fusion to form the main vacuole (unpublished observations), but it may be noted that in the cap the main vacuole initially develops usually from only a few prevacuolar areas and early assumes a prominent part of the cell. However, small vacuolar bodies apparently continue to arise within the cytoplasm and these fuse with the main vacuole during the course of its enlargement, even towards the later stages of differentiation when the cytoplasm becomes restricted to a peripheral lining.

During differentiation changes also occur in the appearance of the vacuolar contents. The young vacuoles of promeristem cells and those of adjacent parts of the cap meristem are usually more electron-dense than the ground substance of the cytoplasm, becoming progressively less dense (Whaley, Mollenhauer & Leech, 1960b) outwards through the cap. At first the contents are finely granular in appearance, but towards the final stages of differentiation they become coarsely granular (Figs. 10, 14). The other small vacuolar bodies which develop during differentiation normally possess denser contents than the main vacuole, which indicates their independence at this stage.

PLASTIDS — Plastids undergo marked changes on differentiation, the most conspicuous being an increase in size and the accumulation of storage products

—→

FIGS. 8, 9 (*ER*, endoplasmic reticulum; *II*, unidentified cytoplasmic body; *M*, mitochondria; *T*, tonoplast; *V*, vacuole; *W*, wall). Fig. 8. The cytoplasm of an enlarged root cap cell from a root tip of slow growth; compared with similarly situated cells of actively growing root caps the *ER* is poorly represented (*cf.* Fig. 6); other organelles are also more scattered and smaller in size. $\times 19000$. Fig. 9. Appearance of cytoplasm after cell enlargement when the vacuole encroaches increasingly on the cytoplasm; in the lowermost cell long *ER* profiles are 'compacted' near the wall while nearer the vacuole the profiles are shorter. $\times 18000$.



FIGS. 8, 9

within the organelle (Figs. 4, 11). They become steadily larger from the meristem out through the cap to where the cells reach maximum size; over this range they increase as much as eight times the size of plastids of promeristem cells. After cell enlargement the plastids become reduced, especially in the outermost root cap cells. Discrete plastids containing starch grains are, nevertheless, often visible in cells which are moribund (Figs. 13, 14).

As the plastids enlarge starch accumulates within them. Starch grains are occasionally present in proplastids of promeristem cells but here they are small and few per organelle (Fig. 2). On differentiation the granules increase in size and number until they come to occupy almost the entire organelle with few of the original internal membrane system still being visible (Fig. 11). Not all plastids of differentiated cap cells become amyloplasts; a few may remain undifferentiated as proplastids, as also noted in companion cells by Esau (1963).

Although plastids appear pleomorphic and are probably subject to differences in distribution in the cell depending on conditions within the protoplast, counts from many sections over several root tips suggest that they become numerous on differentiation; sometimes as many as a third more on the average are present in fully enlarged cells compared with those of the promeristem. Besides a size and probable number increase, changes also occur in the form of plastids. Generally on differentiation they become more oblong in outline but in some instances irregular forms are noted, one type being occasionally noted where the plastid was 'cup-shaped'. Figure 11 shows a group of plastids of this form sectioned differently; one is cut longitudinally and shows some ER profiles and a mitochondrion within the hollow of the organelle; the others are sectioned transversely and appear ring-shaped. They surround some cytoplasm which contains mitochondria.

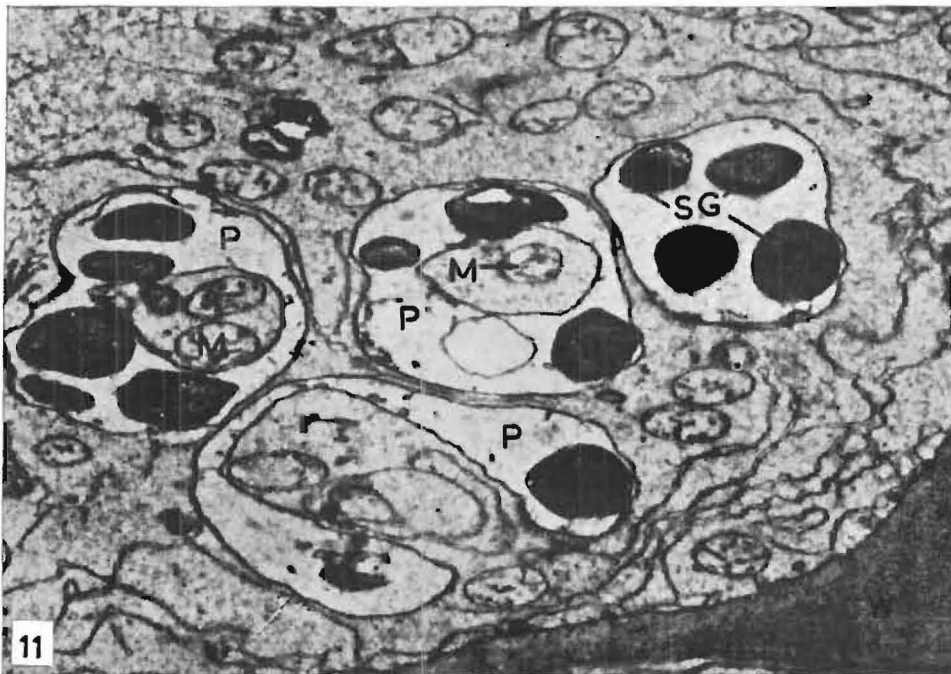
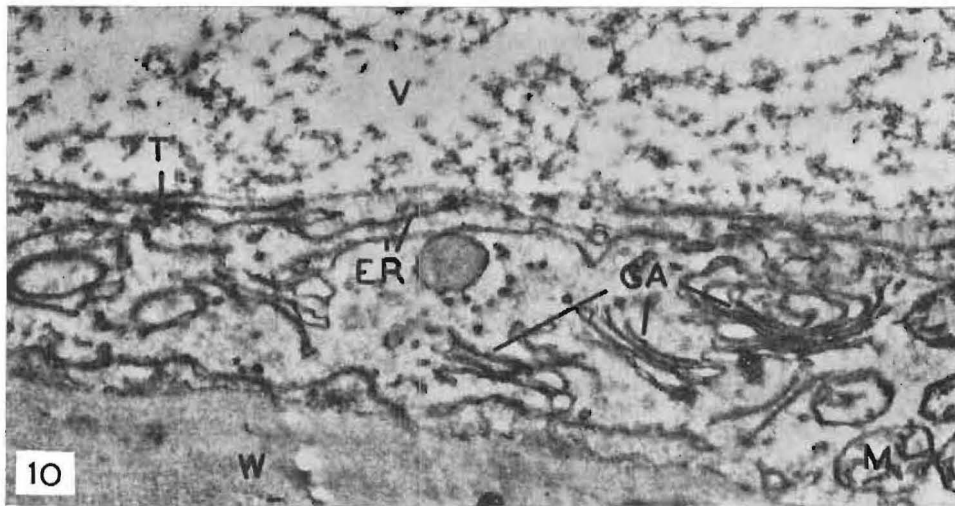
Regarding association of plastids with other organelles, a grouping towards the nucleus is sometimes discerned prior to the cytoplasm becoming reduced through

vacuolation. An association with the nucleus, however, is often more clearly seen in the procambium than in the cap (unpublished observations).

MITOCHONDRIA — Observations from many root tips indicate that mitochondria become larger and numerous on differentiation, increasing in size to about twice that of mitochondria from promeristem cells. After cell enlargement the mitochondria become smaller and eventually disappear on death of the protoplast. The shape of mitochondria, in general, alters little on differentiation, the round to oval forms predominating in interphase cells while internally there is only a moderate tendency for the cristae to become numerous. During final stages of differentiation mitochondria often assume an irregular 'shrunken' appearance with a more disorganized arrangement of cristae (Figs. 10, 12).

GOLGI APPARATUS — Comparisons of micrographs taken at the same magnification indicate a small increase in the size of golgi structures in cells outward through the cap. Counts also suggest an increase in the number of organelles per cell on differentiation. Structurally, however, the average number of cisternae remains much the same, but there is a tendency for a more strongly curved arrangement of them as seen in transection through the lamella stacks (Figs. 4, 7, 8, 10, 12). Towards the later stages of differentiation golgi vesicles also become more numerous and enlarged, particularly in outer root cap cells (Figs. 9, 12). Some differences in orientation of golgi structures are also noted on differentiation, the organelles becoming more randomly arranged compared with those in promeristem cells where they usually lie roughly parallel to the wall.

ENDOPLASMIC RETICULUM — In actively differentiating root cap tissue ER profiles become longer and more concentrated within the cytoplasm, the increase first becoming noticeable in cells in the region of the root cap initials (Fig. 4). From here out through the cap the ER increases progressively reaching its greatest elaboration in cells which have almost completed enlargement. In such cells long winding profiles occur 'folded'



FIGS. 10, 11 (*ER*, endoplasmic reticulum; *GA*, golgi apparatus; *M*, mitochondria; *P*, plastid; *SG*, starch grain; *T*, tonoplast; *V*, vacuole; *W*, wall). Fig. 10. Cytoplasmic lining of a highly vacuolated cell near the surface of root cap, the organelles show some changes on approaching senescence of protoplast; the mitochondria (bottom right) are irregular in outline and internally their cristae show a less ordered arrangement; the *ER*, however, is still represented by longish profiles and the tonoplast is also intact. $\times 22500$. Fig. 11. A group of plastids from an enlarged cap cell with abundant cytoplasm, the plastids contain well-developed starch grains (black), three of the plastids shown have a 'cup-shaped' form and each encloses a small portion of cytoplasm containing various organelles. $\times 15000$.

within the cytoplasm (Fig. 6), sometimes more concentrated in certain parts of the cytoplasm. In less actively growing root tips the ER fails to show the same degree of elaboration, as observed by comparing Fig. 8 with that of a similarly situated cell (Fig. 6) from an actively differentiating cap.

After cell enlargement the ER becomes progressively reduced in amount and in the visible length of its profiles. Frequently reduction first takes place near the vacuole and nucleus leaving the longer profiles more concentrated near the wall (Figs. 9-11). During reduction it is also noted that some profiles apparently break down into tubules or vesicles (Fig. 7), but seldom to the extent that the cisternoid form of the ER is no longer predominant (Figs. 9, 10).

Discussion

In *R. hirtus* every component of the cell undergoes some change on differentiation of root cap tissue compared with cells from the promeristem. Similar general conclusions have recently been reached independently by Juniper & Clowes (1964) for the root tip of *Zea*.

In the present material most components increase during the phase of growth of the cell, this being shown especially by plastids and the ER and to a lesser degree by mitochondria and the golgi apparatus. It is perhaps significant in terms of cellular metabolism that the increase occurs during growth of the cell with little increase after cell enlargement.

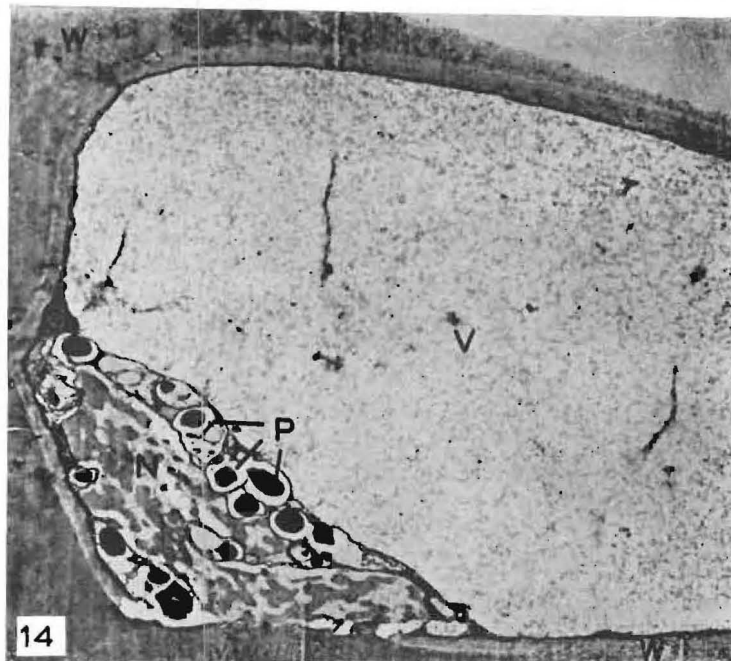
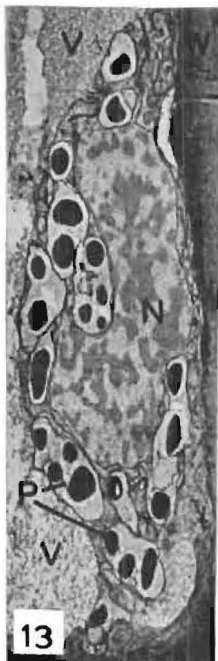
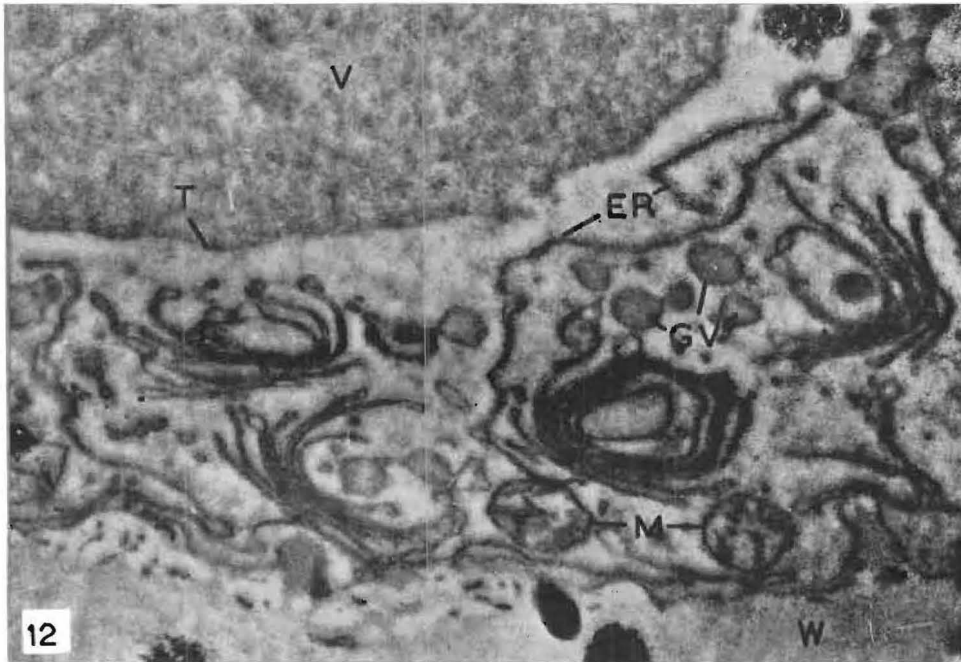
That the elaboration is an expression of the amount of activity within the cell (Buvat, 1963), is shown also by the smaller degree of change in the organelles in root tips where growth is slow.

In general, the organelles become structurally more developed on differentiation of the root cap, this being shown particularly in the massing of the ER, accumulation of starch within plastids, and tendency of the golgi apparatus to proliferate more vesicles. Besides an elaboration in size and structure, preliminary counts also suggest that most organelles become numerous on differentiation of the tissue.

The increase in the ER is perhaps the most striking change occurring in the cap apart from that of vacuolation. In the present material the increase is progressively outward through the cap to where the cells reach full enlargement. Such an increase is in accord with that reported for various tissues by Whaley *et al.* (1962), Esau (1963, 1964), Buvat (1963) and Setterfield (1961). In an earlier paper, however, Buvat (1961) reported little change during vacuolation of root parenchyma of *Triticum*. Mercer & Rathgeber (1962) have also observed a smaller elaboration in parenchyma compared with nectary cells in *Abutilon*. The various forms assumed by the ER in differentiated tissues suggest that the membranes are of some importance in differentiation as a means of increasing the internal metabolic surface within the cell (Palade, 1956; Whaley, Mollenhauer & Kephart, 1959). Some have even suggested an association with the

—→

Figs. 12-14 (ER, endoplasmic reticulum; GV, golgi vesicles; M, mitochondria; N, nucleus; P, plastid; T, tonoplast; V, vacuole; W, wall). Fig. 12. Cytoplasm of an outer root cap cell showing the form of golgi apparatus, in its vicinity occur several round electron-dense bodies apparently derived from the ends of cisternae; at this stage all the organelles are reduced compared with those of fully enlarged cells. $\times 30000$. Fig. 13. A cell from near the surface of root cap showing the nucleus and some cytoplasm lying against the wall, the nucleus is greatly reduced and irregular in outline but the nuclear envelope is still intact surrounding some chromatin; in this cell a thin peripheral mass of cytoplasm (not shown) lined the rest of the cell. $\times 10000$. Fig. 14. A surface cell from the tip of root cap showing the condition of cytoplasm towards the end of differentiation, the nucleus and its surrounding cytoplasm have shrunk into one corner of the cell; around the rest of the cell the peripheral cytoplasmic lining has disappeared leaving only a residual film against the wall; various organelles can still be discerned in the cytoplasm surrounding the nucleus. $\times 12500$.



Figs. 12-14

growth of the cell wall (Porter & Machado 1960; Juniper & Clowes, 1964).

Preliminary counts and measurements in *R. hirtus* indicate a small increase in size and number of the golgi apparatus during enlargement of the cell. An increase in number is referred to by Juniper & Clowes (1964) in root tips of *Zea* where it was found that the golgi apparatus increased with increased rate of division and in later stages of differentiation. An increase is also inferred by Setterfield (1961) in differentiating coleoptile cells of oat. The golgi apparatus also shows some changes in organization on differentiation of cap tissue in *R. hirtus*. The cisternae become generally more crescentic in shape and more vesicles are associated with the ends of the cisternae, especially in outer cap cells (Fig. 12). Mollenhauer *et al.* (1961) have described large vesicles in outermost cap cells of maize produced by hypertrophied portions of the golgi cisternae which later accumulate against the cell wall. Vesicles of an apparent similar nature have also been noted in outer cap cells of present material (Fig. 12), but so far they have not been observed collecting near the wall. Mollenhauer & Whaley (1962) have further considered the function of the golgi apparatus in outer root cap cells and state that the accumulated material against the wall is apparently incorporated into a greatly increased volume of wall and becomes part of the slime surrounding the root cap cells. The thickened outer portion of the wall in Fig. 14 may represent such a slimy cover. That the golgi vesicles are vehicles of transport of secretory products is supported by Schnepf (1964) for carnivorous plants and in the mucilage ducts of *Laminaria*.

Mitochondria are reported as undergoing various changes on differentiation. Buvat (1958, 1963), for example, found that they remained relatively undifferentiated in starch storage cells as compared with metabolically active tissues. In mature sieve elements a poorly developed internal organization is also reported by Esau & Cheadle (1962a). In root cap tissue of *R. hirtus* there is only a moderate tendency for mitochondria to become

larger, more numerous and internally elaborate on differentiation as also observed by Setterfield (1961) in coleoptile tissue. Similarly, in root cap tissue of *Mentha citrata* (unpublished observations) and maize (Juniper & Clowes, 1964), photosynthetic tissue of *Elodea* (Buvat, 1958), phloem parenchyma and companion cells of *Cucurbita* (Esau & Cheadle, 1962a), mitochondria have been shown to become variously elaborate on differentiation. According to Juniper & Clowes (1964) there may be a correlation between the number of organelles per unit volume of cytoplasm and the amount of cristae within the organelle. Esau (1963, 1964) considered that the elaboration appeared to vary depending on the metabolic state of the cell. This is supported by the present observations where the mitochondria are generally more elaborate in actively differentiating caps than in root tips with lesser growth.

The nucleus has been shown to undergo various changes during cell development (Lyndon, 1964). In cap tissue of *R. hirtus* it becomes more elongated in outline, and in some instances two nucleolar areas may also be discerned. Such general changes as these are not confined to cap cells but occur in other tissues, particularly in the procambium (unpublished observations). Towards the end of differentiation the nucleus becomes reduced in size and somewhat irregular in outline which has been noted in maize (Whaley, Mollenhauer & Leech, 1960a) and other tissues (Esau, 1963).

In the present material vacuolation is a striking feature of differentiating root cap tissue. The process begins early with enlargement of the vacuoles taking place over a comparatively short series of cells from the meristem to near the surface of the cap. In this respect cap cells resemble those from the mid-cortex but differ from those of the procambium, where the process spans several millimeters from the meristem region (unpublished observations). In different root tips the state of vacuolation can also vary slightly outwards through the cap, apparently depending on the state of differentiation within the particular root cap. Dormant root tip cells, for

example, have large vacuoles nearer the meristem than is the case with actively growing caps where the final delimitation of cytoplasm is in the outermost cap cells far removed from the meristem. Throughout the course of root cap differentiation the tonoplast is persistent and of a similar thickness (Marinos, 1963), disappearing only in the final stage of senescence of the cytoplasm. Thus, it differs from the behaviour in sieve elements of *Cucurbita* as described by Esau & Cheadle (1962b), where the tonoplast became more or less disorganized after maturity of the cell. Regarding the origin of the vacuole in *R. hirtus*, there is no conclusive evidence to support the suggestion that they arise through some modification of the ER (Buvat, 1962, 1963; Buvat & Mousseau, 1960; Poux, 1961, 1962). However, it seems that they are independent and of separate origin as suggested by other workers (Manton, 1962; Mühlethaler, 1960). According to Marinos (1963) the vacuole arises through enlargement of whole cisternal elements of the golgi apparatus. Current sections of *R. hirtus*, however, do not show this.

Summary

The fine structure of promeristem and differentiating root cap cells in *Ranun-*

culus hirtus is described. The promeristem cells possess organelles typical of the root meristem of other higher plants. In actively differentiating root tips every organelle undergoes some change during growth of the cell. Excluding the vacuole, which comes to occupy the greater part of the cell towards the end of differentiation, the most noticeable increase is that shown by the plastids and the endoplasmic reticulum. Preliminary counts also suggest that most organelles increase in number during growth of the cell. In dormant and less actively growing tips the changes shown by the organelles are much less. After cell enlargement the organelles become reduced and finally disappear on the death of the cell.

I am grateful to the Department of Scientific and Industrial Research, New Zealand, for the opportunity to study electron microscopy at the Physics and Engineering Laboratory, Lower Hutt. In particular, I wish to thank Mr W. S. Bertaud and Mr D. M. Hall for their instruction and encouragement in the arts of electron microscopy, and Mr L. Adamson for preparation of the electron micrographs. I am also indebted to Professor W. R. Philipson of this Department for arranging for this work to be undertaken.

Literature Cited

- ANDRĚ, J. 1962. Presentation d'un appareil destiné à préparer les copeaux de verre. *J. Ultrastruct. Res.* **6**: 437-448.
- BUTTROSE, M. S. 1960. Submicroscopic development and structure of starch granules in cereal endosperms. *J. Ultrastruct. Res.* **4**: 231-257.
- BUVAT, R. 1958. Recherches sur les infrastructures du cytoplasme, dans les cellules du méristème apical, des ébauches foliaires et des feuilles développées d'*Elodea canadensis*. *Annls Sci. nat. Bot.* **19**: 121-161.
- 1961. Le réticulum endoplasmique des cellules végétales. *Ber. dt. bot. Ges.* **74**: 261-267.
- 1962. L'Origine et le développement des vacuoles, des cellules végétales. *In* BREESE S. S. (ed.) Fifth Int. Congr. for Electron Microscopy, Philadelphia. Vol. 2: W-1. New York.
- BUVAT, R. 1963. Electron microscopy of plant protoplasm. *In* BOURNE, G. H. & DANIELLI, J. F. (eds) *Int. Rev. Cytol.* Vol. 14: 41-155. New York.
- & MOUSSEAU, A. 1960. Origine et Evolution du Système vacuolaire dans la Racine de *Triticum vulgare* relation avec l'ergastoplasme. *C. R. Acad. Sci., Paris* **251**: 3051-3053.
- ESAU, K. 1963. Ultrastructure of differentiated cells of higher plants. *Am. J. Bot.* **50**: 495-506.
- 1964. Aspects of differentiation at the ultrastructural level. *Abstr.* 489, 10th Int. Bot. Congr., Edinburgh.
- & CHEADLE, V. I. 1962a. Mitochondria in the phloem of *Cucurbita*. *Bot. Gaz.* **124**: 79-85

- ESAU, K. & CHEADLE, V. I. 1962b. An evaluation of studies on ultrastructure of tonoplast in sieve elements. Proc. natn. Acad. Sci. U.S.A. **48**: 1-8.
- FREY-WYSSLING, A., GRIESHABER, E. & MÜHLETHALER, K. 1963. Origin of spherosomes in plant cells. J. Ultrastruct. Res. **8**: 506-516.
- JUNIPER, B. E. & CLOWES, F. A. L. 1964. The fine structure of the quiescent centre and neighbouring tissues in root meristems. Abstr. 491, 10th Int. Bot. Congr., Edinburgh.
- KARNOVSKY, J. 1961. Simple methods for staining with lead of high Ph in electron microscopy. J. biophys. biochem. Cytol. **11**: 729-732.
- KAWAMATU, S. 1962. Electronmicroscopy of the root-cap of *Azolla imbricata* Nakai. Bot. Mag., Tokyo **75**: 114.
- LANCE, A. 1958. Infrastructure des cellules du méristème apical et jeunes ébauches foliaires de *Chrysanthemum segetum* L. (Composées). Annls Sci. nat. Bot. **19**: 167-207.
- LEDBETTER, M. C. & PORTER, K. R. 1963. A microtubule in plant cell fine structure. J. Cell Biol. **19**: 239-250.
- LEVER, J. D. 1960. A method of staining sectioned tissue with lead for electron microscopy. Nature, Lond. **186**: 810-811.
- LUFT, J. H. 1961. Improvements in epoxy resin embedding methods. J. biophys. biochem. Cytol. **9**: 409-414.
- LYNDON, R. F. 1964. The nucleus in cell development. Abstr. 464, 10th Int. Bot. Congr., Edinburgh.
- MANTON, I. 1962. Observations on stellate vacuoles in the meristem of *Anthoceros*. J. exp. Bot. **13**: 161-167.
- MARINOS, N. G. 1963. Vacuolation in plant cells. J. Ultrastruct. Res. **9**: 177-185.
- MERCER, F. V. & RATHGEBER, N. 1962. Nectar secretion and cell membranes. In BREESE, S. S. (ed.) Fifth Int. Congr. for Electron Microscopy, Philadelphia. Vol. 2: WW-11. New York.
- MOLLENHAUER, H. H. 1959. Permanganate fixation of plant cells. J. biophys. biochem. Cytol. **6**: 431-436.
- & WHALEY, W. G. 1962. A secretory function of the golgi apparatus in certain plant cells. In BREESE, S. S. (ed.) Fifth Int. Congr. for Electron Microscopy, Philadelphia. Vol. 2: YY-3. New York.
- & — 1963. An observation on the functioning of the golgi-apparatus. Cell Biol. **17**: 222-225.
- , — & LEECH, J. H. 1960. Cell ultrastructure responses to mechanical injury—A preliminary report. J. Ultrastruct. Res. **4**: 473-481.
- , — & — 1961. A function of the golgi apparatus in outer root cap cells. J. Ultrastruct. Res. **5**: 193-200.
- MÜHLETHALER, K. 1960. Die Entstehung des Vacuolensystems in Pflanzenzellen. In BARGMANN, W., PETERS, D. & WOLPERS, C. (eds) Fourth Int. Congr. for Electron Microscopy, Berlin. Vol. 2: 491-494. Berlin.
- PALADE, G. E. 1956. The endoplasmic reticulum. J. biophys. biochem. Cytol. **2**: 85-98.
- PORTER, K. R. & MACHADO, R. D. 1960. Studies on the endoplasmic reticulum. IV. Its form and distribution during mitosis in cells of onion root tips. J. biophys. biochem. Cytol. **7**: 167-180.
- POUX, N. 1961. Observations sur les rapports des vacuoles avec les structures réticulaires du cytoplasme. C.R. Acad. Sci., Paris **235**: 2395-2397.
- 1962. Nouvelles observations sur la nature et l'origine de la membrane vacuolaire des cellules végétales. J. Microscope **1**: 55-66.
- SCHNEFF, E. 1964. Relations between ultrastructure and secretion in gland cells of plants. Abstr. 202, 10th Int. Bot. Congr., Edinburgh.
- SETTERFIELD, G. 1961. Structure and composition of plant-cell organelles in relation to growth and development. Can. J. Bot. **39**: 469-489.
- SITTE, P. 1958. Die Ultrastruktur von Wurzelmeristemzellen der Erbse (*Pisum sativum*). Protoplasma **49**: 447-522.
- SUN, C. N. 1962. Fine structure of root cells of *Phaseolus vulgaris*. I. Structure of the meristematic cell. Cytologia **27**: 204-211.
- WHALEY, W. G. 1959. Dynamics of cell ultrastructure in development and growth. Science **130**: 1425-1426.
- , KEPHART, J. E. & MOLLENHAUER, H. H. 1959. Developmental changes in the golgi apparatus of maize root cells. Am. J. Bot. **46**: 743-751.
- , — & — 1964. The form and function of the golgi apparatus in the plant cell. Abstr. 466, 10th Int. Bot. Congr., Edinburgh.
- & MOLLENHAUER, H. H. 1963. The golgi apparatus and cell plate formation—A postulate. Cell Biol. **17**: 216-221.
- , — & KEPHART, J. E. 1959. The endoplasmic reticulum and the golgi structures in maize root cells. J. biophys. biochem. Cytol. **5**: 501-506.
- , — & — 1962. Developmental changes in cytoplasmic organelles. In BREESE, S. S. (ed.) Fifth Int. Congr. for Electron Microscopy, Philadelphia. Vol. 2: W-12. New York.
- , — & LEECH, J. H. 1960a. Some observations on the nuclear envelope. J. biophys. biochem. Cytol. **8**: 233-245.
- , — & — 1960b. The ultrastructure of the meristematic cell. Am. J. Bot. **47**: 401-449.
- , —, STALLARD, W. A. & KEPHART, J. E. 1964. Cell divisions and cell differentiation in a linear growth system. Abstr. 490, 10th Int. Bot. Congr., Edinburgh.

APPENDIX II

The following negatives were used to prepare the illustrations. Plates deposited in the Electron Microscope Laboratory, Department of Botany, University of Canterbury are denoted by a numerical and alphabetical listing e.g. 629/b. Negatives housed in the Electron Microscope Unit, Physics and Engineering Laboratory, D.S.I.R., Lower Hutt, are catalogued by a numerical system only e.g. 2223/18.

| <u>Figure</u> | <u>Negative No.</u> | <u>Figure</u> | <u>Negative No.</u> | <u>Figure</u> | <u>Negative No.</u> |
|---------------|---------------------|---------------|---------------------|---------------|---------------------|
| 1 | 627/2 | 41 | 2229/7 | 80 | 311/b |
| 2 | 624/28 | 42 | 2229/8 | 81 | 316/a |
| 4 | 624/29 | 43 | 523/b | 82 | 1126/a |
| 5 | 624/26 | 44 | 708/b | 83 | 429/a |
| 6 | 626/29 | 45 | 866/b | 84 | 436/b |
| 7 | 624/25 | 46 | 807/b | 85 | 433/a |
| 8 | 626/27 | 47 | 813/b | 86 | 280/b |
| 9 | 626/16 | 48 | 852/b | 87 | 478/a |
| 10 | 626/3 | 49 | 892/a | 88 | 474/a |
| 11 | 626/30 | 50 | 2231/12 | 89 | 440/a |
| 12 | 627/29 | 51 | 243/a | 90 | 420/a |
| 13 | 621/4 | 52 | 673/b | 91 | 440/b |
| 14 | 621/3 | 53 | 251/b | 92 | 444/a |
| 15 | 2221/8 | 54 | 242/b | 93 | 446/b |
| 16 | 2220/23 | 55 | 687/b | 94 | 451/a |
| 17 | 593/a | 56 | 723/a | 95 | 365/b |
| 18 | 735/b | 57 | 247/a | 96 | 368/a |
| 19 | 2222/13 | 58 | 668/b | 97 | 369/a |
| 20 | 2232/24 | 59 | 674/b | 98 | 316/b |
| 21 | 589/b | 60 | 664/a | 99 | 326/b |
| 22 | 588/a | 61 | 720/a | 100 | 325/a |
| 23 | 2223/15 | 62 | 721/a | 101 | 341/b |
| 24 | 2223/16 | 63 | 715/a | 102 | 350/b |
| 25 | 2228/13 | 64 | 677/a | 103 | 358/a |
| 26 | 2229/24 | 65 | 776/a | 104 | 357/b |
| 27 | 597/b | 66 | 815/b | 105 | 365/a |
| 28 | 2224/12 | 67 | 773/a | 106 | 360/a |
| 29 | 633/b | 68 | 772/a | 107 | 364/a |
| 30 | 730/b | 69 | 766/a | 108 | 255/a |
| 31 | 2232/19 | 70 | 809/a | 109 | 211/a |
| 32 | 2230/29 | 71 | 2228/12 | 110 | 1252/b |
| 33 | 2229/22 | 72 | 2231/7 | 111 | 1260/a |
| 34 | 2228/1 | 73 | 2230/25 | 112 | 1236/a |
| 35 | 2223/26 | 74 | 629/b | 113 | 1266/a |
| 36 | 2229/4 | 75 | 2231/4 | 114 | 1144/a |
| 37 | 2229/10 | 76 | 2232/3 | 115 | 1145/a |
| 38 | 548/b | 77 | 2230/24 | 116 | 1146/b |
| 39 | 544/b | 78 | 731/a | 117 | 1147/b |
| 40 | 576/a | 79 | 310/a | 118 | 1156/a |

| <u>Figure</u> | <u>Negative No.</u> | <u>Figure</u> | <u>Negative No.</u> | <u>Figure</u> | <u>Negative No.</u> |
|---------------|---------------------|---------------|---------------------|---------------|---------------------|
| 119 | 1166/a | 164 | 874/a | 213 | 963/a |
| 120 | 1176/b | 165 | 875/a | 214 | 966/b |
| 121 | 1170/a | 166 | 919/a | 215 | 966/a |
| 122 | 1170/b | 167 | 893/b | 216 | 960/b |
| 123 | 1179/b | 168 | 920/a | 217 | 965/b |
| 124 | 1180/a | 169 | 920/a | 218 | 775/a |
| 125 | 1189/b | 170 | 920/a | 219 | 984/a |
| 126 | 1187/a | 171 | 886/a | 220 | 984/a |
| 127 | 1201/b | 172 | 886/b | 221 | 974/a |
| 128 | 1206/a | 173 | 929/a | 222 | 979/a |
| 129 | 1237/a | 174 | 929/b | 223 | 970/a |
| 130 | 1235/b | 175 | 2230/26 | 224 | 989/a |
| 131 | 1236/a | 176 | 869/b | 225 | 755/a |
| 132 | 1239/b | 177 | 869/b | 226 | 991/a |
| 133 | 1242/b | 178 | 623/a | 227 | 980/a |
| 134 | 934/a | 179 | 872/b | 228 | 991/a |
| 135 | 866/a | 180 | 870/a | 229 | 971/a |
| 136 | 870/b | 181 | 845/b | 230 | 982/b |
| 137 | 891/a | 182 | 846/a | 231 | 975/b |
| 139 | 848/b | 183 | 2230/24 | 232 | 985/b |
| 140 | 851/a | 184 | 890/a | 233 | 971/b |
| 141 | 881/b | 185 | 890/b | 234 | 741/b |
| 142 | 848/a | 186 | 2231/9 | 235 | 994/a |
| 143 | 848/b | 187 | 2231/26 | 236 | 760/b |
| 144 | 886/b | 188 | 877/b | 237 | 762/b |
| 145 | 870/b | 189 | 2230/21 | 238 | 759/a |
| 146 | 881/b | 190 | 924/b | 239 | 993/a |
| 147 | 866/a | 191 | 923/a | 240 | 1002/a |
| 148 | 892/b | 192 | 887/b | 241 | 999/b |
| 149 | 545/b | 193 | 2231/5 | 242 | 1017/b |
| 150 | 896/b | 194 | 848/b | 243 | 1017/a |
| 151 | 579/b | 195 | 904/a | 244 | 1016/b |
| 152 | 562/b | 196 | 889/b | 245 | 1018/b |
| 153 | 563/a | 197 | 2231/31 | 246 | 1026/a |
| 154 | 904/b | 198 | 681/a | 247 | 1008/b |
| 155 | 904/a | 199 | 721/b | 248 | 254/b |
| 156a | 902/b | 200 | 719/b | 249 | 1008/a |
| 156b | 897/a | 201 | 685/b | 250 | 220/b |
| 156c | 897/b | 202 | 680/a | 251 | 1015/b |
| 156d | 576/a | 203 | 939/b | 252 | 1011/b |
| 157a | 899/a | 204 | 943/a | 253 | 1021/b |
| 157b | 900/a | 205 | 938/a | 254 | 1026/b |
| 157c | 898/a | 206 | 940/b | 255 | 1022/b |
| 157d | 903/a | 207 | 1032/b | 256 | 1005/a |
| 158a | 907/a | 208 | 956/a | 257 | 814/a |
| 158b | 907/b | 209 | 962/b | 258 | 917/a |
| 159 | 904/a | 210 | 963/b | 259 | 776/a |
| 160 | 563/a | 211 | 716/a | 260 | 1028/b |
| 163 | 925/b | 212 | 715/a | 261 | 1029/b |

Figure Negative No.

| | |
|-----|--------|
| 262 | 1027/a |
| 263 | 1033/b |
| 264 | 985/a |
| 265 | 1042/b |
| 266 | 953/a |
| 267 | 1036/a |
| 268 | 1039/a |
| 269 | 983/a |
| 270 | 1037/a |
| 271 | 1038/a |
| 272 | 914/a |
| 273 | 1045/b |
| 274 | 1050/b |
| 275 | 1052/a |
| 276 | 1037/b |
| 277 | 949/a |
| 278 | 1042/a |
| 279 | 911/a |
| 280 | 742/a |
| 281 | 739/b |
| 282 | 909/b |
| 283 | 917/b |
| 284 | 916/a |
| 285 | 1053/a |
| 286 | 740/b |
| 287 | 1202/b |
| 288 | 777/a |
| 289 | 722/b |
| 290 | 933/a |
| 291 | 719/a |